PRODUCTION, PURIFICATION AND CHARACTERIZATION OF CELLULASE FROM BACTERIA

By

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of
Masters in Biotechnology

Department of Mathematics and Natural Sciences
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Declaration

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Abstract

Microorganisms isolated from soil samples that showed growth on carboxymethylcellulose (CMC) agar media were selected for enzyme production by utilizing carbon sources such as CMC, leaves and paper. Biochemical analysis results indicated a possibility of the isolates being *Pseudomonas fluorescens biovar* and *Streptococcus sp*.

Cellulase produced from the symbiotic effect of the obtained microorganisms was found to be exponentially more potent than the enzymes produced by them separately.

The enzyme activity on using CMC as carbon source was noted as 0.55 U/m. The enzyme activity obtained from using leaves and paper as carbon source was slightly less.

The enzyme activity of the purified protein was found to show an seven-fold increase. The purified protein showed good activity within a pH range of 2-12, with an optimum ph of 6.5. The enzyme activity was maintained with negligible variation within a temperature range of 4°C to 43°C. High salinity did not affect the efficiency of the enzyme either.

The enzyme seems to possess good potential for use in the paper, textile and medicine industry.

Keywords: Carboxymethylcellulose (CMC) Agar; Cellulolytic bacteria; Cellulase; Enzymatic Activity; Protein purification; Synergistic effect.

Dedication

Dedicated to

My parents, without whose love and support I could never have dreamed of embarking on my quest for knowledge

My brother, for providing me mental and technical assistance throughout the project

My respected teachers, without whose guidance and support I could never have come this far

And my family and friends, for always encouraging me to proceed further

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Table of Contents

Declaration .... **II**

Approval .... **III**

Abstract .... **IV**

Dedication .... **V**

Acknowledgement .... **VI**

Table of Contents .... **VII**

List of Tables .... **X**

List of Figures .... **XI**

List of Acronyms .... **XIII**

Chapter 1 Introduction **1**

1.1 Cellulose 2

1.2 Cellulase 4

1.3 Common sources of Cellulase and its Usage 6

1.4 Usage of bacteria as cellulase producers 8

1.5 Symbiotic effect and Antagonistic effect 9

Chapter 2 Materials and Methods **10**

2.1 Collection of Samples and Identification of Cellulolytic Bacteria 10

2.3 Enzyme production and extraction 11

2.4 Enzyme Assay 11

2.5 Protein Estimation 13

2.6 Optimization of Enzyme production 14

2.7 Enzyme production and extraction from Plant sources 15

2.8 Enzyme production and extraction from Paper sources 15

2.9 Protein Purification 16

2.9.1 Ammonium Sulphate Precipitation 16

2.9.2 Protein Dialysis 16

2.10 Protein Characterisation 17

2.10.1 Protein Size 17

2.10.2 Temperature Curve 17

2.10.3 Salinity Curve 18

2.10.4 pH Curve: 18

2.11 DNA Extraction 18

2.12 DNA Quantification 18

2.13 DNA Quality Testing 19

Chapter 3 Results 20

3.1 Identification of Cellulolytic Bacteria 20

3.2 Biochemical Analysis 21

3.3 Enzyme production and extraction 25

3.4 Enzyme Assay 25
3.5 Protein Estimation 27
3.6 Enzyme Optimization 29
3.7 Enzyme production and extraction from Plant sources 33
3.8 Enzyme production and extraction from Paper sources 33
3.9 Protein Purification 34
3.9.1 Ammonium Sulphate Precipitation 34
3.9.2 Protein Dialysis 34
3.10 Protein Characterisation 34
3.10.1 Protein Size 34
3.10.2 Temperature Curve 35
3.10.3 Salinity Curve 36
3.10.4 pH Curve 37
3.11 DNA Quantification 38
3.12 DNA Quality Testing 38
Chapter 4 Discussion 39
Chapter 5 Conclusion 46
References 48

List of Tables

Table 1 *: Results of Biochemical Analysis* 21

Table 2 *: Difference in Enzyme Activity based on Incubation Temperature* 26

Table 3 *: Difference in Enzyme Activity based on Organism* 29

Table 4 *: Difference in Enzyme Activity based on interval of Time* 29

Table 5 *: Difference in Enzyme Activity based on Temperature* 30

Table 6 *: Difference in Enzyme Activity based on pH* 30

Table 7 *: Difference in Enzyme Activity based on Extraction Temperature* 31

Table 8 *: Difference in Enzyme Activity based on Concentration of Carbon Source* 32

Table 9 *: Variation of enzyme activity based on amount of leaf used* 33

Table 10 *: Variation of enzyme activity based on amount of paper used* 33

Table 11 *: Variation of enzyme activity based on temperature* 36

Table 12 *: Variation of enzyme activity based on salinity* 36

Table 13 *: Variation of enzyme activity based on pH* 37

List of Figures

Figure 1 *: Structural representation of cellulose* 3

Figure 2 *: Ribbon representation of Cellulase Structure* 4

Figure 3 *: Chemical representation of Cellulase Structure* 4

Figure 4 *: Mechanism of action of Cellulase* 5

Figure 5 *: Colonies showing clear zones after flooding with Gram's Iodine* 20

Figure 6 *: Colonies showing no zones after flooding with Gram's Iodine* 20

Figure 7 *: Left: Colonies demonstrating positive results for FPase test; Right: Colonies demonstrating negative results for FPase test* 20

Figure 8 *: Gram Staining (a) Strain A and (b) Strain B* 22

Figure 9 *: Phenol Red Carbohydrate Broth (a) positive result and (b) negative result* 22

Figure 10 *: Indole Test (a) positive result and (b) negative result* 23

Figure 11 *: Vogues- Proskauer Test (a) positive result and (b) negative result* 23

Figure 12 *: Citrate Test (a) positive result and (b) negative result* 23

Figure 13 *: Catalase Test (a) positive control, (b) positive result and (c) negative result* 24

Figure 14 *: Oxidase Test - positive results* 24

Figure 15 *: Starch Hydrolysis Test (a) negative result and (b) positive result* 24

Figure 16 *: Motility Indole Urease Test (a) motile and (b) non-motile* 24

Figure 17 *: Standard Curve of Glucose Liberation Assay* 25

Figure 18 *: Liberation of glucose by Bacterial Enzyme: Left: Positive result; Right: Blank* . 26

Figure 19 *: Standard Curve of Biuret's Assay* 27

Figure 20 *: Protein Concentration by Biuret's Assay: Left: Negative Result; Right: Posit*ive result 28

Figure 21 *: Protein Band Size* 35

List of Acronyms

BSA	Bovine Serum Albumin
CMC	Carboxymethylcellulose
DNA	Deoxyribonucleic Acid
DNS	Dinitrosalicylic Acid
mL	Milliliter
RNA	Ribonucleic Acid
SDS	Sodium Dodecyl Sulphate

Chapter 1

Introduction

From almost the dawn of civilization enzymes have been inadvertently, though incognizantly, used in the production of various wines or cheeses (Minussi et al., 2002). However, the first known discovery of enzymes (Philips et al.,) was in the year 1833 by Anselme Payen (Payne et al., 2015). Nonetheless, it was not until the year 1914 that enzymes were isolated and extracted for industrial purposes. The 1960's saw a flourish in the production of microbial enzymes. Since then, a revolution has taken place and more and more industries have been incorporating the use of enzymes (Kuhad & Singh, 1993). An enzyme that has been increasingly used is cellulase, which acts as a catalyst to catabolize cellulosic materials.

About 6 decades prior, in the year 1957, Trichoderma reesei was isolated by Mandels and Reese for its massive ability to produce extracellular cellulases (Zhang et al., 2013). Back then, the main purpose in mind was to catalyse the production of fuel from the lignocellulosic mass. Nevertheless, cellulases soon found other purposes such as being used in detergents, wines (Baker et al., 1996) and other foods (Minussi et al., 2002). Textile industries and paper industries also took up the use of cellulases to decrease chemical usage and for economical profits. Furthermore, new discoveries claim cellulase to be an effective antibiotic against biofilms of Pseudomonas sp. origin.

This resulted in a mass worldwide demand for cellulases. In order to meet demands, researchers all over the world have painstakingly worked to extract cellulases from various sources. Fungal sources were viewed as more easily accessible (Wang et al., 2010).

Nonetheless, in order to reduce costs and accrue more easily accessible cellulases, research turned to utilising bacteria as enzyme sources (Wang et al., 2010). Extraction of cellulases from bacteria had long been a far-fetched dream for the scientific community. However, the advent of new techniques made it possible in recent years to extract bacterial cellulase (Moraïs et al., 2010). The methods and techniques are being researched every day and being improved world-wide (Bailey et al., 1992).

