Characterization of cellulase from *Bacillus subtilis* N15

A
DISSERTATION REPORT

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CERTIFICATE

This is to certify that the thesis entitled "**Characterization of cellulase from *Bacillus subtilis* N15**" submitted by Mr. Dinesh Choudhary in partial fulfillment of the requirements for the award of degree of Master of Science in Microbiology to Thapar University, Patiala, is a record of student's own work carried out by his under his supervisor. The report has not been submitted for the award of any other degree or certificate in this or any other University or institute.

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Dated: 11-07-2013

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CANDIDATE'S DECLARATION

I, hereby declare that the work presented in the dissertation entitled **"Characterization of cellulase from *Bacillus subtilis* N15"** in partial fulfillment of the requirements for the award of the degree of Master of Science in Microbiology, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is an authentic record of my own work during the period of six months from Jan 2013 to July 2013, under the supervision of Dr. Dinesh Goyal, Professor, Department of Biotechnology & Environmental Sciences, Thapar University. The report has not been submitted for the award of any other degree or certificate in this or any other university.

Dated: 11-7-2013

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Abbreviations

BSA	Bovine serum albumin
CMC	Carboxymethyl cellulose
°C	Degree Celsius
DNS	3, 5-dinitrosalicylic acid
et al	and others
$FeSO_4.7H_2O$	Ferrous sulphate heptahydrate
g	Gram
h	Hour
K_2HPO_4	Di potassium hydrogen phosphate
L	Liter
µg	Microgram
µl	Microliter
min	Minute
mM	Millimolar
ml	Milliliter
$MgSO_4.7H_2O$	Magnessium sulphate heptahydrate
nm	Nanometer
O.D.	Optical Density
%	Percent
rpm	Rotation per minute
s	Second
U/ml	Units per ml

ABSTRACT

Bacillus subtilis N15 is an isolate from sugarcane bagasse was checked for carboxymethyl cellulose (CMCase) activity of cell free supernatant using 2% carboxymethyl cellulose. Optimum enzyme activity was 0.15 U/ml at pH 7.0 and 0.11 U/ml at 40°C. Crude cellulase from *Bacillus sp.* was stable at pH ranging from 5.0-7.0 and was thermally stable up to 50°C. The enzyme activity of the partially purified cellulases was higher at 30-90 mM $MgSO_4.7H_20$ and $FeSO_4.7H_2O$, while EDTA inhibited the enzyme activity. Cellulase was partially purified by ammonium sulphate precipitation and exhibited 6 fold increase in CMCase activity with a molecular weight of 35 kDa as confirmed by zymography.

INTRODUCTION

Cellulose is one of the most abundant organic macromolecules in the ecosystem (Ross et al, 1991) and is totally insoluble in water (Lederberg, 1992). It is a linear, unbranched homopolysaccharide consisting of glucose subunit joined together via β 1-4 glycosidic linkages. Individual cellulose molecules vary widely in length and are usually arranged in bundles or fibrils (Walsh, 2002). Within the bundles, cellulose molecules can occur in crystalline or paracrystalline (amorphous) structures (Walter, 1998). Cellulose is usually the dominant structural polysaccharide of plant cell walls (35–50%), followed by hemicelluloses (20–35%) and lignin (10–25%). Cellulolytic enzymes are synthesized by a number of microorganisms. Fungi and bacteria are the main natural agents of cellulose degradation (Lederberg, 1992). The cellulose utilizing population includes aerobic and anaerobic mesophilic bacteria, filamentous fungi, thermophilic and alkaliphilic bacteria, actinomycetes and certain protozoa.

Bioconversion of cellulose to soluble sugars and glucose is catalyzed by a class of enzymes called cellulases. Microorganisms including bacteria, fungi and actinomycetes produce mainly three types of cellulase components - endo-1, 4-β-D-glucanase, exo-1, 4-β-D-glucanase and β-glucosidase. Bacterial cellulases exist as discrete multi-enzyme complexes, called cellulosomes that consist of multiple subunits that interact with each other synergistically and degrade cellulosic substrates efficiently (Bayer et al, 2004). Although a large number of microorganisms can degrade cellulose, only a few them produce significant quantities of free enzyme capable of completely hydrolyzing crystalline cellulose (Koomnok, 2005).

Cellulose is often crystalline in the native stage and is surrounded by a mixture of amorphous cellulose (non-organized chains), hemicellulose and lignin. Because of its structural rigidity, crystalline cellulose is resistant to the action of individual cellulases. The commonly described mode of action for cellulases on polymers is either exo- or endo-cleavage. Using this classification system, cellobiohydrolases (exoglucanases) were classified as exo-acting based on the assumption that they all cleave β-1, 4-glycosidic bonds from chain ends. Endoglucanases on the other hand, are often classified as endo-acting cellulases because they are thought to cleave β-1, 4-glycosidic bonds internally only.

Cellulose degradation occurs in three general steps (Bisariaet al, 1989). First, the long chain polymer is degraded into random lengths of 4 to 6 glucose units by an endoglucanase, which is often exported to the environment outside the cell. Second, an exoglucanase cleaves the shortened chains into dimers. This enzyme is exported or held in close association with the outer membrane. The final step is cleavage of the dimer into glucose by a β-glucosidase, most commonly located within the cell. 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.

Lignocelluloses are the building blocks of all plants and are ubiquitous to most regions of our planet. Their chemical properties make it a substrate of enormous biotechnological value. Lignocellulose is a complex substrate and its biodegradation is not dependent on environmental conditions alone, but also the degradative capacity of the microbial population (Waldrop et al, 2000).Chemically, lignocellulose is a combination of two linear polymers, cellulose and hemicellulose and a nonlinear, three-dimensional polymer lignin (Perez et al, 2002). Cellulose is surrounded by matrix like hemicellulose and encrusting lignin. A variety of microorganisms and mechanisms are involved in the complete biodegradation of lignocellulose in natural environments ranging from soil and rumen ecosystems to the termite hindgut. The primary objective of lignocellulose pretreatment by the various industries is to access the potential of the cellulose and hemicellulose encrusted by lignin within the lignocellulose matrix. Cellulases has a wide range of industrial applications. The main applications include textile, paper and pulp, food, animal feed, fuel and chemical industry. Additionally they can be used in waste management, pharmaceutical industry, protoplast production and genetic engineering (Bhat, 2001).The utilization of cellulosic biomass continues to be a subject of worldwide interest in view of fast depletion of our oil reserves and food shortages (Kuhadet al, 1997).

