

ISOLATION, SCREENING AND PRODUCTION 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
Bachelor in Biotechnology

Department of Mathematics and Natural Sciences
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Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

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Abstract

Cellulase finds uses as raw material in various industries. The current sources of cellulase are highly expensive. However, using bacteria as cellulase producers can bring the cost down and make our industries self-sufficient. This experiment was carried out bearing this thought in mind. The experiment was started by culturing microorganisms obtained from various soil and municipal samples on carboxymethylcellulose agar media. Colonies that showed clear zones after being flooded with Gram's Iodine were further analysed to investigate their ability to utilize filter paper as a carbon source. Positive samples were selected for enzyme production. Biochemical analysis was used to characterize the bacterial isolates. The results indicated a possibility of the isolates being *Pseudomonas fluorescens biovar* and *Streptococcus sp.* Bacteria were cultured in carboxymethylcellulose broth. The cultured broth was then centrifuged and the supernatant was used as crude enzyme. The enzyme activity was tested using DNS assay to check glucose liberation after the degradation of cellulosic bonds.. Protein concentration was estimated to be 0.09 mg/mL after performing Biuret's Assay. Enzyme production was then optimized using a range of temperature, time, pH and carbon source. Cellulase produced from the synergistic effect of the obtained microorganisms was found to be almost five times more potent than the enzymes produced by them separately.

The enzyme activity was noted as 0.175 U/mL, whereas, the specific enzyme activity was found to be 1.82 U/mL. Thus, there was a tenfold increase in the value which indicates that the enzyme activity would be increased at least ten times upon concentration and purification of the enzyme.

Keywords: Carboxymethylcellulose 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*

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

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List of Acronyms

BSA	Bovine Serum Albumin
CMC	Carboxymethylcellulose
DNA	Deoxyribonucleic Acid
DNS	Dinitrosalicylic Acid
mL	Milliliter
RNA	Ribonucleic Acid

Chapter 1

Introduction

1.1 Background

From almost the dawn of civilisation enzymes have been inadvertently, though incognizantly, used in the production of various wines or cheeses. However, the first known discovery of enzymes¹ was in the year 1833 by Anselme Payen². 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. 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. Back then, the main purpose in mind was to catalyse the production of fuel from lignocellulosic mass. Nevertheless, cellulases soon found other purposes such as being used in detergents, wines³ and other foods⁴. 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*.

¹ M. Phillips, Anselme Payen, distinguished French chemist and pioneer investigator of the chemistry of lignin, vol. 30, Washington Academy of Sciences, pp. 65-71

² Payne, C., Knott, B., Mayes, H., Hansson, H., Himmel, M., Sandgren, M., . Beckham, G. (2015, 2 11). Fungal cellulases. *Chemical Reviews*, 115(3), 1308-1448. American Chemical Society

³ Baker, R., Technology, L.-T., & 1996, u. (n.d.). *Current and potential applications of enzyme infusion in the food industry*. Elsevier

⁴ Youn, K., Hong, J., Bae, D., Kim, S., & Kim, S. (2004). Effective clarifying process of reconstituted apple juice using membrane filtration with filter-aid pretreatment. *Journal of Membrane Science*. 228, pp. 179-186. Elsevier

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 sources⁵. Fungal sources were viewed as more easily accessible. Nonetheless, in order to reduce costs and accrue more easily accessible cellulases, research turned to utilising bacteria as enzyme sources⁶. 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 cellulase⁷. The methods and techniques are being researched every day and being improved world-wide^{8,9}.

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 country¹⁰. 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.

⁵ Gupta, R., Mehta, G., Khasa, Y., & Kuhad, R. (2011, 7). Fungal delignification of lignocellulosic biomass improves the saccharification of celluloses. *Biodegradation*, 22(4), 797-804

⁶ Ito, S., Kobayashi, T., Ara, K., Ozaki, K., Kawai, S., & Hatada, Y. (1998, 8). Alkaline detergent enzymes from alkaliphiles: Enzymatic properties, genetics, and structures. *Extremophiles*, 2(3), 185-190

⁷ Ash, C., Priest, F., & Collins, M. (1993, 9 1). Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test - Proposal for the creation of a new genus *Paenibacillus*. *Antonie van Leeuwenhoek*, 64(3-4), 253-260

⁸ S. Sethi, A. Datta, B. L. Gupta and S. Gupta, "Optimization of Cellulase Production from Bacteria Isolated from Soil," *ISRN Biotechnology*, vol. 2013, pp. 1-7, 2013

⁹ M. J. Bailey, P. Biely and K. Poutanen, "Interlaboratory testing of methods for assay of xylanase activity," *Journal of Biotechnology*, vol. 23, no. 3, pp. 257-270, 1992.