In Bangladesh, cellulases, which are rather expensive, have to be imported for use in various industries. This results in increased prices for the goods produced. However, it is not impossible to utilise bacteria to produce cellulase in our very own country (Mehadi et al., 2013). This would result in a drastic reduction of expenses and help make our industries self-sufficient.

Hence, this study aims to extract cellulases from bacterial sources.

With that thought in mind, a brief about the substrate, cellulose and the enzyme cellulose, is given below.

1.1 Cellulose

Cellulose is an insoluble straight chain polysaccharide. The structure is composed of glucose monomers linked by beta-1,4-glycosidic bonds. Cellulose can be represented by the formula $(C_6H_{10}O_5)_n$. The chain can be formed of several hundred to many thousands of $\beta(1\rightarrow4)$ linked D-glucose units arranged in a linear chain structure (O'Sullivan, 1997).

Cellulose synthase, an enzyme, helps in the formation of cellulose. This enzyme can be found in organisms ranging from bacteria to higher plants.

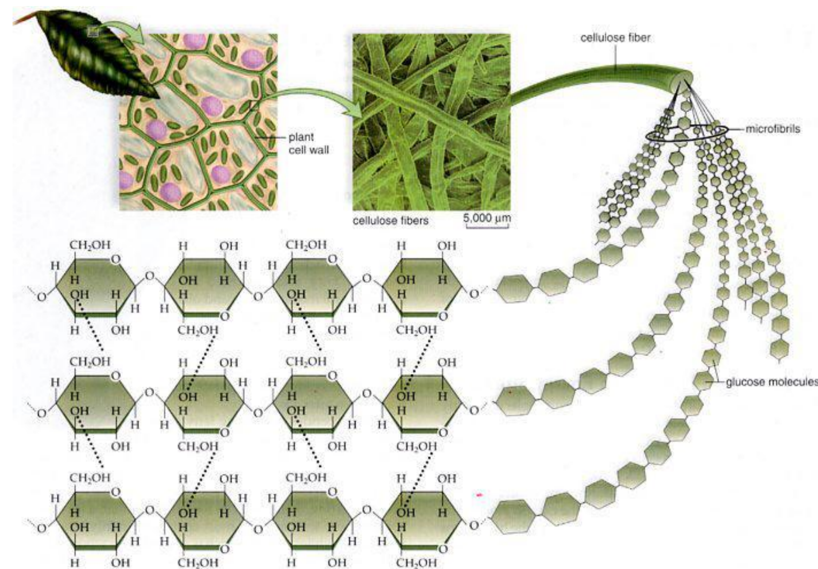


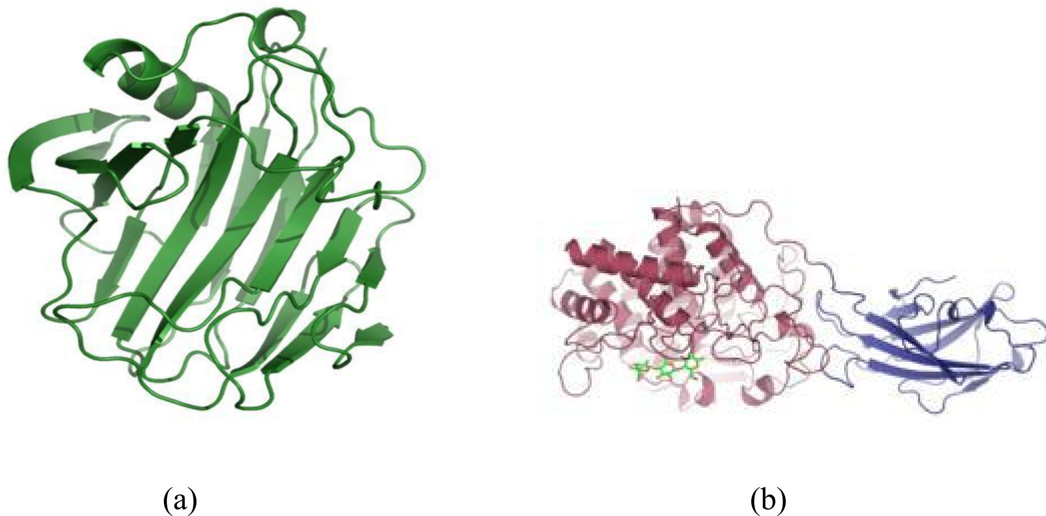
Figure 1 : Structural representation of cellulose

Cellulose has abundant purposes in nature as well as in industries (Moraïs et al., 2010). Cellulose is thought to be the most abundant organic compound on earth. The structural base of plants is formed from cellulose which, in turn, forms the source of energy for herbivores. In humans, cellulose forms the bulk of the diet and aids in the smooth working of the intestines. Moreover, it serves as a raw material in various industries such as paper industries, textile industries. Cellulose derivatives also find uses in the manufacturing of explosives, plastics and films (Minussi et al., 2002).

The structural bonds of cellulose can be broken down using the enzyme cellulase. The final product of cellulose hydrolysis by cellulase enzyme is usually glucose.

1.2 Cellulase

Cellulase is used to refer to a class of enzymes that have the ability to hydrolyzese cellulose. It breaks down complex cellulosic material into monosaccharides or into shorter polysaccharides and oligosaccharides. The molecular formula of cellulase is stated to be $C_{18}H_{32}O_{16}$.



(a) (b)

Figure 2 : Ribbon representation of Cellulase Structure

(a)Catalytic domain of beta-1,4-endoglucanase produced by Streptomyces lividans (b) Representation of Cellulase produced by Thermomonospora fusca

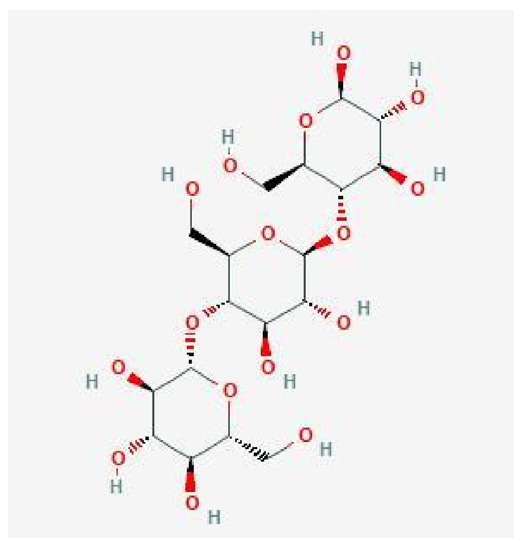


Figure 3 : Chemical representation of Cellulase Structure

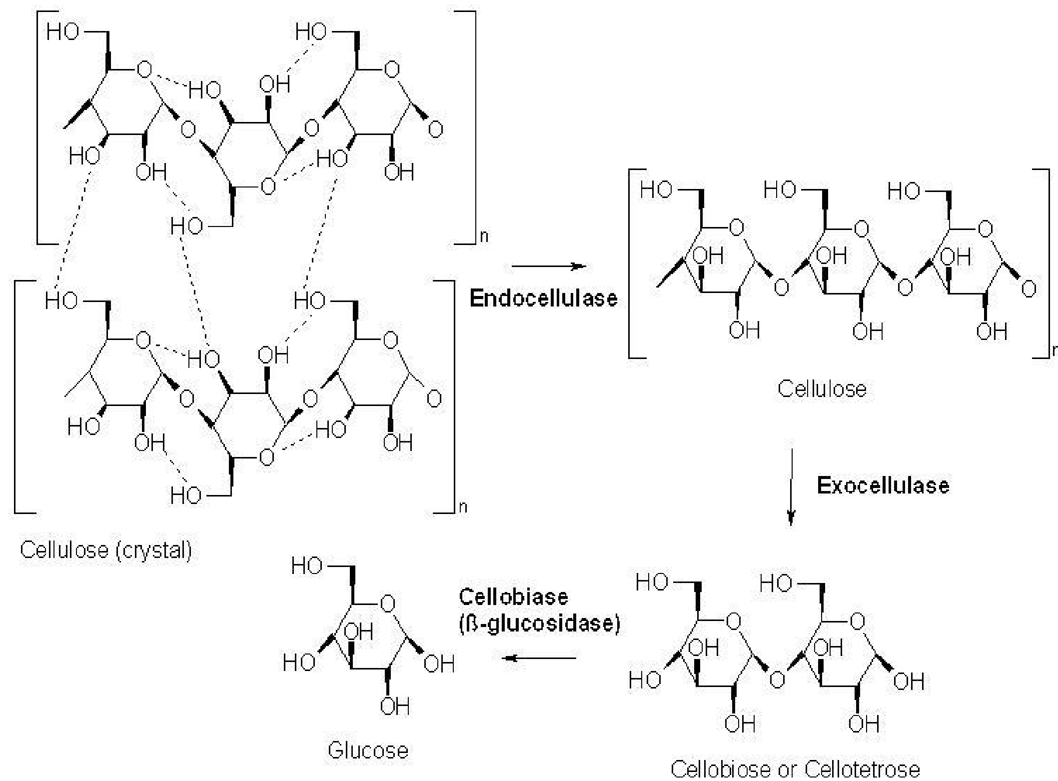


Figure 4 : Mechanism of action of Cellulase

Cellulases can catalyze different types of reactions: (a) Endocellulase activity (b) Exocellulase activity (c) Cellobiose activity - formation of glucose (beta-glucosidase) units

Based on activity and the type of reaction they catalyse, cellulases can be classified into five types:

- Endocellulases: They randomly cleave the cellulose at random sites and create new chain ends.
- Exocellulases: They are also referred to as cellobiohydrolases. They cleave two to four units at the chain end, from where the endocellulase has cleaved. Tetrasaccharides or disaccharides are usually formed.
- Cellobiases: Also referred to as β-glucosidases, they form monosaccharides by cleaving the products left behind by the action of exocellulase.