Currently, ethanol production from cellulosic biomass conversion is one of the most studied and promising alternative. In addition, ethanol production from lignocellulose wastes is very

attractive because of their low cost and abundance. Therefore, a large variety of lignocellulosic materials including wood, straws, agricultural wastes, and crop residues have been evaluated for use in this bioconversion process. The basic process steps in producing ethanol from cellulose biomass consist in an initial treatment (for example, diluted acid, alkaline or steam explosion) to render cellulose more accessible to the subsequent step of enzymatic hydrolysis, which break down polysaccharides to simple sugars. The glucose solution obtained can be fermented to ethanol by microorganisms; *Saccharomyces cerevisiae*being the most currently used since it gives high ethanol yields from glucose.

In the present project work, carboxymethylcellulase (CMCase) activity in cell free supernatant of *Bacillus subtilis* N15was optimized for different parameters such as pH, temperature, checked for thermal stability and cellulose activity after partial purification.

Literature Review

Cellulose

Cellulose, the major chemical component of the fiber wall, is a homopolysaccharide composed entirely of D-glucose linked together by β-1,4-glycosidic bonds with degree of polymerization ranging from 1,000 in bleached kraft pulps to 10,000 in native wood (Schmidt, 2006). It is a linear structure that has a strong tendency to form intra or intermolecular hydrogen bonds resulting in the formation of cellulose microfibrils. 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, 2003).

Cellulose degrading enzymes

The enzymes for the degradation of celluloses belong predominantly to the hydrolases which cleave glycosidic bonds by hydrolysis; therefore the enzymes that catalyze 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) (Kuhadet 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 (Harhangiet 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 (Gilkeset al, 1991).

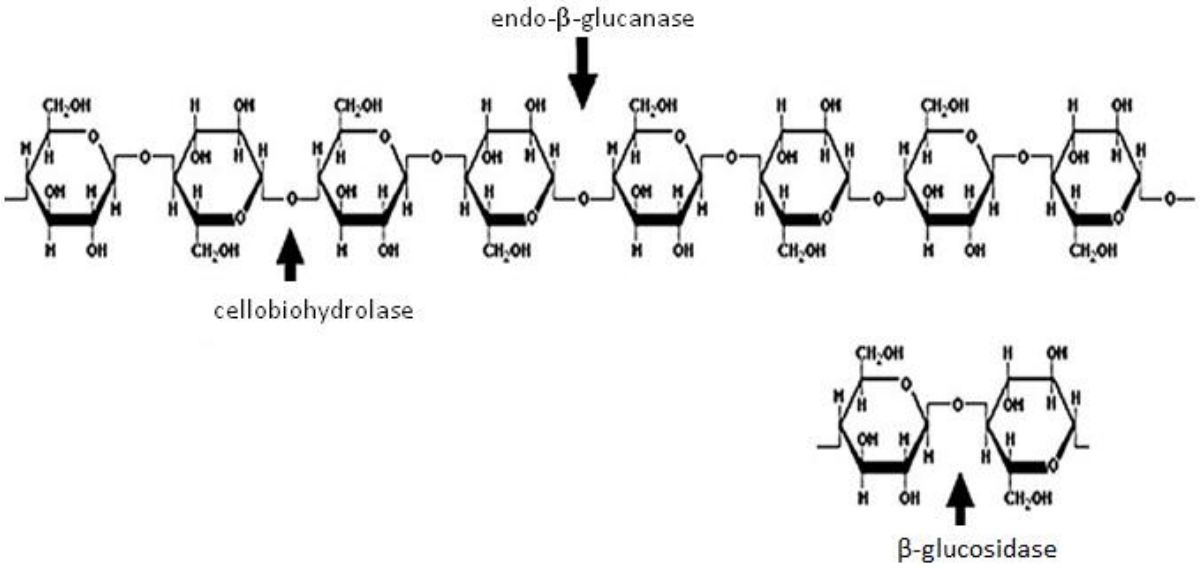

Figure 1. Molecular structure of cellulose and site of action of endoglucanase, cellobiohydrolase and β-glucosidase (Kumar et al, 2008).

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

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*) and fungi (e.g. *Penicillium, Aspergillus, Trichoderma*) 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 (Perssonet 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 C-30 (Montenencourtet al, 1979) still preserves its leading role as the major test organism in fundamental cellulase research.

Table 1. Microorganisms producing cellulose degrading enzymes.

Microorganism	**Substrate**	**Enzyme**	**Reference**
Trichoderma harzianum	CMC,birchwo odXylan	Exoglucanase,endoglucan ase and β-glucosidase	Ahmed et al, 2009
Pseudomonas fluorescens	CMC	Endoglucanase	Bakare et al,2005
Aspergillus niger	pNPG	β-glucosidase	Bailey et al,1981
Tricoderma reseii	Filter paper	Endoglucanase	Jahangeer et al,2005
Bacillus subtilis	CMC	β-1,3-1,4-D-Glucanase	Robert et al,2007
Penicillium echinulatum	CMC	β-glucosidase	Sharma et al,2001
Bacillus licheniformis	Cellobiose	Endoglucanase	Aygan et al,2011

Applications of Cellulase

Previous research has revealed the application of cellulolytic bacteria in biotechnology and industry field such as food, brewery and wine, animal feed, textile and laundry, pulp and paper and also in agriculture waste industry. Some of thesevarious applications are shown in Table.

Nowadays, cellulolytic bacterium is widely used in many industrial sites particularly in enzyme production, which has been increased due to the demand for more stable, highly active and specific enzymes.

Table 2. Applications of cellulase in different industries.

Industry	**Function**	**Application**	**References**
Textile	Remove of excess dye from denim fabrics; soften the cotton fabrics without damaging the fibre.	Bio-stoning of denim fabrics: production of high quality and Environmentally friendly washing powders.	Galanteet al,1998,Godfrey, 1996, Uhlig, 1998.
Animal Feed	Partial hydrolysis of Lignocellulosic materials, hydrolysis of β-glucans.	Improvement in the nutritional quality of animal feed.	Beauchemin et al, 1995, Chesson, 1987, Graham and Balnave, 1995.
Pulp and Paper	Enzymatic modification of unrefined wood chips.	Increase tensile strength and high fibre qualities, energy consumption reduced.	Pere et al, 1996, Akhtar1994, Leatham et al, 1990.
Beer and Wine	Hydrolysis of plant cell wall polysaccharides.	Improvement in skin maceration and color extraction of grapes, quality, stability, filtration and clarification of wines.	Galante et al, 1998,Uhlig, 1998.