¹⁰ M. Mehadi, H. Sohag, M. M. Hasan, J. Ahmed and M. M. Hoq, "Production and Partial Characterization of Cellulase from *Pseudomonas* Isolates Obtained from Cow Dung and Municipal Solid Wastes SNP study of insulin receptor View project Mass Production of Efficient Bt. Biopesticides View project," *Article in Bangladesh Journal of Industrial Microbiology and Biotechnology*, 2013.

1.2 Enzymes

Enzymes are biological molecules that act as biological catalysts and enhance the speed of a particular reaction. In some cases, enzymes can speed up a reaction by even a million times. Without the intervention of enzymes, some of life's processes would be too slow to actually sustain life. Thus, enzymes form an essential and indispensable part of the biochemical reactions required for supporting life. Enzymes are present in every biological reaction and they catalyse biochemical reactions in the body ranging from DNA replication to protein folding to the digestion of food. They are typically proteins, though they can also be RNA molecules called ribozymes.

Industries require a lot of different chemicals in order to perform a multitude of functions. However, most of these chemicals are extremely hazardous and are not easily degraded. They pose a threat to human health as well as to the surrounding flora and fauna. In order to tackle such issues, enzymes are being used extensively in many industries. Not only do enzymes not pose a threat to health or the environment, they are much more efficient than commonly used chemicals as well. Moreover, they are only required in small quantities. Furthermore, being catalysts, enzymes can also be used for speeding up reactions without the enzymes themselves getting fully used up. Hence, they can essentially be used over and over again. This inadvertently helps reduce costs as well. As the cost of production goes down, it can help to lower the prices of goods as well¹¹.

Data supports that industrial usage of enzymes has increased substantially. Many industries have replaced the usage of chemicals with the usage of enzymes.

¹¹ Leisola, M., Jokela, J., Pastinen, O., Turunen, O., & Schoemaker, H. (n.d.). INDUSTRIAL USE OF ENZYMES

Industry	Chemicals Used	Replacement Enzymes
Leather Industry	Chromium, Sodium Sulphite, Aldehydes, Vegetable tannin	Keratinase, Protease, Lipase, Pepsin
Paper Industry	Dextrin, Oxidised starch, Styrene-butadiene latex, Styrene acrylic	Cellulase, Xylanase, Hemicellulase
Textile Industry	Acetic acid, Oxalic Acid, Soda ash, Sulphuric acid, Sodium hypo chloride	Cellulase, Pectinase, Protease, Catalase, Amylase, Lipase
Detergent Industry	Soda ash, Carboxymethyl chloride, Phosphates, Sodium lauryl sulphate	Cellulase, Protease, Amylase, Lipase
Food Industry	Sodium Benzoate, Potassium bromate, Sodium Caseinate	Isomerase, Carbohydrase, Protease, Pepsin

Table 1: Chemicals and their replacement enzymes

1.3 Cofactors

Most enzymes are present in an inactive state, called an apoenzyme. In order to form a fully functional enzyme molecule, called a holoenzyme, a cofactor is required. When paired with their cofactor, enzymes get activated to the holoenzyme state. Some cofactors bind to the apoenzyme and change its active site so that it is of the correct alignment for the substrate. Cofactors are usually non-protein molecules that are specific to each enzyme. However, the cofactors can fall in the range of either organic molecules or inorganic molecules. Elements such as Zinc, Copper or Iron function as common cofactors.

1.4 Mechanism of action of enzyme

Enzymes are very specific and can only catalyse the substrate they are made for. They work by lowering the required activation energy for a reaction to take place. Moreover, the chemical equilibrium of the reaction is not affected during catalysis and the final energy remains the same for the products.

Enzymes have specific sites, called active sites, where the substrate can bind. A substrate is a molecule with which the enzyme binds. Once the substrate binds to the active site, it then undergoes chemical reactions and a product is formed. The product is then released and a new cycle continues with a new substrate molecule.