- Cellulose Phosphorylase: They use phosphates to form monomers, instead of hydrolysing.
- Oxidative Cellulases: They form monomers by using radical reactions.

Every enzyme that has been studied has an Enzyme Commission Number. Based on the chemical reactions catalysed by an enzyme, they are numerically classified. This number is unique to every enzyme and is termed as Enzyme Commission Number (EC number). The EC number of cellulase is 3.2.1.4.

1.3 Common sources of Cellulase and its Usage

The enzymes are mostly produced by fungi, bacteria and protozoans. Although cellulases in the industry are almost exclusively isolated from fungi (Zhang et al., 2013) such as *Trichoderma sp.* or *Aspergillus sp.,* bacteria are viewed as potential mini-factories for production of cellulase. *Bacillus sp., Pseudomonas sp., Serratia sp.*, etc are usually good producers of cellulase. Some plants such as *Cuscuta reflexa* have also at times been used as sources of cellulase. However, extraction of cellulase from fungal and plant sources can be pretty expensive, which leads to the incorporation of bacteria as cellulase producers.

Naturally, industries that require cellulose as raw material would also require cellulase enzyme for easier preparation of raw material. Thus, cellulase also finds usage in various industries (Singh et al., 2013) such as textile industry, paper industry, food industry, animal feeds industry, etc.

In the paper industry, using cellulase reduces costs, provides improved strength properties (Ko et al., 2010), reduces fibre coarseness, enhances bleachability, increases drinkability and provides brightness to the paper produced. (Hakamada et al., 1997)

In textile industries cellulases are used to provide enhanced biopolishing, reduced fibre damage, improved fibre strength, improved water absorbance and softness and accentuates appearance (Moreira et al., 2016).

Production of bioethanol (Kuhad et al., 2010) cannot proceed effectively without first being treated by cellulases to soften the fibres for better fermentation. Microorganism is able to better metabolize the celluloses when they have been bio-converted to smaller molecules.

Cellulases are being increasingly used in wine and beer production for enhanced quality and stability. Filtration, maceration and extraction of colour and aroma are enhanced by the use of cellulase.

In the food industry, cellulases can be utilised to provide better juice yield from fruits and various vegetables. The texture and stability are improved for the extracts. Olive oil extraction may also be improved by utilising a mixture of hemicellulase, pectinase and cellulase. (Carvalho et al., 2008)

Cellulase is used in detergents in order to enhance cleaning capabilities of the detergent. Detergents containing cellulases can improve the feel and brightness of the fabrics after washing. Discoloration due to harsh chemicals can be prevented. Cellulases can provide

glossier and better-maintained fabric. Using cellulase ensures that grass stains and various such stains are easily removed from clothes during washing.

Cellulases can enrich the feed value of animal feeds. The cellulases can effectively hydrolyze the cellulosic material and can thus improve overall nutritional absorption, consequently increasing overall animal health.

1.4 Usage of bacteria as cellulase producers

Bacteria that can produce cellulase in order to break down cellulose are called cellulolytic bacteria (Sethi et al., 2013.)Bacteria previously discovered to be cellulolytic include *Bacillus sp.*, *Pseudomonas sp.* and *Serratia sp.* These bacteria can be isolated from sources such as soil, municipal solid wastes and rice straw wastes. Although mammals do not produce the enzyme cellulase, ruminants usually have a symbiotic relationship with microbes that produce the enzyme. This way, ruminants can utilize energy from the cellulose that they consume. Thus, cellulolytic bacteria can also be isolated from the refuse of ruminants (Singh et al., 2013).

Using bacteria as producers of cellulases would be of a major advantage since bacteria can virtually be grown almost anywhere and require very little care or upbringing. (Kim et al., 2012)Extraction of bacterial enzyme is also less expensive (Hakamada et al., 1997)and the process is a lot easier. Moreover, genetic enhancement of bacteria is much easier than genetically modifying multi-cellular species (Ahmed et al., 2016). Furthermore, cellulases produced by bacteria are often more effective catalysts due to being less inhibited by the presence of hydrolyzed carbon sources. (Ito et al., 1998) Extraction of cellulase from bacteria

would also alleviate the cost to a large extent, thus making the enzyme more accessible and cost-effective. (Ito, 1997)

1.5 Symbiotic effect and Antagonistic effect

Two organisms that enhance the effect of each other are said to be symbiotic with each other. The effect produced in such cases is greater than the result of a normal addition of results or effects. Such organisms are able to boost each other up and produce a final product that is intrinsically better than that produced by the organisms alone (Panchapakesan et al., 2016).

In the event that an organism produces secondary metabolites that hinder or inhibit the growth of any competing organisms, the displayed effect is termed as antagonistic effect (Chorostowska-Wynimko et al., 2001

Chapter 2

Materials and Methods

2.1 Collection of Samples and Identification of Cellulolytic Bacteria

Samples of a few types of soil, cow dung and compost were collected from various locations around Dhaka City.

Samples that were serially diluted up to a dilution of 10^{-5} times were plated on sterile petri dishes containing Carboxymethylcellulose (CMC) agar media. The cultured plates were kept in the incubator at temperatures of 28°C, 30°C, 35°C and 37°C respectively for 24-30 hours. The cultured plates showed growth at a temperature of 37°C. The plates were then flooded with Gram's Iodine in order to identify cellulolytic colonies that showed clear haloes after a duration of 4-5 minutes. The identifiably cellulolytic colonies were then streaked on fresh plates from replica plates.

The isolated cultures were cultured in Luria Bertani broth and CMC both respectively and a copy of each was stored in 30% glycerol as stock culture. The stock cultures were stored at a temperature of - 20 °C.

2.2 Biochemical Analysis

Biochemical analysis (refer to Appendix B) was carried out in order to putatively identify the strains of cellulolytic microbes obtained. The corresponding characteristics displayed by the microbes are charted in the results sub-section.

2.3 Enzyme production and extraction

Flasks containing 1% CMC broth were inoculated with a loopful each of the respective samples. The flasks were kept in a shaker incubator for 36 hours at a temperature of 37°C and 150 rpm.

After the incubation period was over, 40 ml of culture was taken from each sample into fresh centrifuge tubes and were then centrifuged for 20 minutes at 4°C and 11,000 rpm. The supernatant thus obtained was collected into fresh centrifuge tubes and kept for use as crude enzyme.

2.4 Enzyme Assay

The obtained crude enzyme was assayed on its ability to liberate glucose after acting on cellulose as a substrate. Upon acting on the cellulose, the crude cellulase would hydrolyse it into smaller compounds, resulting in the liberation of glucose in the reaction medium.

Dinitrosalicylic Acid (DNSA) was used as an agent to analyze the concentration of glucose liberated during the enzyme-substrate reaction. DNSA reacts with glucose present in the reaction medium and gives color ranging from yellowish-orange to dark red, depending on the concentration of liberated glucose in the reaction medium.

The obtained results can be considered conclusive considering DNSA does not react with complex polysaccharides such as CMC, resulting in yellow color after the completion of the assay.

In order to obtain the standard curve for the absorbance values obtained from the DNSA-Glucose reaction, samples ranging in glucose concentrations of 0.1 mg/ mL to 1.0 mg/mL

with a sequential increase of 0.1 mg/mL were assayed with DNSA. The absorbance values were recorded and charted on a graph paper in order to obtain the standard curve.

The reaction media containing the DNSA-Glucose reaction were tested for enzymatic activity.