MATERIALS AND METHODS

CMCase in cell free supernatant of cellulose degrading *Bacillus subtilis*N15 was optimized for different parameters such as pH, temperature, thermal stability and salt concentration and it was further subjected to partial purification by ammonium sulfate precipitation.

Microorganism: *Bacillus subtilis* N15

Already characterized *Bacillus subtilis* N15 strain was provided by the same laboratory.

1. CMCase activity in cell free supernatant of *Bacillus subtilis* N15 (Miller 1959).

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

Materials

a. Stock: 2 mg/ml glucose
b. DNS reagent (Appendix 1)

Method

1. The overnight grown culture of *B. Subtilis* N15 (2%) was inoculated in flask containing Enriched media (Appendix 2) supplemented with 0.5% CMC and incubated at 37°C.
2. Two ml of culture from flask having *B. Subtilis* N15 was taken and centrifuged in cooling centrifuge at 10,000 rpm for 10 min.
3. In an eppendorf tube 500 µl of CMC dissolved in 50 mM sodium phosphate buffer (pH 7) was added.
4. Incubated in water bath at 50°C for 10 minutes.
5. After incubation 500 µl of sample supernatant was added.
6. Incubated in water bath at 50°C for 30 min.
7. Then 3 ml of DNS reagent (Appendix 1) was added and placed in boiling water bath for 10 min.
8. Cool the above mixture at room temperature.
9. OD was taken at 540 nm.
10. A standard graph was drawn and concentration of sugar was calculated.

$$CMC = \frac{0.185}{\text{Critical enzyme concentration}} \text{ units/ml}$$

Table 3. Standard curve of Glucose.

Stock (ml)	Distilled water (ml)	Glucose conc. (mg/ml)	DNS (ml)	Boiled for 10 minutes	O.D taken at 540 nm
0	1.0	0	3		
0.2	0.8	0.4	3		
0.4	0.6	0.8	3		
0.6	0.4	1.2	3		
0.8	0.2	1.6	3		
1.0	0	2.0	3		

Biochemical characterization of cellulose in cell free supernatant

Biochemical characterization of CMCase in cell free supernatant of *Bacillus subtilis*N15 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% CMC in the presence of appropriate buffers for different pH like 50 mM sodium citerate (pH 4.0, 5.0 and 6.0), 50mM potassium phosphate (pH 7.0) and 50 mM glycine-NaOH buffer (pH 8.0,9.0 and 10.0). The reaction mixture of various pH buffers 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.

Table 4.Buffers used to determine the optimum pH (Appendix 3).

pH	50 mM buffer
4	Sodium citrate
5	Sodium citrate
6	Sodium citrate
7	Potassium phosphate
8	Glycine-NaOH
9	Glycine-NaOH
10	Glycine-NaOH

Determination of optimum temperature for CMCase activity

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 50mM 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 using CMCase

Thermal stability of cellulase was determined by preincubating the enzyme at various temperatures ranging from 30°C to 80°C for 30 min. After incubating the enzyme for 30 min 2% CMC dissolved in 50mM 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 increased salt concentration and EDTA on CMCase activity.

Materials

Salts

1. $MgSO_4.7H_2O$ (30 - 150 mM)
2. $FeSO_4.7H_2O$ (30 - 150 mM)
3. K_2HPO_4 (30 - 150 mM)
4. EDTA (10 - 50 mM)

METHOD

1. CMC (2%) solution was made in 50 mM potassium phosphate buffer (pH 7.0).
2. Different concentration of salts and EDTA were added in CMC (500 µl) and placed in water bath at 50°C.
3. Cell free supernatant (500 µl) was added into it and mixed.
4. Mixture was incubated in water bath for 10 min at 50°C.
5. DNS reagent (3 ml) was added in above mixture and placed them in a boiling water bath for 10 min.
6. Cool the above mixture at room temperature.
7. O.D. was taken at 540 nm.

2. Protein estimation in *Bacillus subtilis*15by Bradford's method (1976).

Materials

a. Stock BSA – 10mg/ml
   Working stock (500 µl/ml) was made - (50µl of 10 mg/ml BSA stock & 950 µl of distilled water).
b. Bradford Reagent

Method

1. The overnight grown culture of *B. Subtilis* N15 (2%) was inoculated in flask containing Enriched media (Appendix 2) and incubated at 37°C.
2. Two ml of culture from flask having strain N15 was taken and centrifuged in cooling centrifuge at 10,000 rpm for 5 min.
3. From the supernatant solution 20 µl of sample was taken and volume was made upto100µl with distilled water.
4. To this 1 ml of Bradford's reagent was added and mixed well.
5. Incubated the above mixture at 50°C for 10 min.
6. OD was taken at 595 nm.
7. A standard graph was drawn with BSA and calculated amount of protein in the sample.

Table 5. Standard curve of BSA.

BSA working standard (µl)	DW (µl)	Total volume (µl)	Protein amount (µgs)	Protein conc. (µg/ml)	Bradford Reagent (ml)	Incubated for 10 minutes	O.D taken at 595 nm
0	100	100	0	0	1		
0	100	100	0	0	1		
20	80	100	10	100	1		
40	60	100	20	200	1		
60	40	100	30	300	1		
80	20	100	40	400	1		
100	0	100	50	500	1		

3. Ammonium salt precipitation of cell free supernatant of *Bacillus subtilis*N15.

Materials

a. Ammonium Sulphate
b. Potassium phosphate buffer (pH-7)

Method

1. *Bacillus subtilis*N15 were grown in a Enriched media (Appendix 2)containing CMC (0.5%) as a substrate for 24 h.
2. The overnight grown culture was centrifuged at 12000 rpm for 10 min. and take the supernatant.
3. Supernatant was precipitated with the help of Ammonium Sulphate at 4^0C upto 80% saturation.
4. Precipitated sample were stored at 4^0C for overnight.
5. Cool Precipitated sample was centrifuged at 12000 rpm for 10 min.
6. Excess salt present in the protein sample was removed by adding 1 - 3 ml of 50 mm Potassium Phosphate buffer (pH 7) and centrifuge them at 12000 rpm for 10 min.

7. Collect the pellet and dissolve in 100 µl of 50 mm Potassium Phosphate buffer.
8. Protein estimation of partially purified protein was done by Bradford method (1976) as describe earlier.

5. SDS-PAGE and Zymography.

Sodium dodecyl sulfate-polyacylamide gel electrophoresis (SDS–PAGE) was used to separate proteins according to their size, as described by (Laemmli 1970).