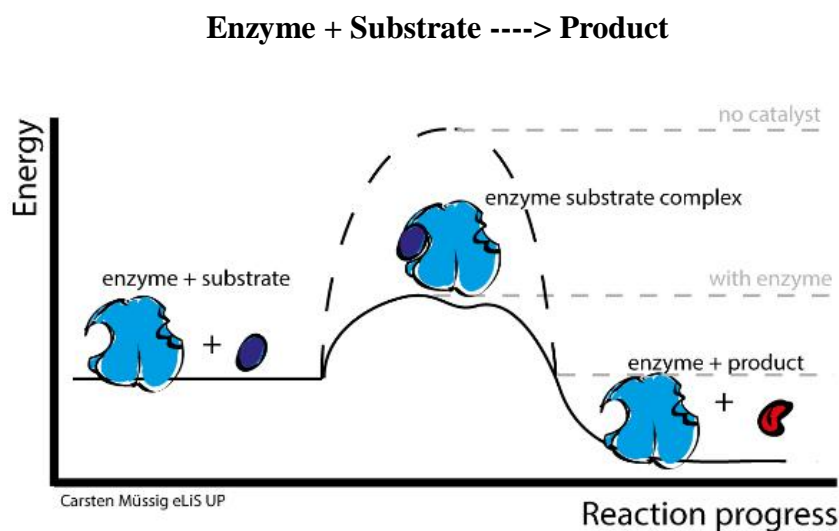


Figure 1: Mechanism of action of Enzyme. The activation energy is lowered and the reaction proceeds by binding of enzyme with substrate to obtain products

Two models have been proposed for the working of enzymes.

The first model, Lock and Key model, was proposed in the year 1894. This model treats the active site of the enzyme as a lock. The specific site is of a particular shape and size, such that only the specific substrate can bind to it. The active site of the enzyme is where the substrate or the "key" fits.

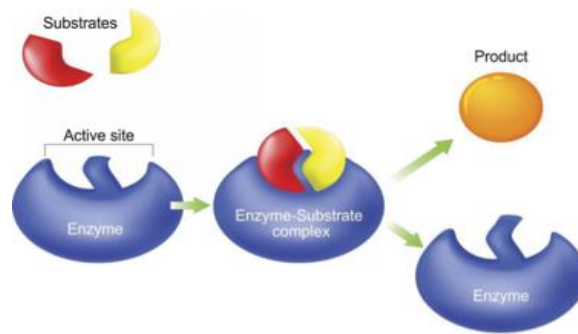


Figure 2: Lock and Key model of Enzyme activity

The second model, which is used nowadays, is the Induced Fit model. In this model, the active site of the enzyme is thought to have a flexible conformation and thought to undergo a change in conformation on binding with the specific substrate. This is thought to let the groups in the active site of the enzyme align better with the substrate. This means that the enzyme has better binding and catalytic effects.

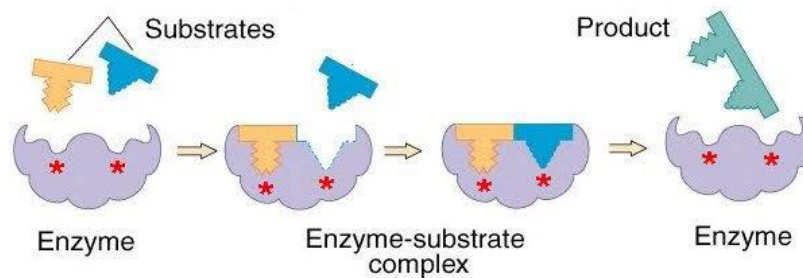


Figure 3: Induced Fit model of Enzyme activity

The temperature and pH of the surrounding environment need to be optimum for an enzyme to function at its best. If the conditions are too harsh, the enzyme has a possibility to get denatured.

1.5 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 structure¹². Cellulose synthase, an enzyme, helps in the formation of cellulose. This enzyme can be found in organisms ranging from bacteria to higher plants.