2 mL of crude enzyme was tested with each of the following as substrates respectively:

- 1 mL of 1% CMC
- 1 ml of 1% Avicel suspension
- 5 mm X 5 mm strip of filter paper soaked in 1 mL of distilled water

and gently vortexed in order to ensure proper and uniform mixing of the substrate and enzyme. The test tubes were then allowed to incubate in a shaker incubator at 37°C for 60 minutes. After that, 1mL of DNSA was added to stop the enzyme-substrate reactions. Test tubes containing the respective substrates, with 2 mL of autoclaved distilled water in place of the crude enzyme were used as blanks. 1mL of DNS was added to the blank reaction tubes. The test tubes were then heated in a water bath at 100°C for 10 minutes and then cooled down to room temperature.

The absorbance of the samples were taken by spectrophotometer at a wavelength of 540 nm.

The obtained absorbance values were checked against the obtained standard curve in order to determine the concentration of liberated glucose due to the action of the crude enzyme.

The formula used for measuring enzyme activity was:

$$\text{Enzyme Activity} = \frac{\text{Product Concentration X 1000 X Dilution factor}}{\text{Molecular weight of glucose X Time of Incubation in Minutes}}$$

2.5 Protein Estimation

Biuret's method was utilised in order to estimate the concentration of protein (enzyme) in the crude enzyme/supernatant.

The method can be used to estimate the concentration of polypeptides containing two or more peptide bonds (tripeptides or higher).

Biuret's test is based on the perceived changes in color upon the reaction of the copper sulfate with the protein in alkaline condition. The intensity of color increases upon increase in concentration of protein in the solution. The color changes from blue to mauve for high concentrations of protein.

Since bonds are formed with the same frequency per amino acid, an idea of the concentration of the protein can be formed from the intensity of colour.

Bovine Serum Albumin (BSA) solutions with sequentially increasing concentrations ranging from 0.1 mg/ mL to 1.0 mg/mL (with a sequential increase in 0.1 mg/mL) were measured at a wavelength of 650 nm. The recorded absorbances were plotted to form a standard curve.

In order to test the samples, 1 mL of crude enzyme was added to a test tube containing 4 mL of biuret's reagent. A test tube containing 4 mL of Biuret's reagent and 1 mL of autoclaved distilled water was used as blank. The test tubes were then kept in a dark room for development of colour for about 20 minutes. The test tubes were retrieved and the absorbances of the samples were taken at a wavelength of 650 nm.

Specific enzyme activity is estimated using estimated protein concentration values. It can be inferred by using the following formula:

$$\text{Specific Enzyme Activity} = \frac{\text{Enzyme Activity}}{\text{Protein Concentration}}$$

2.6 Optimization of Enzyme production

The conditions for enzyme production were optimised by testing the enzyme production based on variation of:

- time
- temperature
- pH
- concentration of carbon source

The activity of the enzyme produced was tested by obtaining crude enzymes at different time intervals to confirm the duration of incubation that produced the best results. The enzyme extracts were obtained at intervals of 24 hours, 48 hours, 72 hours, 96 hours, 120 hours, 144 hours and 168 hours.

Enzyme production was also tested at different temperatures to check which incubation temperature gave the best results. The enzyme extracts were obtained from cultures incubated at temperatures of 30°C, 35°C and 37°C.

The enzyme production was also tested at different pH levels of the culture media to check which pH produced the best results. The enzyme extracts were obtained at pH 5.0, pH 7.0, pH 9.0 and pH 11.0.

Another important factor that was taken into consideration was the effects of increasing the concentration of carbon source in the media. The variations tested were at concentrations of 1%, 2% and 3% of carboxymethylcellulose.

2.7 Enzyme production and extraction from Plant sources

Flasks containing leaf samples in salt broth were inoculated and incubated in a shaker incubator at 37°C and 130 rpm. After 15 days, the leaf was found to have been completely degraded.

The mixture was then centrifuged at 11,000 rpm for about 20 minutes. The obtained supernatant was used as crude enzyme and the enzyme activity was noted using DNSA assay.

2.8 Enzyme production and extraction from Paper sources

Flasks containing paper samples in salt broth were inoculated and incubated in a shaker incubator at 37°C and 130 rpm. After 25 days, the paper was found to have been completely degraded.

The mixture was then centrifuged at 11,000 rpm for about 20 minutes. The obtained supernatant was used as crude enzyme and the enzyme activity was noted using DNSA assay.

2.9 Protein Purification

2.9.1 Ammonium Sulphate Precipitation

The obtained supernatant, which serves as the crude enzyme, was first precipitated with 45% Ammonium Sulphate salt.

The beaker containing the crude enzyme was placed in a larger beaker and the whole setup was placed on a magnetic stirrer. The salt was gently stirred into the crude enzyme by using a small spatula in order to avoid formation of bubbles. After mixing the salt in, the whole setup was left at 4°C overnight in order to let the protein precipitate.

The next day, the mixture was centrifuged at a speed of 13,000 rpm for 20 minutes, allowing the protein to precipitate and get separated from the mixture. The protein was then collected into a separate tube and dialysed.

2.9.2 Protein Dialysis

The precipitated protein was dialysed using a salt buffer. SnakeskinTM Dialysis Tubing, 10K MWCO, 22 mm Inner Diameter was used to dialyse the concentrated protein obtained after Ammonium Sulphate Dialysis. A 10 cm long membrane tube, with an extra 2.5 cm at each end to form the knots, was about 20 % filled with the concentrated protein. The tube was then soaked in the buffer for 12 hours on a magnetic stirrer on low speed. The buffer was changed every two hours. After 12 hours, the buffer was changed one final time and the setup was stored at 4°C overnight.

The next day, the membrane was found to be fully taut and the dialysis was completed.

The concentrated protein was taken out of the dialysis tubing and stored for further use at 4°C.

2.10 Protein Characterisation

2.10.1 Protein Size

SDS-PAGE was performed in order to know the size of the protein.

The protein was mixed with an equal amount of Laemmli buffer and heat shock was applied by heating the protein at 95°C for five minutes.

The raw proteins obtained by varying the carbon sources (CMC, Leaf, Filter Paper) as well as the concentrated proteins were run for SDS-PAGE.

A polyacrylamide gel matrix composed of 10% SDS was used to run the experiment.

The gel was run at 70 volts for 60 minutes. The protein was then compared to the ladder to know the size.

Eco-mini by Analytik Gena was used to perform the SDS-PAGE.

2.10.2 Temperature Curve

A range of temperatures were tested in order to determine the optimum range of pH that the enzyme could function in.

The temperatures tested were:

- 4°C
- 30°C
- 37°C
- 40°C
- 45°C

2.10.3 Salinity Curve

A range of salinities were tested in order to determine the optimum range of pH that the enzyme could function in.

The saline concentrations tested were from 0.1% to 1%, with gradual increments of 0.1%.

2.10.4 pH Curve:

In order to find out the working pH range of the enzyme, the pH of the enzyme was adjusted from pH 2 to pH 13 by gradual increments of 1 sequentially. Hydrochloric Acid was used to decrease the pH, while, Sodium Hydroxide was used to increase the pH.

2.11 DNA Extraction

The DNA was extracted using Boiling method and Phenol-Chloroform method.

2.12 DNA Quantification

The absorbance of the extracted bacterial DNA was quantified by measuring the absorbance of the sample at a wavelength of 260 nm.

20 μL of extracted DNA was mixed with 1980 μL of Tris-EDTA (TE) buffer. The mixture was then transferred to a cuvette and the absorbance at a wavelength of 260 nm was noted.

The quantity of DNA was calculated using the formula:

Total DNA (ug) concentration = 50 μg/mL × OD_{260} × dilution factor

The working DNA solution was then diluted to a concentration of 50 μg/mL

2.13 DNA Quality Testing

The quality of the extracted DNA was tested by calculating the ratio between the absorbances of the DNA at wavelengths of 260 nm and 280 nm.

20 μL of extracted DNA was mixed with 1980 μL of Tris-EDTA (TE) buffer. The mixture was then transferred to a cuvette and the absorbance at a wavelength of 260 nm was noted. Next, the absorbance of the sample at a wavelength of 280 nm was also noted.

The ratio of A260/A280 was calculated.

Chapter 3

Results

3.1 Identification of Cellulolytic Bacteria

The colonies showing clear zones were identified and selected for further enzyme production purposes. The colonies that showed no clear zones were discarded.