Materials

a. RUNNING GEL (10 ml)

1. Distilled water - 4 ml
2. 30% Acrylamide & Bisacrylamide - 3.4 ml
3. 4X Separating gel buffer (pH 8.8) - 2.5 ml
4. 10% SLS - 0.1 ml
5. 10% APS (100mg/ml) - 100 µl
6. Temed - 10 µl

b. STACKING GEL (5 ml)

1. Distilled water - 3 ml
2. 30% Acrylamide & Bisacrylamide - 0.83 ml
3. 4X Separating gel buffer (pH 6.8) - 1.25 ml
4. 10% SLS - 25 µl
5. 10% APS (100 mg/ml) - 50 µl
6. TEMED - 7 µl

c. Protein ladder (Fermentas) - 14.4 – 116 kDa

Method

1. Ingredients of running gel were mixed and poured quickly into gel casting stand and some space was leaved for the stacking gel approx. 2 centimeters below the bottom of the comb.
2. Bubbles were removed by addition of a layer of saturated butanol poured on the top of the gel with water and left for 30 minutes for the gel to polymerize completely.
3. Butanol layer was discarded and washed with distilled water 3 times.

4. Ingredients of stacking gel were mixed and poured on the top of the running gel.
5. Comb was inserted in gel after pouring stacking gel and allowed another 30 min - 1 hour for complete polymerization.
6. Gel was clamped in the SDS-PAGE apparatus and buffer chambers were filled with gel running buffer.
7. Partially purified protein was taken in 4:1 with the sample buffer and put the sample mixture in boiling water bath for minutes at 95°C.
8. Protein sample and standard marker were loaded in the separate wells and current was applied (80 volt, 25 Ma).
9. Current supply was stopped when sample reaches at 0.5 cm above the end of gel.
10. Separated proteins were visualized using (0.15% W/V) Coomassie Brilliant Blue R-250.

Zymography

Zymography was done using protocol as per Schwarz et al,(1987).

1. Gel was prepared by including 0.1% CMC before polymerization in the 12% SDS-PAGE resolving gel mixture. Gel mixtures were heated at 30 °C and CMC was added slowly to prevent aggregation.
2. APS and TEMED were added when CMC was dissolved and gel was allowed to polymerize at room temperature.
3. Commercial grade *Aspergillus niger cellulose* (100 µg/ml) was used as a positive control.
4. Partially purified protein was taken in 4:1 with the sample buffer and loaded in the wells and current supply (80 volt, 25 Ma).
5. Current supply was stopped when sample reaches at 0.5 cm from top of the gel.
6. After electrophoresis, gel was washed five times at room temperature; each wash for 30min with 50 mL of wash buffer (50 mm potassium phosphate buffer).
7. Remaining CMCin the gel was stained with 0.1% Congo red (Teather and Wood, 1984) solution and incubated for 10min at room temperature.
8. Gel was destained by washing in 50mL of 1 M NaCl until cellulase bands became visible as clear zones where CMC was degraded due to CMCase activity.
9. After destaining for 20 min, 100µl of glacial acetic acid was added to the gel for improved band visualization (Waeonukul et al. 2007).

RESULTS AND DISCUSSION

Bacillus subtilis N15 an isolate from sugarcane bagasse was checked for its CMCase activity. The CMCase activity of cell free supernatant was optimized at various pH and temperature. Thermal stability of *Bacillus subtilis* N15 was also determined at various temperature ranges. The effect of various salts like$MgSO_4.7H_20$, $FeSO_4.7H_2O$, $K_2HPO_4$and EDTA at different concentration on CMCase activity was checked. Enzyme purification was done by ammonium sulphate precipitation of cell free supernatant and molecular weight of cellulose was determined by SDS-PAGE and confirmed by zymography.

1. CMCase activity in cell free supernatant of *Bacillus subtilis*N15(Miller 1959).

CMCase activity was determined by estimating the amount of reducing sugar using DNS (3,5-Dinitrosalicyclic acid) method as per Miller (1995) using glucose as the standard.

Table 6. Glucose Standard curve readings.

Stock (ml)	Distilled Water (ml)	Glucose concentration (mg/ml)	DNS (ml)	O.D. (540 nm)
0	1.0	0	3.0	0
0.2	0.8	0.4	3.0	1.373
0.4	0.6	0.8	3.0	1.634
0.6	0.4	1.2	3.0	2.251
0.8	0.2	1.6	3.0	2.673
1.0	0	2.0	3.0	3.051

Absorbance of *Bacillus subtilis* N15 – 1.632 at 540 nm.

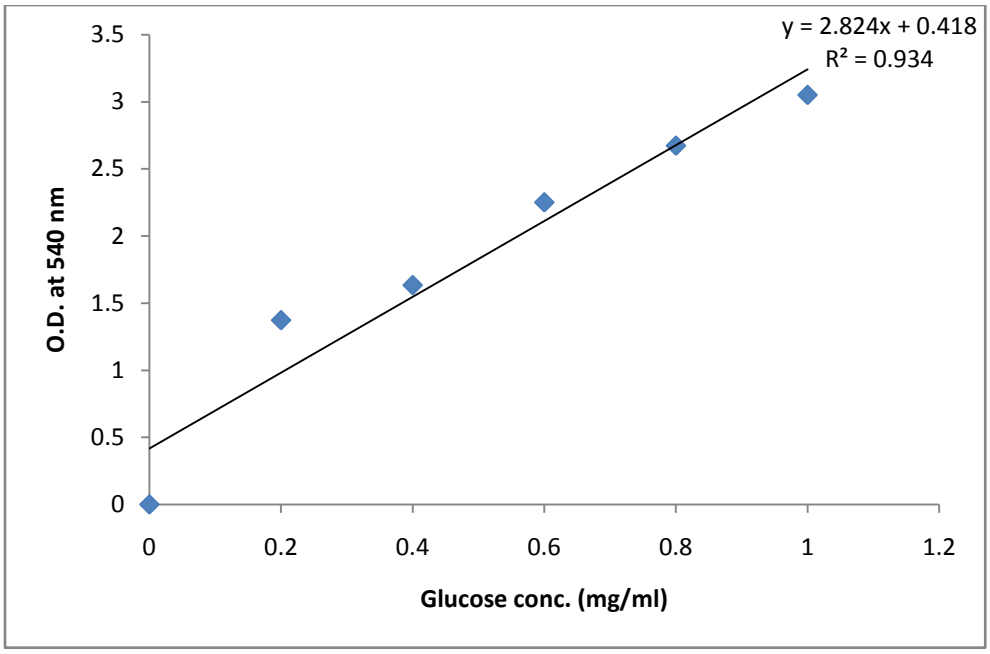

Figure 2. Standard curve for Enzyme assay.