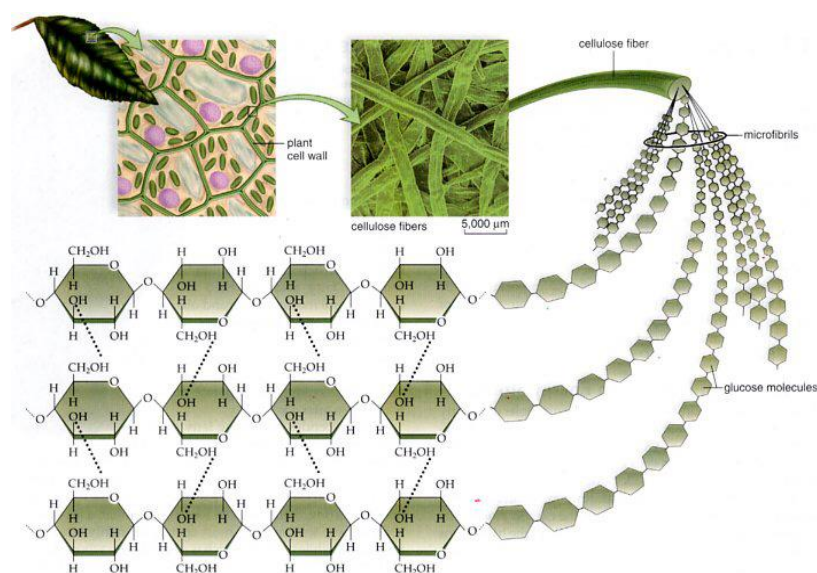


Figure 4: Structural representation of cellulose

Cellulose has abundant purposes in nature as well as in industries¹³. 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

¹² O'Sullivan, A. (1997). Cellulose: The structure slowly unravels. *Cellulose*, 4(3), 173-207

¹³ "Cellulose - humans, body, used, water, process, Earth, plants, chemical, form," [Online]. Available: <http://www.scienceclarified.com/Ca-Ch/Cellulose.html>.

intestines. Moreover, it serves as raw material in various industries such as paper industries, textile industries. Cellulose derivatives also find uses in the manufacturing of explosives, plastics and films.

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.