Figure 5 : Colonies showing clear zones after flooding with Gram's Iodine

Figure 6: Colonies showing no zones after flooding with Gram's Iodine

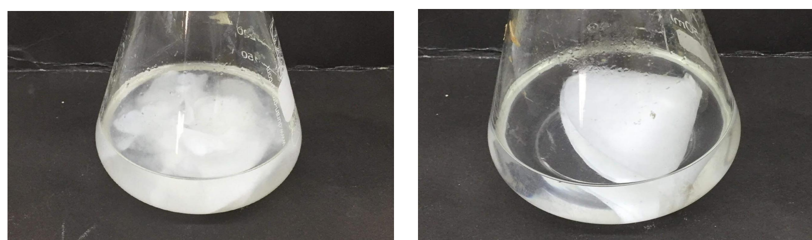


Figure 7: Left: Colonies demonstrating positive results for FPase test; Right: Colonies demonstrating negative results for FPase test

3.2 Biochemical Analysis

Results obtained after performing biochemical analysis are charted below:

Test	Bacteria A	Bacteria B
Bacterial Shape	Rods	Cocci
Gram Strain	Negative	Positive
Glucose utilization	Positive	Positive
Sucrose utilization	Positive	Positive
Lactose utilization	Negative	Positive
Mannitol utilization	Positive	Negative
Indole	Negative	Negative
Methyl Red	Negative	Positive
Vogues Proskauer	Negative	Negative
Citrate	Positive	Negative
Hydrogen Sulphide	Negative	Negative
Catalase	Positive	Negative
Oxidase	Positive	Negative
Starch Hydrolysis	Positive	Positive
Urease	Negative	Negative
Motility Test	Non-Motile	Non-Motile

Table 1: Results of Biochemical Analysis

The biochemical analysis suggests that the two strains of bacteria possibly are *Pseudomonas fluorescens biovar* and *Streptococcus sp.*

Gram Staining

Of the two isolated bacteria, one tested negative during gram staining and appeared rod-shaped, whereas, the second tested positive and appeared to have streptococcal shape. appears to have.

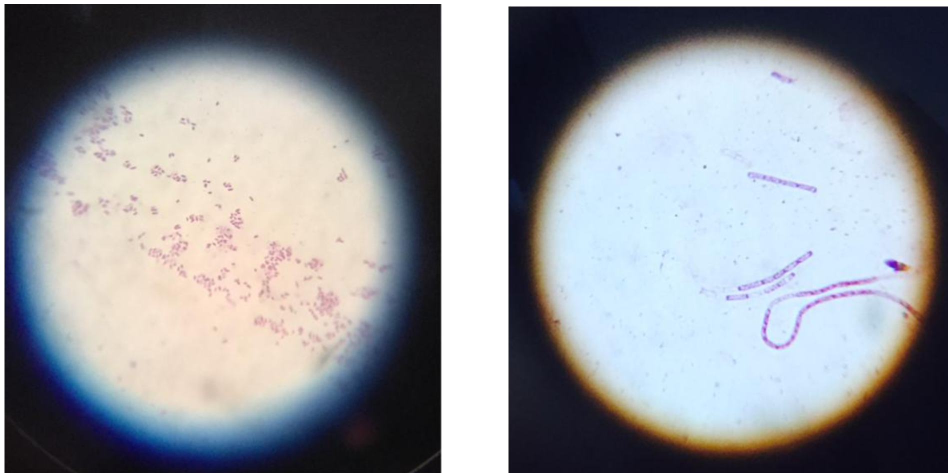


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Figure 8: Gram Staining (a) Strain A and (b) Strain B

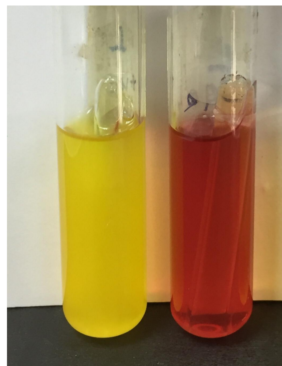

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Figure 9: Phenol Red Carbohydrate Broth (a) positive result and (b) negative result

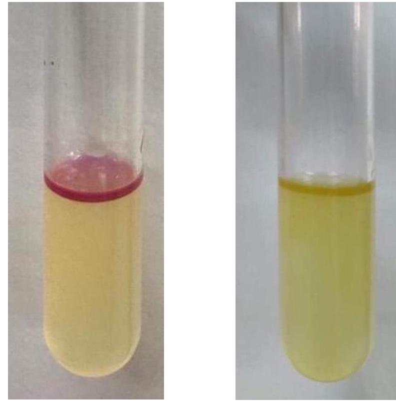

Figure 10: Indole Test (a) positive result and (b) negative result

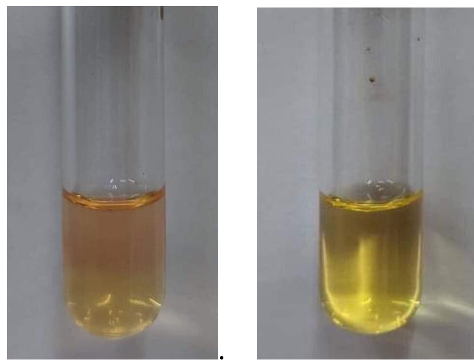

Figure 11: Vogues- Proskauer Test (a) positive result and (b) negative result

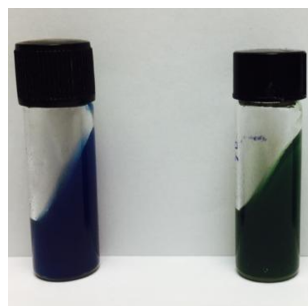

Figure 12: Citrate Test (a) positive result and (b) negative result

Figure 13: Catalase Test (a) positive control, (b) positive result and (c) negative result

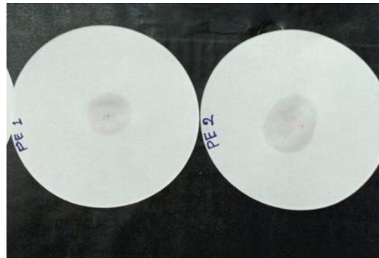

Figure 14: Oxidase Test - positive results

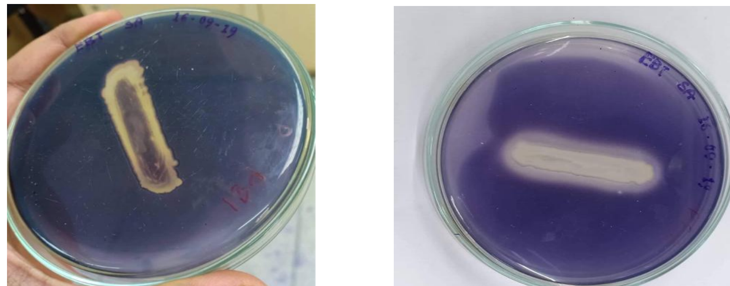

Figure 15: Starch Hydrolysis Test (a) negative result and (b) positive result

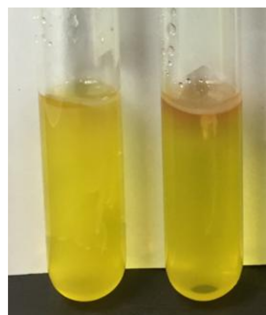

Figure 16: Motility Indole Urease Test (a) motile and (b) non-motile

3.3 Enzyme production and extraction

The supernatant obtained after centrifugation of the 36 hour liquid cultures was used as crude enzyme.

3.4 Enzyme Assay

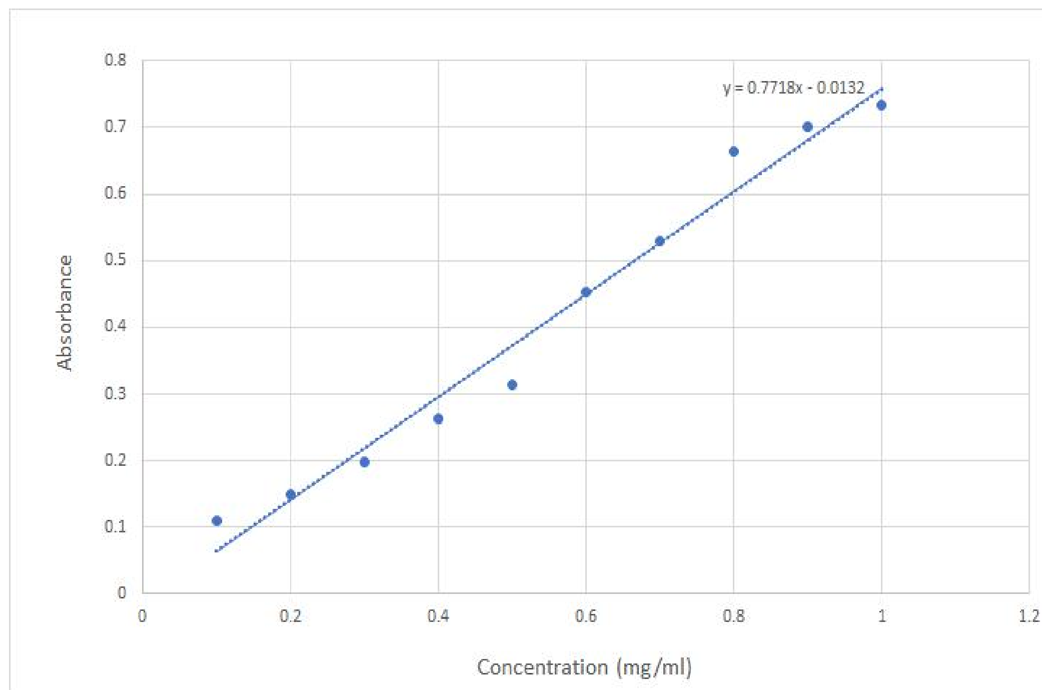

Figure 17: Standard Curve of Glucose Liberation Assay

Regression coefficient, r = 0.987

Hence, $R^2 = 0.974$

The activity of the enzyme showed an increase with the increase in temperature. However, increasing the temperature above 43 °C showed the activity deteriorating.