CMCase activity of *Bacillus subtilis*N15 is = 0.24 U/ml.

Biochemical characterization of CMCase in cell free supernatant

Determination of optimum pH for CMCase activity

CMCase activity in cell free supernatant of 24 h old *Bacillus subtilis*N15was found maximum (0.15 U/ml) in the presence of 2% CMC dissolved in 50 mM potassium phosphate buffer having pH 7.0 (Table 7, Figure 3).

The optimum pH for the *Bacillus subtilis*N15 found to be 7.0, which was in accordance with the previous study by (Ray et al. 2007), where pH 6.5 - 7.0was more suitable for cellulose produced by *B. subtilis* and *B.circulans*. In contrast to that, *Bacillus subtilis*N15 strain showed maximum activity (0.15 U/mL) at pH 7.0 which suggesting its neutral in nature. 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 having an optimum pH of 4.0 - 6.0 (Ariffin et al. 2006).

Table 7. CMCase assay of bacterial isolate at different pH (Appendix 3).

pH	OD (540 nm)	#CMCase (U/ml)
4	1.557	0.074 ± 0.009
5	2.432	0.132 ± 0.008
6	2.734	0.148 ± 0.005
7	2.685	0.152 ± 0.004
8	1.730	0.086 ± 0.005
9	1.631	0.079 ± 0.008
10	1.305	0.058 ± 0.003

#Values are means of ± SE.

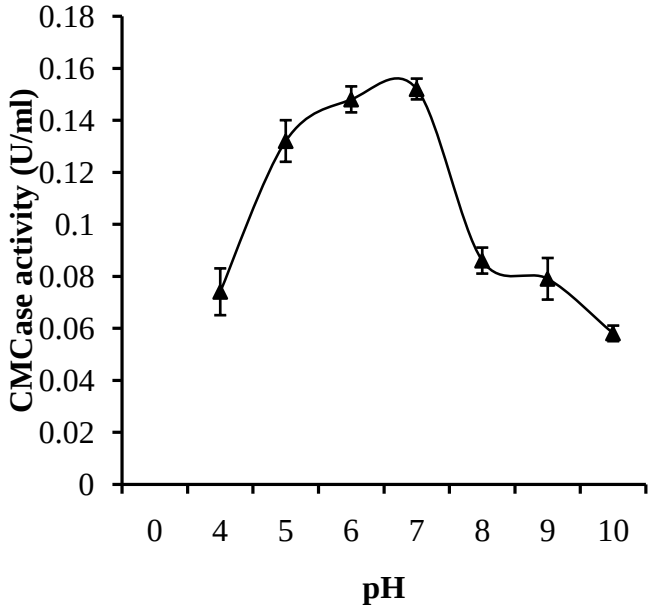

Figure 3.CMCase activity in cell free supernatant of 24 h old culture at different pH in presence of 2% CMC dissolved in 50 mM sodium phosphate buffer (Values are means of triplicate, bars shows the standard error).

Determination of optimum temperature for CMCase activity

Maximum CMCase activity of 0.109 U/ml was found at assay temperature of 40°C using 2% CMC dissolved in 50 mM potassium phosphate buffer (Table 8, Figure 4).Beyond 40°C there was gradual decline in enzyme activity and at 80°C very low activity was observed (Table 8, Figure 4).

Table 8.CMCaseactivity of bacterial isolate at different temperature.

Temperature (°C)	OD (540 nm)	#CMCase (U/ml)
30	1.513	0.072 ± 0.001
40	2.085	0.109 ± 0.002
50	1.764	0.088 ± 0.001
60	1.113	0.046 ± 0.003
70	0.835	0.027 ± 0.002
80	0.734	0.021 ± 0.003

#Values are means of ±SE.

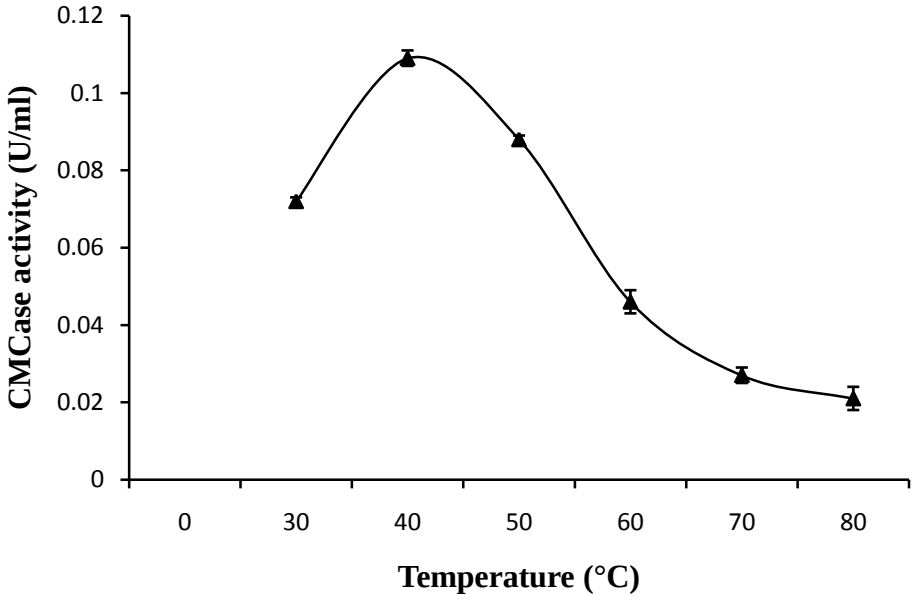

Figure 4.CMCase activity of cell free supernatant of 24 h old culture at different temperatures in presence of 2% CMC dissolved in 50 mM sodium phosphate buffer (Values are means of triplicate, bars shows the standard error).

Enzyme activity at 40°C was optimum for *B. subtilis* and *B.circulans*(Ray et al, 2007) and maximum endoglucanase activity was observed in *Cellulomonas*, *Bacillus*, and *Micrococcus* sp.

at 40°C and neutral pH (Immanuel et al. 2006). Enzyme activity was optimum at 50°C in case of *B. amyloliquefaciens* DL-3 and found stable at 40–80°C (Lee et al. 2008).