1.6 Cellulase

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

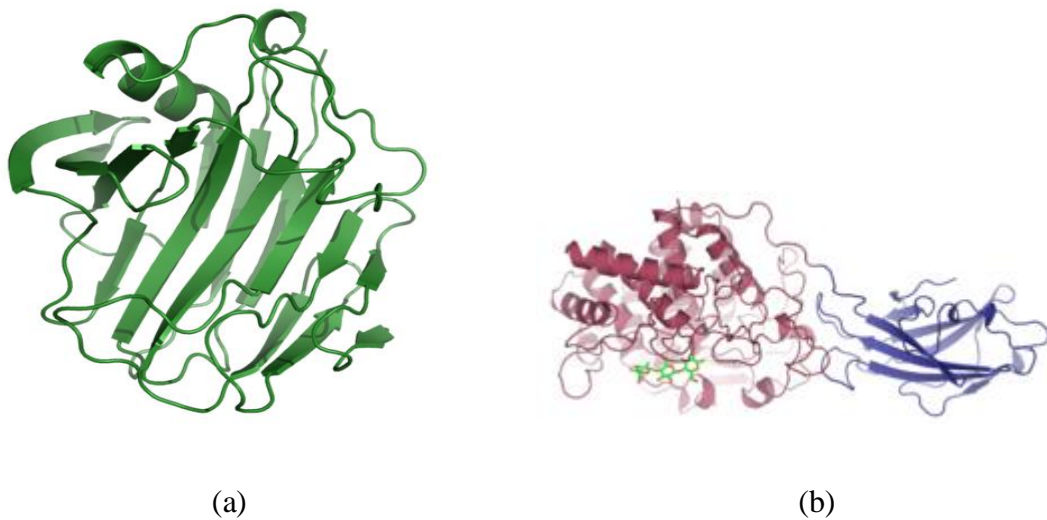


Figure 5: Ribbon representation of Cellulase Structure

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

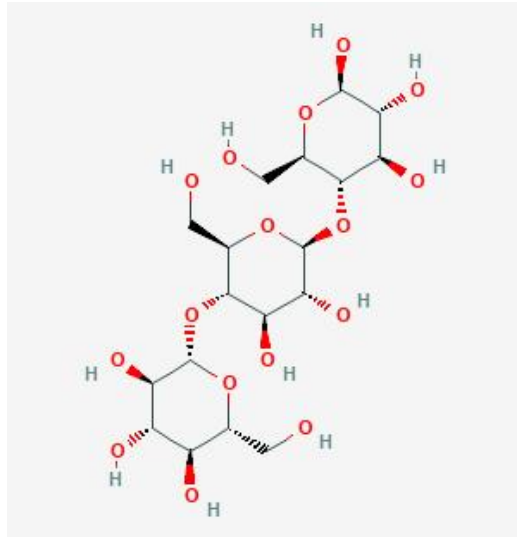


Figure 6: Chemical representation of Cellulose Structure

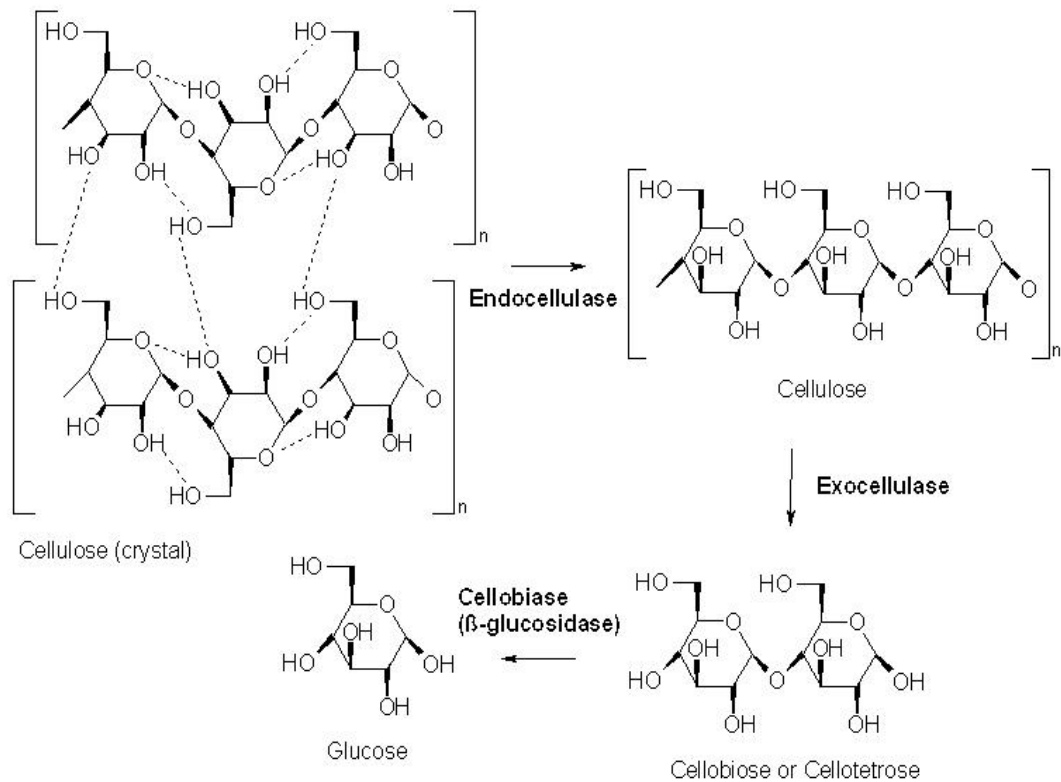


Figure 7: Mechanism of action of Cellulase

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.

1.7 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 such as *Trichoderma sp.* or *Aspergillus sp.*¹⁴, bacteria are viewed as potential mini-factories for

¹⁴ A. Sherief, A. El-Tanash, N. A. -. R. J. o. Microbiology and u. 2010, "Cellulase production by *Aspergillus fumigatus* grown on mixed substrate of rice straw and wheat bran," docsdrive.com

production of cellulase enzyme. *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 such as textile industry, paper industry, food industry, animal feeds industry, etc.

In the paper industry, using cellulase reduces costs, provides improved strength properties¹⁵, reduces fiber coarseness, enhances bleachability, increases deinkability and provides brightness to the paper produced¹⁶.

In textile industries cellulases are used to provide enhanced biopolishing, reduced fibre damage, improved fiber strength, improved water absorbance and softness and accentuates appearance¹⁷.