Results below show the variation of enzyme activity according to the incubation temperature: for enzyme and substrate.

Enzyme tested with substrate:	Concentration of Glucose Liberated	Enzyme Activity (U/mL)
Avicel	0.54 mg/mL	0.313 U/mL
Filter Paper	0.83 mg/mL	0.537 U/mL
Carboxymethylcellulose	0.85 mg/mL	0.55 U/mL

Table 2: Difference in Enzyme Activity based on Incubation Temperature

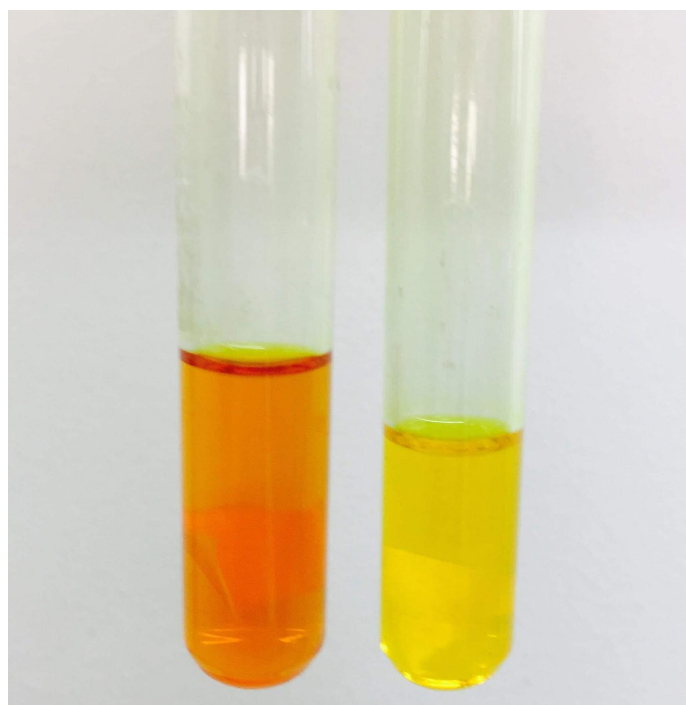

Figure 18: Liberation of glucose by Bacterial Enzyme: Left: Positive result; Right: Blank

3.5 Protein Estimation

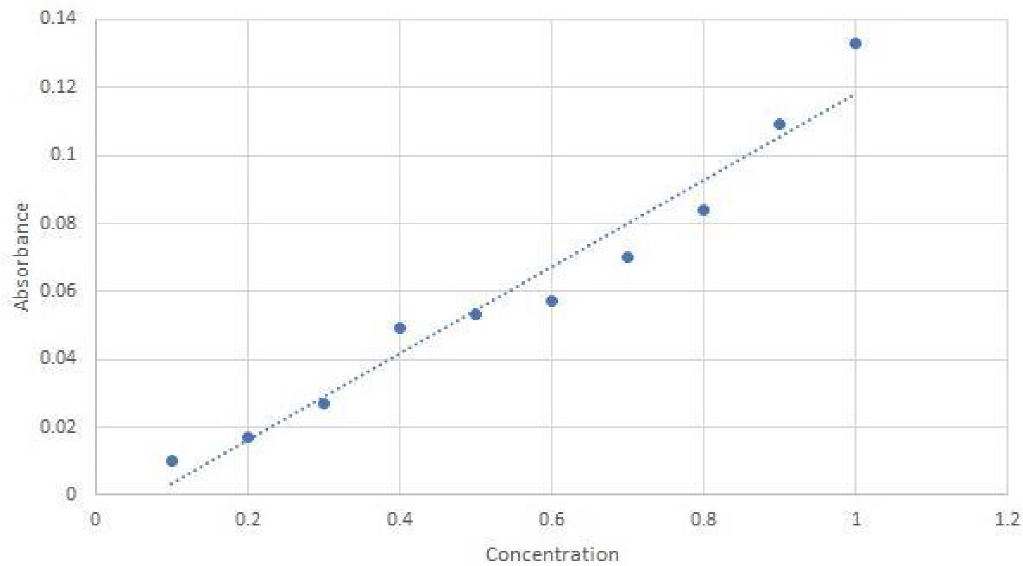

Figure 19: Standard Curve of Biuret's Assay

Regression coefficient, r = 0.978

Hence, $R^2 = 0.956$

Estimating protein concentration is necessary to find out specific activity of the enzyme.

However, no result could be obtained after conducting biuret's assay on the protein sample. This might be due to the absence of cysteine residues forming disulphide bonds.

*Figure 20: Protein Concentration by Biuret's Assay: Left: Negative Result; Right: Posit*ive result

3.6 Enzyme Optimization

The obtained strains of bacteria were all tested for enzyme production. However, the best results were obtained from the symbiotic effect of two strains of bacteria isolated from a soil sample isolated from the area near Hazaribagh tannery. Hence only those isolates were selected for further enzyme production.

Bacterial source of Enzyme	Concentration of Glucose Liberated	Enzyme Activity (U/mL)
Strain A	0.28 mg/mL	0.26 U/mL
Strain B.	0.31 mg/mL	0.27 U/mL
Strain A + Strain B	0.85 mg/mL	0.55 U/ml

Table 3: Difference in Enzyme Activity based on Organism

The enzyme activity was tested at intervals of 24 hours, 48 hours, 72 hours, 96 hours, 120 hours, 144 hours and 168 hours. The best activity was obtained after a duration of 168 hours.

Duration	Concentration of Glucose Liberated	Enzyme Activity (U/mL)
24 hours	0.54 mg/mL	0.313 U/mL
48 hours	0.825 mg/mL	0.53U/mL
72 hours	0.83 mg/mL	0.537 U/mL
96 hours	0.83 mg/mL	0.537 U/mL
120 hours	0.84 mg/mL	0.545 U/mL
144 hours	0.84 mg/mL	0.545 U/mL
168 hours	0.85 mg/mL	0.55 U/mL

Table 4: Difference in Enzyme Activity based on interval of Time

The results below show the variation of enzyme activity according to the incubation temperature of the bacteria:

Enzyme tested at temperature:	Concentration of Glucose Liberated	Enzyme Activity (U/mL)
30 °C	0.4 mg/mL	0.258 U/mL
35 °C	0.76 mg/mL	0.537 U/mL
37 °C	0.85 mg/mL	0.55 U/mL

Table 5: Difference in Enzyme Activity based on Temperature

The results obtained due to different pH of the broth used during incubation are summarised in the following table:

pH of Enzyme	Concentration of Glucose Liberated	Enzyme Activity (U/mL)
5.0	0.4 mg/mL	0.074 U/mL
6.5	0.85 mg/mL	0.55 U/mL
7.0	0.76 mg/mL	0.537 U/mL
9.0	0.35 mg/mL	0.064 U/mL
11.0	0.2 mg/mL	0.037 U/mL

Table 6: Difference in Enzyme Activity based on pH

Enzyme activity also showed variation depending on temperature during extraction.

Extraction temperature:	**Glucose Liberation**	**Enzyme Activity (U/mL)**
4 °C	0.85 mg/mL	0.55 U/mL
25 °C	0.76 mg/mL	0.537 U/mL

Table 7: Difference in Enzyme Activity based on Extraction Temperature

The results obtained by using different carbon sources for enzyme production are summarised in the following table:

Carbon Source of Enzyme:	**Concentration of Glucose Liberated**	**Enzyme Activity (U/mL)**
Avicel	0.45 mg/mL	0.282 U/mL
Carboxymethylcellulose	0.85 mg/mL	0.55 U/mL
Filter Paper	0.76 mg/mL	0.537 U/mL
Vegetable Waste	0.84 mg/mL	0.545 U/mL

Table 10: Difference in Enzyme Activity based on Carbon Source

The results obtained by using different concentrations of carbon sources for enzyme production are summarised in the following table:

Carbon Source of Enzyme:	**Concentration of Glucose Liberated**	**Enzyme Activity (U/mL)**
1% Carboxymethylcellulose	0.85 mg/mL	0.55 U/mL
2% Carboxymethylcellulose	1.33 mg/mL	0.835 U/mL
3% Carboxymethylcellulose	1.76 mg/mL	0.974 U/mL

Table 8: Difference in Enzyme Activity based on Concentration of Carbon Source

3.7 Enzyme production and extraction from Plant sources

Plant leaves were utilised as a source of carbon and the microbes were able to produce cellulase enzyme which displayed good cellulolytic activity.