Thermal stability of CMCase

The enzyme was found to be thermally stable between temperature 30-60°C (Table 9, Figure 5). However, the enzyme activity decreased above 60ºC and was negligible at 80ºC, due to denaturation of the enzyme. Thermal stability of CMCase produced by *Geobacillus sp.* dropped rapidly beyond 70°C (Tai et al. 2004). Enzyme activity was optimum at 50°C in case of *B.amyloliquefaciens* DL-3 and found stable at 40–80°C (Lee et al. 2007).

Table 9. Thermal stability of CMCase.

Temperature (°C)	OD (540 nm)	#CMCase (U/ml)
30	1.598	0.077 ± 0.001
40	1.610	0.078 ± 0.002
50	1.201	0.032 ± 0.001
60	0.687	0.018 ± 0.003
70	0.655	0.016 ± 0.002
80	0.625	0.013 ± 0.001

#Values are means of ±SE

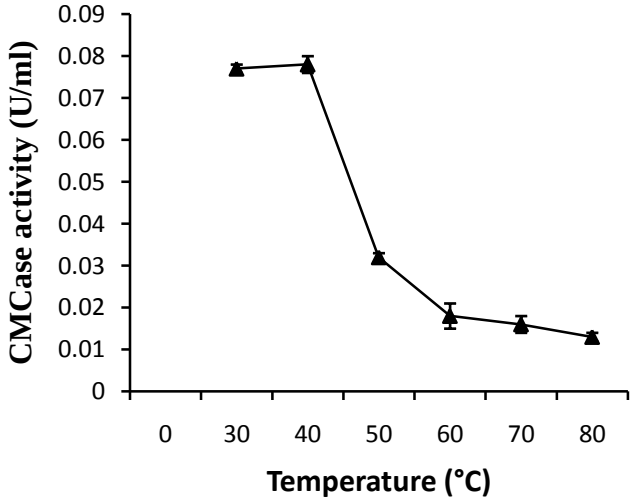

Figure 5.Thermal stability of CMCase in cell free supernatant of 24 h old culture in presence of 2% CMC dissolved in 50 mM sodium phosphate buffer. (Values are means of triplicate, bars shows the standard error).

Effect of salt concentration and EDTA on CMCase activity.

In presence of $MgSO_4.7H_2O$, increase in enzyme activity was observed in the range of 30- 90 mM concentration. It was found maximum (0.351 U/ml) in presence 90 mM concentration and beyond 90 mMit decreased (Table 10, Figure 6). It indicates that the addition of higher concentration $MgSO_4.7H_2O$ leads to increase in CMCase enzyme activity.

Table 10. Absorbance at various concentration of $MgSO_4.7H_2O$ (mM).

$MgSO_4.7H_2O$ (mM)	**OD (540 nm)**	**#CMCase (U/ml)**
30	1.495	0.211 ± 0.001
60	2.108	0.332 ± 0.002
90	2.204	0.351 ± 0.001
120	2.082	0.327 ± 0.002
150	1.662	0.245 ± 0.001

#Values are means of ±SE

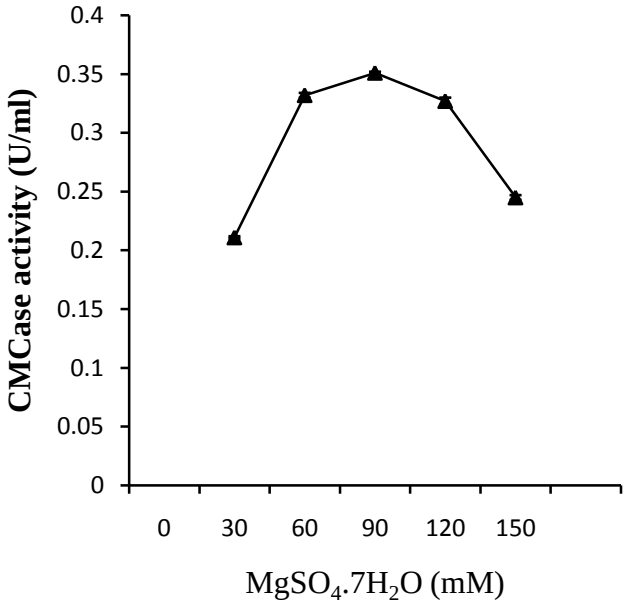

Figure 6. Effect of $MgSO_4.7H_2O$ on cellulase activity (Values are means of triplicate, bars shows the standard error).

$FeSO_4.7H_2O$ have also effected CMCase activity as maximum activity was found at 90 mM concentration. However there was slight decrease beyond 90 mM , which indicates that addition of higher concentration of $FeSO_4.7H_2O$ leads to increase in cellulose CMCase activity.

Table 11. CMCase activity at various concentration of$FeSO_4.7H_2O$ (mM).

$FeSO_4.7H_2O$ (mM)	**OD (540 nm)**	**#CMCase (U/ml)**
30	2.513	0.412 ± 0.001
60	3.227	0.552 ± 0.002
90	3.334	0.574 ± 0.001
120	3.179	0.543 ± 0.003
150	3.205	0.548 ± 0.002

#Values are means of ± SE.

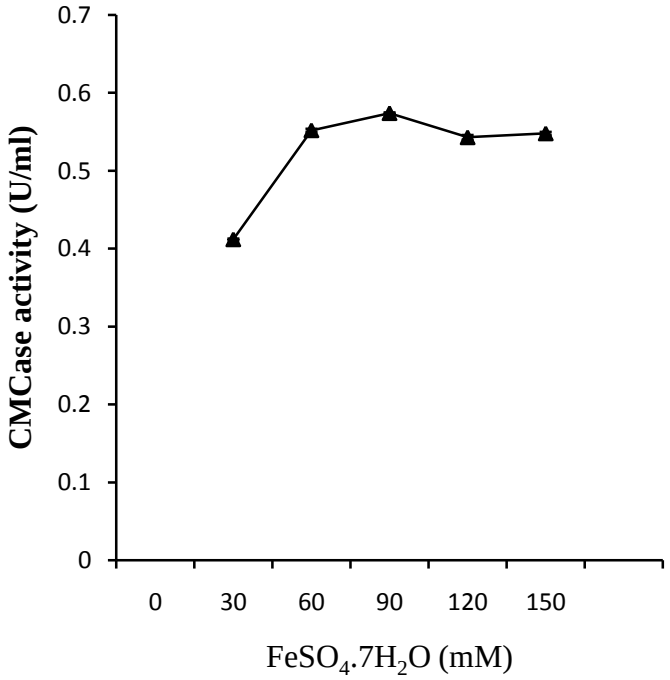

Figure 7. Effect of $FeSO_4.7H_2O$ (mM) on CMCase activity(Values are means of triplicate, bars shows the standard error).