Production of bioethanol cannot proceed effectively without first being treated by cellulases to soften the fibers for better fermentation¹⁸. Microorganisms are able to

¹⁵ Ko, C., Tsai, C., Lin, P., Chang, K., ..., J.-B., & 2010, u. (n.d.). Characterization and pulp refining activity of a *Paenibacillus campinasensis* cellulase expressed in *Escherichia coli*. Elsevier

¹⁶ Gupta, R., Mehta, G., Khasa, Y., & Kuhad, R. (2011, 7). Fungal delignification of lignocellulosic biomass improves the saccharification of cellulose. *Biodegradation*, 22(4), 797-804

¹⁷ de Souza Moreira, L., Lo Sciuto, D., & Filho, E. (2016). An Overview of Cellulose-Degrading Enzymes and Their Applications in Textile Industry. In L. de Souza Moreira, D. Lo Sciuto, & E. Filho, *New and Future Developments in Microbial Biotechnology and Bioengineering: Microbial Cellulase System Properties and Applications* (pp. 165-175). Elsevier Inc

¹⁸ Bailey, M., Biely, P., & Poutanen, K. (1992). Interlaboratory testing of methods for assay of xylanase activity. *Journal of Biotechnology*, 23(3), 257-270

better catabolize the celluloses when they have been bioconverted to smaller molecules¹⁹.

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

In the food industry, cellulases can be utilized 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 utilizing a mixture of hemicellulase, pectinase and cellulase.²⁰

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 washing²¹.

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.

¹⁹ Kuhad, R., Gupta, R., Khasa, Y., technology, A.-B., & 2010, u. (n.d.). Bioethanol production from lantanacalara (red sage): pretreatment, saccharification and fermentation. Elsevier

²⁰ Carvalho, L., , I.-J., & 2008, u. (n.d.). A study of retention of sugars in the process of clarification of pineapple juice (*Ananas comosus*, L. Merrill) by micro-and ultra-filtration. Elsevier

²¹ Ito, S. (1997). Alkaline cellulases from alkaliphilic *Bacillus*: Enzymatic properties, genetics, and application to detergents. *Extremophiles*, 1(2), 61-66. Springer Japan

1.8 Usage of bacteria as cellulase producers

Bacteria that can produce cellulase in order to break down cellulose are called cellulolytic bacteria. 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 ruminants²².

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. Extraction of bacterial enzyme is also less expensive and the process is a lot easier²³. Moreover, genetic enhancement of bacteria is much easier than genetically modifying multi-cellular species. Furthermore, cellulases produced by bacteria are often more effective catalysts due to being less inhibited by the presence of hydrolyzed carbon sources. Extraction of cellulase from bacteria would also alleviate the cost to a large extent, thus making the enzyme more accessible and cost-effective.

²² Mehadi, M., Sohag, H., Hasan, M., Ahmed, J., & Hoq, M. (2013). Production and Partial Characterization of Cellulase from *Pseudomonas* Isolates Obtained from Cow Dung and Municipal Solid Wastes SNP study of insulin receptor View project Mass Production of Efficient Bt

²³ J. Ahmed, M. H. Sohag, K. Islam and A. K. Azad, "Isolation, Screening and Characterization of Cellulase Producing Bacterial Isolates from Municipal Solid Wastes and Rice Straw Wastes," J Bioprocess Biotech, vol. 6, no. 4, p. 280, 2016

1.9 Synergistic effect

Two organisms that enhance the effect of each other are said to be synergistic with each other²⁴. 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 alone²⁵.

1.10 Enzymatic activity assessment

The enzyme activity can be checked by assaying with 3,5 - dinitrosalicylic acid (DNS) for glucose liberation. Once the cellulase enzyme reacts with the cellulose, glucose is liberated. DNS can react with the released glucose and gives orange colour. The intensity of the colour is an indicator of the concentration of glucose liberated. The obtained result can be compared to a standard curve to obtain results²⁶.

The concentration of protein can be checked by using Biuret's reagent. The absorbance can be checked against a standard Bovine Serum Albumin (BSA) curve.

²⁴ B. Schink, "Synergistic interactions in the microbial world," *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, vol. 81, no. 1-4, pp. 257-261, 2002

²⁵ J. Chorostowska-Wynimko, M. Krotkiewski, D. Radomska-Leśniewska, I. Sokolnicka and E. Skopińska-Różewska, "The synergistic effect of lactic acid bacteria and alkylglycerols on humoral immunity in mice.," *International journal of tissue reactions*, vol. 23, no. 3, pp. 81-7, 2001

²⁶ Miller, G. (1959, 3 1). Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Analytical Chemistry*, 31(3), 426-428