Carbon Source of Enzyme:	Concentration of Glucose Liberated	Enzyme Activity (U/mL)
0.5 grams of leaf	0.835 mg/mL	0.54U/mL
1.0 gram of leaf	0.84 mg/mL	0.545 U/mL
1.5 grams of leaf	0.85 mg/mL	0.55 U/mL

Table 9: Variation of enzyme activity based on amount of leaf used

3.8 Enzyme production and extraction from Paper sources

Paper was utilised as a source of carbon and the microbes were able to produce cellulase enzyme which displayed good cellulolytic activity.

Carbon Source of Enzyme:	Concentration of Glucose Liberated	Enzyme Activity (U/mL)
0.5 grams of paper	0.835 mg/mL	0.54U/mL
1.0 gram of paper	0.84 mg/mL	0.545 U/mL
1.5 grams of paper	0.85 mg/mL	0.55 U/mL

Table 10: Variation of enzyme activity based on amount of paper used

3.9 Protein Purification

3.9.1 Ammonium Sulphate Precipitation

A gelatinous mass of concentrated protein was obtained upon precipitation with ammonium sulphate.

3.9.2 Protein Dialysis

The gelatinous mass was purified and a concentrated solution of protein was obtained. The protein thus obtained was sevenfold concentrated and gave much higher efficiency against cellulose.

3.10 Protein Characterisation

3.10.1 Protein Size

After the protein was run for an SDS-PAGE, the band size of the protein was found to be 60 kDa.

Upon concentration, the protein amount did not cross the threshold and thus no band was obtained.

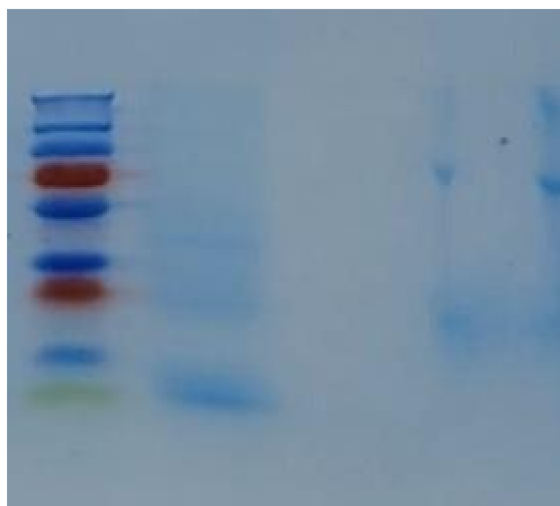

Figure 21: Protein Band Size

3.10.2 Temperature Curve

The enzyme was found to function optimally at 42°C. The results varied slightly when the temperature was reduced or increased slightly. Thus, the protein was found to function well at a temperature range of 4°C to 50°C.

Enzyme tested at temperature:	Concentration of Glucose Liberated	Enzyme Activity (U/mL)
4°C	0.825 mg/mL	0.52 U/mL
25°C	0.825 mg/mL	0.53U/mL
35°C	0.825 mg/mL	0.53U/mL
37°C	0.83 mg/mL	0.537 U/mL
40°C	0.835 mg/mL	0.54U/mL
42 °C	0.85 mg/mL	0.55 U/mL

45 °C	0.84 mg/mL	0.545 U/mL
47 °C	0.84 mg/mL	0.545 U/mL
50 °C	0.84 mg/mL	0.545 U/mL

Table 11: Variation of enzyme activity based on temperature

3.10.3 Salinity Curve

The enzyme seems to function pretty well at elevated salinity. The salinity does not seem to affect the function of the enzyme in any significant way.

Enzyme tested at salinity (mg/mL:	**Concentration of Glucose Liberated**	**Enzyme Activity (U/mL)**
0.1 mg/mL	0.85 mg/mL	0.55 U/mL
0.2 mg/mL	0.85 mg/mL	0.55 U/mL
0.3 mg/mL	0.85 mg/mL	0.55 U/mL
0.4 mg/mL	0.85 mg/mL	0.55 U/mL
0.5 mg/mL	0.85 mg/mL	0.55 U/mL
0.6 mg/mL	0.85 mg/mL	0.55 U/mL
0.7 mg/mL	0.84 mg/mL	0.545 U/mL
0.8 mg/mL	0.845 mg/mL	0.549 U/mL
0.9 mg/mL	0.84 mg/mL	0.545 U/mL
1.0 mg/mL	0.844 mg/mL	0.547 U/mL

Table 12: Variation of enzyme activity based on salinity

3.10.4 pH Curve

Optimal pH condition for the enzyme to carry out its function properly was found to be 6.5.

However the effect of pH variation was not very widely pronounced other than at extreme pH.

Enzyme tested at pH:	Concentration of Glucose Liberated	Enzyme Activity (U/mL)
2	0.415 mg/mL	0.176U/mL
3	0.716 mg/mL	0.476U/mL
4	0.83 mg/mL	0.537 U/mL
5	0.84 mg/mL	0.545 U/mL
6	0.84 mg/mL	0.545 U/mL
6.5	0.85 mg/mL	0.55 U/mL
7	0.84 mg/mL	0.545 U/mL
8	0.84 mg/mL	0.545 U/mL
9	0.83 mg/mL	0.537 U/mL
10	0.714 mg/mL	0.472 U/mL
11	0.716 mg/mL	0.476U/mL
12	0.716 mg/mL	0.476U/mL

Table 13: Variation of enzyme activity based on pH

3.11 DNA Quantification

The absorbance of the extracted bacterial DNA was found to be 0.484 at a wavelength of 260 nm.

Using the formula:

Total DNA (ug) concentration = 50 µg/mL × OD_{260} × dilution factor

The DNA concentration of the extracted DNA sample was found to be 2420 µg/mL.

In order to adjust the concentration of the working DNA solution, the DNA was diluted by a factor of 48.4 times.

3.12 DNA Quality Testing

The absorbance of the extracted bacterial DNA was found to be 0.484 at a wavelength of 260 nm, whereas, the absorbance of the extracted bacterial DNA was found to be 0.283 at a wavelength of 280 nm.

The ratio of the absorbances was found to be 1.71, thus indicating protein contamination.

Chapter 4

Discussion

Numerous strains of cellulolytic bacteria were found abounding in the various samples of municipal wastes, bovine refuse and soil samples. The best results were obtained when the samples were cultured at 37°C for 36 hours.

A few different recipes of CMC agar were tried for bacterial growth. The bacteria did not show growth when the agar concentration used was too high (more than 1.5%). On the other hand, when the agar concentration was brought down to around 1.2% - 1.3%, bacterial growth was observed.

The obtained colonies that were cultured separately were tested for cellulolytic activity using Gram’s Iodine. The colonies which showed clear halos after being flooded with Gram's Iodine proved themselves able to hydrolyse the cellulose in the media. Since iodine is unable to bind with hydrolyzed cellulose due to the absence of substrate, clear zones were only obtained when the organisms could hydrolyse cellulose.

The samples of cow dung were expected to give the best results considering cellulolytic bacteria have a major chance of being present in the guts of ruminating herbivores. However, contrary to expected results, bacterial strains obtained from a soil sample collected from Hazaribagh tannery showed exemplary results.

The colonies were then further tested for their ability to degrade filter paper and utilise it as an energy source. The colonies that were able to utilise filter paper proved themselves to have FPase activity – the ability to degrade filter paper. The microbial strains which could not utilize filter paper were unable to display FPase activity. Thus, they would not be suitable for usage in the paper industry.

The colonies able to utilize both carboxymethylcellulose and filter paper as carbon sources were selected to be used in further enzyme production. Two strains of microbes were eventually observed to give good results and were then identified using biochemical tests. The biochemical analysis suggested that the first strain of bacteria might be Pseudomonas fluorescens. The certain indicators were utilisation of mannitol and sucrose as carbon sources and being positive for catalase and oxidase. Being able to hydrolyse starch was also an indicator of the microorganism being Pseudomonas fluorescens. The results showed the second microbial strain being unable to utilise mannitol as a carbon source. This is an indicator of the microbe being of streptococcus species. Further test results proved the microorganism to be Streptococcus sp.