Increase in the concentration of K_2HPO_4leads to decreases in CMCase activity, which indicates that the addition of higher concentration K_2HPO_4leads to decrease in activity (Table 12, Figure 8).

Table 12. CMCase activity at various concentration of K_2HPO_4.

K_2HPO_4 (mM)	OD (540 nm)	#CMCase (U/ml)
30	1.294	0.172 ± 0.001
60	1.275	0.169 ± 0.002
90	1.249	0.163 ± 0.001
120	1.235	0.161 ± 0.003
150	1.202	0.154 ± 0.002

#Values are means of ± SE.

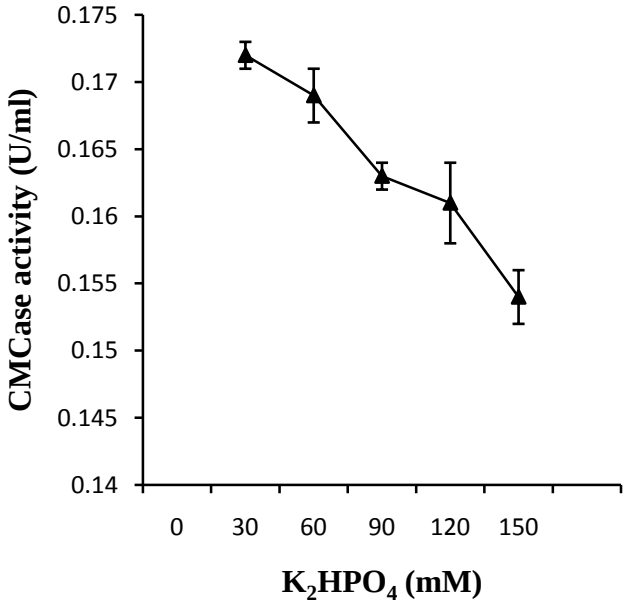

Figure 8. Effect of K_2HPO_4 on CMCase activity(Values are means of triplicate, bars shows the standard error).

A gradual decrease in CMCase activity was observed in presence of varying concentration of EDTA10 - 50 mM (Table 13, Figure 9). EDTA inhibits the CMCase activity, which was also reported by Lee et al. (2007) at 5mM.

Table 13. CMCase activity at various concentration of EDTA.

EDTA (mM)	OD (540 nm)	#CMCase (U/ml)
10	0.760	0.067 ± 0.001
20	0.757	0.066 ± 0.002
30	0.734	0.062 ± 0.001
40	0.712	0.058 ± 0.003
50	0.639	0.043 ± 0.002

#Values are means of ± SE.

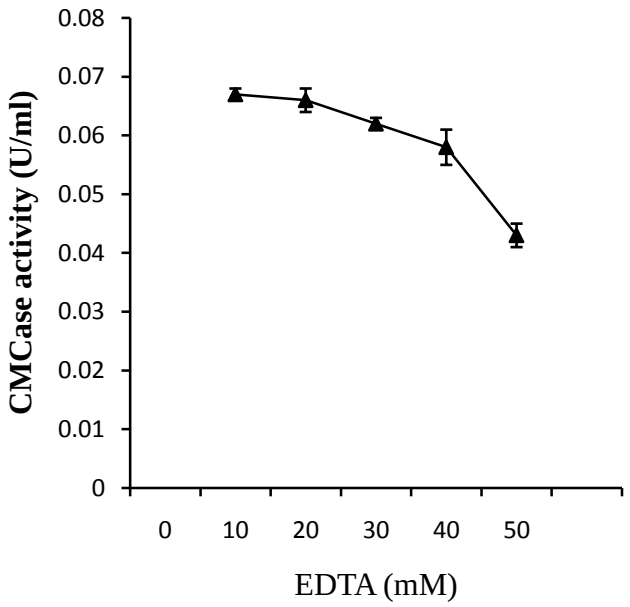

Figure 9. Effect of EDTA on CMCase activity(Values are means of triplicate, bars shows the standard error).

2. Protein estimation in cell free supernatant of *Bacillus subtilis*N15

Protein content was estimated in cell free supernatant of 24 h old grown culture in order to determine the amount of protein present in the sample. The amount of protein can thus be determined in the sample by using standard curve of BSA having equation y = 0.002x + 0.099 and value of R^2=0.94.

Table 14. Standard curve of BSA.

Sr.No.	BSA working standard (µl)	Distilled Water (µl)	Total volume (µl)	Protein amount (µgs)	Protein (µg/ml)	Bradford Reagent (ml)	O.D. (595 nm)
1	0	100	100	0	0	1	0
2	20	80	100	10	100	1	0.431
3	40	60	100	20	200	1	0.701
4	60	40	100	30	300	1	0.843
5	80	20	100	40	400	1	0.947
6	100	0	100	50	500	1	1.13

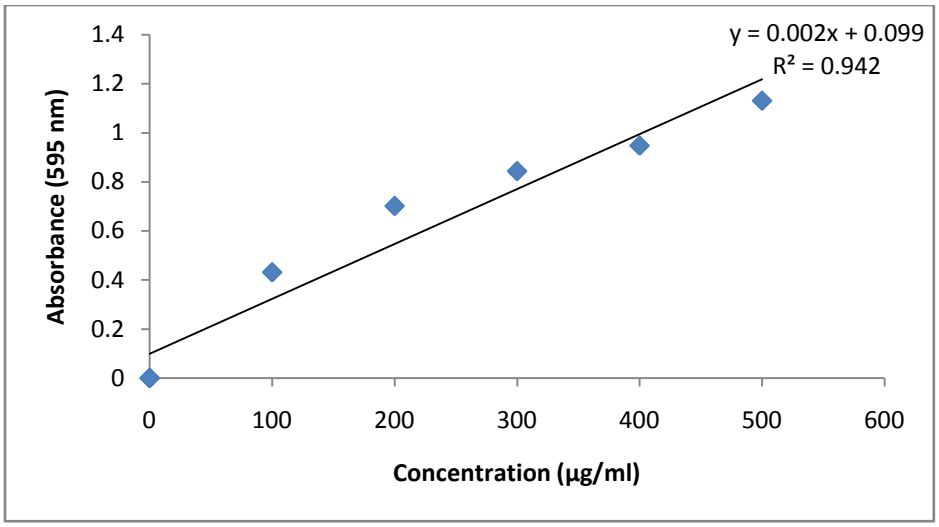

Figure 10. Standard curve of BSAhaving (y=0.002x + 0.099and R^2=0.94).