Chapter 2

Materials and Methods

2.1 Apparatus and Reagents

Apparatus	Reagents
<ul style="list-style-type: none">• Petri Dishes	<ul style="list-style-type: none">• Carboxymethylcellulose
<ul style="list-style-type: none">• Erlenmeyer Flasks	<ul style="list-style-type: none">• Gram's Iodine
<ul style="list-style-type: none">• Test Tubes	<ul style="list-style-type: none">• Crystal Violet Solution
<ul style="list-style-type: none">• Eppendorf Tubes	<ul style="list-style-type: none">• Safranin Solution
<ul style="list-style-type: none">• Falcon tubes	<ul style="list-style-type: none">• 95% Ethanol
<ul style="list-style-type: none">• Reagent Bottles	<ul style="list-style-type: none">• Phenol Red
<ul style="list-style-type: none">• Glass Rods	<ul style="list-style-type: none">• Dextrose
<ul style="list-style-type: none">• Burner	<ul style="list-style-type: none">• Sucrose
<ul style="list-style-type: none">• Magnetic Stirrer	<ul style="list-style-type: none">• Lactose
<ul style="list-style-type: none">• Laminar Air Flow	<ul style="list-style-type: none">• Mannitol
<ul style="list-style-type: none">• Fume hood	<ul style="list-style-type: none">• Peptone
<ul style="list-style-type: none">• Shaker Incubator	<ul style="list-style-type: none">• Sodium Chloride
<ul style="list-style-type: none">• Multipurpose Centrifuge Machine	<ul style="list-style-type: none">• Potassium Phosphate
<ul style="list-style-type: none">• Water Bath	<ul style="list-style-type: none">• Hydrogen Peroxide
<ul style="list-style-type: none">• Spectrophotometer	<ul style="list-style-type: none">• N,N-dimethyl-p-phenylenediamine
	<ul style="list-style-type: none">• 40% Urease Solution
	<ul style="list-style-type: none">• Dinitrosalicylic Acid
	<ul style="list-style-type: none">• Biuret's Reagent

2.2 Methodology

2.2.1 Collection of Samples and Identification of Cellulolytic Bacteria

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

The obtained samples were serially diluted up to 10^{-5} times. The samples were then spread on petri dishes containing CMC agar media. After a growth period of 24 - 30 hours, the plates were then flooded with Gram's Iodine. The colonies which showed a clear halo after 4-5 minutes were selected. Those colonies were taken from replica plates and streaked to obtain pure cultures.

The selected colonies were then further tested for FPase production by mixing a loopful of colony of each respective sample into broth containing minimal salts and Filter paper as the sole carbon source.

Stock cultures (in 30% glycerol) were prepared for the respective bacterial strains and stored at a temperature of - 20 °C.

2.2.2 Biochemical Analysis

In order to identify the obtained strains of microbes, a few biochemical tests (refer to Appendix B) were carried out. The corresponding characteristics observed were checked against a chart and the microbial strains could thus be identified²⁷.

²⁷ Microbiology Lab: MOLB 2210. (n.d.). Retrieved from https://www.uwyo.edu/molb2210_lab/info/biochemical_tests.htm?fbclid=IwAR1rf9GTh1gXq4-823iTQy_3XrB5frc2NzWKOq_YN1hBzFIB4iUWqiYLyrE#sulfur

2.2.3 Enzyme production and extraction

A loopful of colony from the fresh culture of each respective sample was mixed into flasks containing CMC broth. The flasks were kept in a shaker incubator at a temperature of 37°C and 150 rpm.

Afterward, 1 ml of each sample was taken in eppendorf tubes and centrifuged for 15 minutes at 4°C and 5000 rpm. The supernatant obtained was used as crude enzyme.

2.2.4 Enzyme Assay

The obtained crude enzyme was assayed using DNS to check for glucose liberation. DNS assay can be used to measure the concentration of glucose in a particular sample. Since the cellulase hydrolyses the cellulose compounds and releases glucose, the enzyme activity can be estimated using DNS assay²⁸.

Samples containing glucose in concentrations ranging from 0.1 mg/ mL to 1.0 mg/mL (with an increase on 0.1 mg/mL sequentially) were assayed and the absorbances were recorded to form a standard curve. Next, the samples obtained from enzymatic activity were tested.

1 mL of crude enzyme was mixed with

- 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

²⁸ Miller, G. (1959, 3 1). Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Analytical Chemistry, 31(3), 426-428

and allowed to incubate at 37°C for 30 minutes. After that, 1mL of DNS was added to stop the reactions. A test tube containing 2 mL CMC and 1mL DNS was used as a blank. The test tubes were then boiled in a water bath at 100°C for 10 minutes. The tubes were then cooled to room temperature and 9 mL distilled water was added to each test tube.