In the enzyme production stage it was found that even though cellulolytic bacteria could be abundantly found in various sources, not all of them could produce enough cellulase to be commercially viable. Although they were able to consume cellulose and showed excellent growth, the enzyme produced was not substantial enough to be considered for production. In fact, a strain of bacteria that showed vigorous growth on CMC agar media and could easily hydrolyse concentrated CMC broth, did not produce any substantial amount of cellulase. Contrary to expectations, the little amount of cellulase produced by it did not even demonstrate any significant activity.

The reason might have been due to the microbes producing endocellulase, which remains inside the bacterial cell and needs to be extracted through cell lysis. Thus, when centrifugation was performed, the enzyme might not have been extracted.

However, since the main objective was to obtain copious amounts of exocellulase from microbes, those producing endocellulase were opted out for production.

Thus, two strains of microbes were selected for their relatively better enzyme production capability. Surprisingly, it was found that the microbial strains showed a synergistic effect. On their own, the bacteria did not produce enzymes with any noteworthy activity. However, on culturing a flask of carboxymethylcellulose with a combination of both the microbial strains, a large increase was observed in the enzyme production. The enzyme activity showed an increase of as much as ten-fold. Thus, the bacteria were then used together during later stages of enzyme production. Although, whether the increased efficiency could be attributed to synergistic effect or antagonistic effect remains debatable. The reason for this is that enzymes extracted after about a week showed greater efficiency than the enzymes extracted earlier. There remains a possibility of secondary metabolites being released. Since the nature of possible secondary metabolites has not been established, it cannot be clearly said whether or not the increase in efficiency can be attributed to synergistic or antagonistic effect.

The source of the enzyme was also found to be a determining factor about the quality of enzyme. The results suggested that carboxymethylcellulose seemed to be the best carbon source for production of cellulase enzyme. Carboxymethylcellulose might have given better results since the water-soluble formula is more easily degraded by microorganisms. Furthermore, another factor might have been that carboxymethylcellulose is known to act as a stimulant to enhance cellulase production. However, enzymes produced from plant leaves or from filter paper were not far behind in demonstrating efficient enzymatic activity.

It was found that an incubation time of 168 hours in carboxymethylcellulose broth gave the best results for enzymatic activity. The enzyme activity showed gradual decrease as the incubation time increased. This might have been due to some of the

enzymes staying bound to the leftover substrates in the media and thus not remaining free during centrifugation, thus preventing their presence in the crude enzyme .

However, in the case of using leaves, filter paper or avicel as carbon sources, the enzyme activity was found to the most after extracting enzyme after a three week interval. This might have been due to the fact that filter paper and avicel are not water soluble. Hence it might have proven a bit difficult to degrade these carbon sources.

The temperature at which the enzyme was extracted also had an effect on the enzyme activity. Extraction of enzyme at 4°C was found to give maximum enzyme activity. Enzymes are known to be affected by temperature. With increase in temperature, there is a chance for the enzymes to get denatured. Thus, increasing extraction temperature beyond 4°C lowered the enzyme activity to a certain extent.

An enzyme works best when in an environment that provides optimum pH of the enzyme. Similar to changes brought about by temperature, a change in pH can also cause an enzyme to be denatured. The enzyme activity was found to be highest at a pH of 6.5. However, the enzyme could work quite well within a pH range of 2-12. The activity was slightly lowered when the pH reached the extremes. However, the extent of lowering of activity was not too much and fell in a rather acceptable range.

Increasing the concentration of carbon source was found to increase the enzyme activity. This might have been due to higher enzyme production due to higher concentration of carbon source. Since more resources were available, more enzymes must have been produced by the microorganisms.

The temperature at which the enzyme and substrate were incubated during DNS assay did not prove to be a very determining factor for enzyme activity. Although every enzyme has their own optimum temperature at which they show best efficiency and

the same holds true for this enzyme too, altering temperatures did not seem to affect the activity beyond a barely acknowledgable degree. Maximum activity was obtained at a temperature of 43°C. However, increasing the incubation temperature beyond 55°C affected the enzyme activity inversely. Thus, the optimum temperature for the extracted cellulase was found to be around 43°C.

The activity of the enzyme could be measured by assaying with Dinitrosalicylic Acid to check the amount of glucose liberation. When cellulase degrades cellulose, glucose is liberated. This liberated glucose can then bind themselves to the DNS molecules and can produce more intense colours upon being heated. The intensity of colour during the assay indicates the amount of glucose liberated by the enzyme. The higher the glucose liberated, the better the activity of the enzyme. The concentrations of glucose liberated were checked against a previously formulated standard concentration curve of glucose.

The protein concentration was measured by using Bovine Serum Albumin as a standard for Biuret's assay. This assay measures the intensity of color produced upon reacting with the polypeptide bonds of a protein. Presence of protein lends a mauve colour to the solution. Since enzyme is also considered to be a protein, this assay can also be used to quantify protein. The higher the number of peptide bonds, the more intense the mauve colour of the solution. Estimating the protein concentration was necessary in order to find the specific activity of the enzyme. The specific activity of an enzyme is a measure of purity of enzyme in the obtained crude enzyme. This enables one to estimate the amount of enzyme truly present in the solution and thus gives a sort of qualitative analysis of the enzyme before it is concentrated using a viable method.

A problem encountered while using carboxymethylcellulose as a carbon source is the relative thickness of the broth if higher concentrations of carboxymethylcellulose are used, which might cause some difficulties during enzyme extraction. A possible way to solve this issue might be by using avicel as a carbon source in the broth. Since the unused avicel would get sedimented at the bottom of the flask, the supernatant can be easily collected for enzyme extraction. However, the enzyme production would get lowered and the time required would be more. Furthermore, due to economic considerations, using vegetable wastes or paper based wastes as carbon source might bring down the cost of enzyme production. The cost would thus make the use of the enzyme industrially profitable.

The DNA concentration of the extracted DNA sample was found to be 2420 μg/mL. However, the working concentration of DNA is 50 μg/mL. In order to adjust the concentration of the working DNA solution, the DNA was diluted by a factor of 48.4 times.

The DNA was found to have protein contamination upon quality testing. Protein, being a bigger unit, absorbs less light. Thus, the absorbance is less than it would be for pure DNA. On the other hand, RNA molecules being single stranded, would have absorbed more light and increased the optical density.

Protein purification increased the concentration of protein sevenfold, thereby increasing the enzyme activity exponentially.

SDS results indicated the protein to have a size of 60 kDa. However, upon performing SDS-PAGE on the concentrated protein, protein band could not be obtained. This most likely resulted from the fact that the amount of protein had reduced upon

concentration and did not reach the threshold amount, thus resulting in the absence of a visible band.

The protein functioned really well within a wide range of temperatures, pH and salinity. This would significantly improve the economic value of the enzyme.

Furthermore, the enzyme demonstrated really good shelf life. It was found to be functional even after a span of 36 months.

Chapter 5

Conclusion

Enzymes have been found to be more efficient than chemicals in every way. Enzymes are required in very small amounts, do not get used up in the reactions, and are safer for the environment since enzymes are biochemicals and they can easily be degraded in the environment.

For further studies, the genes responsible for cellulase production in the microbial strains might be identified and manipulated to increase the expression of the bacterial genes that produce the enzyme cellulase.

The chemical structure of the enzyme might also be studied to see if the possible symbiotic effect of the microorganism actually simply increases enzyme production, or rather, whether there is a change in the amino acid sequence which increases the efficiency of the enzyme.

Transportation of any enzyme is tough since the correct temperature and environment has to be maintained. However, producing cellulase in our own country can bring down the expenses associated with importing the enzyme from foreign countries. Furthermore, self-sufficiency will also be promoted by lowering the cost of associated commodities. Furthermore, if production exceeds demand, Bangladesh can then export enzymes in the global market. This would also further the country's economic development.

The enzyme can find further uses as a possible agent to dissolve biofilm matrices.

Alongwith deinkase enzyme, cellulase would find great uses during paper recycling. The combined usage of these enzymes would lead to better quality of recycled paper.

The enzyme might have potential use as an antimicrobial agent, either on its own or in combination with other antimicrobial agents. That would be a great step to reduce the usage of antibiotics, thus proving to be a step against antibiotic resistance of microbes.

Moreover, usage of leaves and plant material to produce cellulase is beneficial since it will yield a two-fold benefit of enzyme production as a result of bioremediation. On one hand, the production of cellulase shall be able to aid industries, while on the other hand, waste management would be upgraded. This would further bring down the amount of waste, at least partially, in the environment by utilising an innovative way of cellulolytic waste management.

In retrospect, self-sufficiency in enzyme production in our country would indeed provide an economic boost to the various industrial sectors while promoting a safer environment for future generations. This can certainly improve the living standards of the country and promote national development as a whole.

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