Absorbance of *Bacillus subtilis*N15= 0.701 at 595 nm.

Therefore, the concentration of protein in cell free supernatant of *Bacillus subtilis* N15 was 1.575 mg/ml.

3. Ammonium salt precipitation for protein sample preparation.

Ammonium sulphate precipitation of cell free supernatant of 24 h old grown culture was done upto 80% 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 °C and was found to be 0.24 U/ml which was6.02fold higher than CMCase activity in crude protein.

5. SDS-PAGE and Zymography

SDS-PAGE of partially purified protein showed different bands which was subjected to Zymography which confirms the protein band of CMCase. In zymogram, a clear band was 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 35kDa.

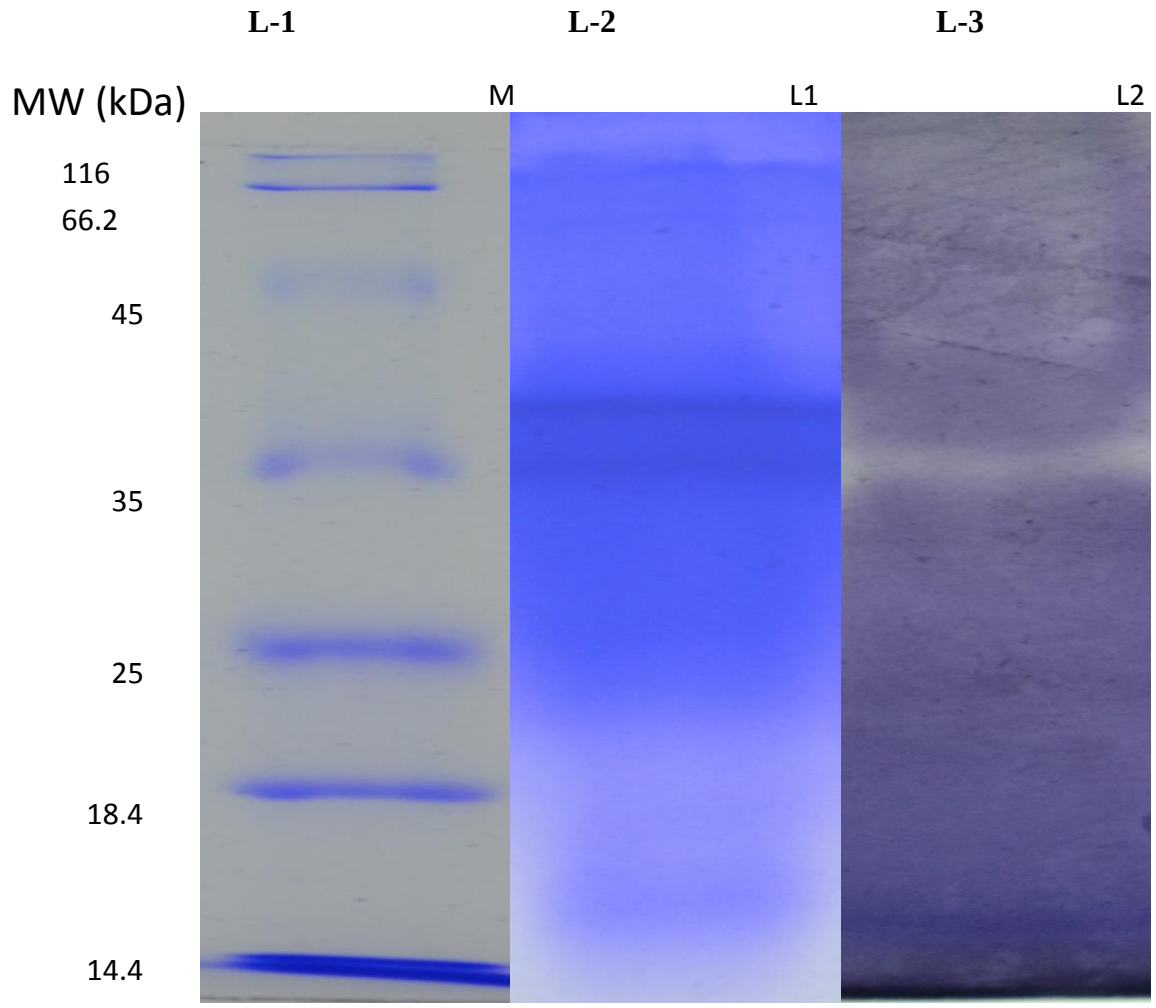

Figure 11.SDS-PAGE and zymography of protein isolated from cell free supernatant of *Bacillus subtilis*N15. Lane (L-1) Protein ladder 14.4-116 kDa (L-2) SDS-PAGE of isolated crude protein (L-3) Zymography of isolated crude protein.

It was reported that in *Bacillus sp.* the molecular weight of protein was determined to be 97 kDa by SDS-PAGE (Sadhu et al, 2013) and in cellulase enzyme of *Trichoderma viride* the molecular weight of protein is 58 kDa (Iqbal et al, 2011). *Caldibacillus cellulovorans* also showed the band of molecular weight 85.1 kDa (Huang et al, 2004).

CONCLUSION

1. A potential cellulase producing *Bacillus subtilis* N15 was checked for its CMCase activity in cell free supernatant which was optimum at pH 7.0 and temperature 40°C.
2. Cellulase produced by *Bacillus subtilis* N15 was thermally stable up to 50°C and activity decreased drastically beyond 50°C and was negligible at 80°C.
3. CMCase activity was optimum at 30–90mM salt concentration, whereas $K_2HPO_4$and EDTA completely inhibited the enzyme activity.
4. Cell free supernatant was subjected to precipitation using ammonium sulphate upto 80% saturation. Six fold increase in CMCase activity of partially purified enzyme was observed with respect to crude enzyme in cell free supernatant.
5. Partially purified enzyme was separated on SDS-PAGE and different bands were seen which was subjected to Zymography for affinity staining. A hazy band of CMCase of approximately 35 kDa was observed which needs to be further purified by column chromatography.

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Appendix I

DNS reagent

Mix:	Distilled Water	1416 ml
	3, 5-Dinitrosalicyclic acid	10.6 g
	NaOH	19.8 g

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

Appendix II

Enriched Media

Media composition (g/l)

Tryptone	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

Appendix III

Buffers

Sodium Citerate buffer (pH 4.0, 5.0 and 6.0)

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

(0.1 M) Potassium phosphate buffer (pH 7.0).

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

A (ml)	B (ml)	pH
61.5	38.5	7

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	pH
2.0	8
6.0	9
19.3	10