The samples were then measured for absorbance by spectrophotometer. The wavelength was set at 540 nm.

The obtained absorbances were checked against a standard curve to find out the concentration of glucose liberated by the enzyme.

The enzyme activity was measured using the formula:

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

2.2.5 Protein Estimation

Protein concentration can be estimated by Biuret's method. It is a generally used method for polypeptides containing at least two peptide bonds (tripeptides) or more. The basis of biuret's test is the formation of a mauve compound when biuret is treated with copper sulphate in alkaline condition. The intensity of colour gives an idea of the concentration of the protein since bonds are formed with the same frequency per amino acid. This is in coordination with Beer-Lambert law. The copper (II) binds with the free nitrogen of the peptides. In a later reaction, the copper (II) gets reduced to copper (I). The copper in the reagent is what gives out the blue-purple colour.

Despite what the name suggests, biuret (a derivative obtained by heating urea to 180°C) is not actually a part of the test reagent. The name only derives its origins from the fact that the same results are also obtained when the peptide-like bonds of biuret molecule react with the compound.

Samples containing Bovine Serum Albumin (BSA) in concentrations ranging from 0.1 mg/ mL to 1.0 mg/mL (with an increase in 0.1 mg/mL sequentially) were assayed at 650 nm and the absorbances were recorded to form a standard curve. Next, the enzyme samples were tested.

1 mL of enzyme was added to a test tube. Next, 4 mL of biuret's reagent was added in the test tube and the test tube was kept in a dark room. After about 20 minutes, the test tubes were retrieved and the absorbances of the samples were taken at a wavelength of 650 nm.

Protein estimation is required in order to obtain specific enzyme activity. It can be found by using the following formula:

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

2.2.6 Optimization of Enzyme production

The optimum conditions for enzyme production were tested on the basis of:

- time variation
- temperature variation
- pH variation
- carbon source variation
- variation of concentration of carbon source

The enzyme produced was tested at different time intervals to check which incubation time produced the best results. The enzyme extracts were obtained at intervals of 8 hours, 16 hours, 24 hours and 48 hours.

The enzyme was also produced at different temperatures to check which incubation temperature produced the best results. The enzyme extracts were obtained at temperatures of 30 °C, 35 °C and 37 °C.

The enzyme was produced at different pH 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.

The source of enzyme was also taken into consideration. The impact of using different carbon sources for the purpose of enzyme production was tested. The different carbon sources tested were carboxymethylcellulose, filter paper, avicel and vegetable waste.

The effects of increasing the concentration of carbon source in the media was also observed. The variations tested were at concentrations of 1%, 2% and 3% of carboxymethylcellulose.

Chapter 3

Results

3.1 Protein Isolation and Identification of Cellulolytic Bacteria

A few cellulolytic strains were identified after primary selection (flooding with Gram's Iodine) and the bacterial samples since that provided no clear zones were discarded.



Figure 8: Colonies showing positive results for Gram's Iodine test



Figure 9: Colonies showing negative results for Gram's Iodine test

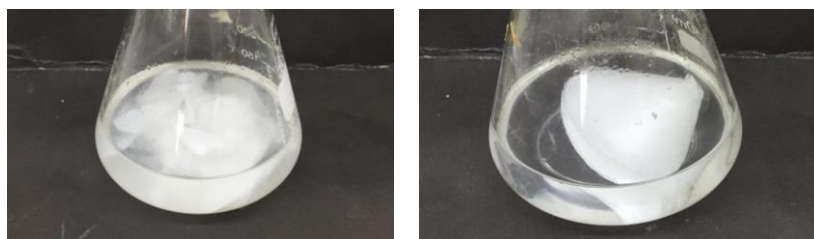


Figure 10: Left: positive results for FPase test; Right: negative results for FPase test

3.1 Biochemical Analysis

Results obtained after performing biochemical analysis are charted below:

Test	Strain PE1	Strain PE2
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
Voges 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 2: Results of Biochemical Analysis

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

Gram Staining

Of the two isolated bacteria, one tested negative during gram staining, whereas, the second tested positive. The first strain appears to be of rod shape, while, the second strain appears to have streptococcal shape.

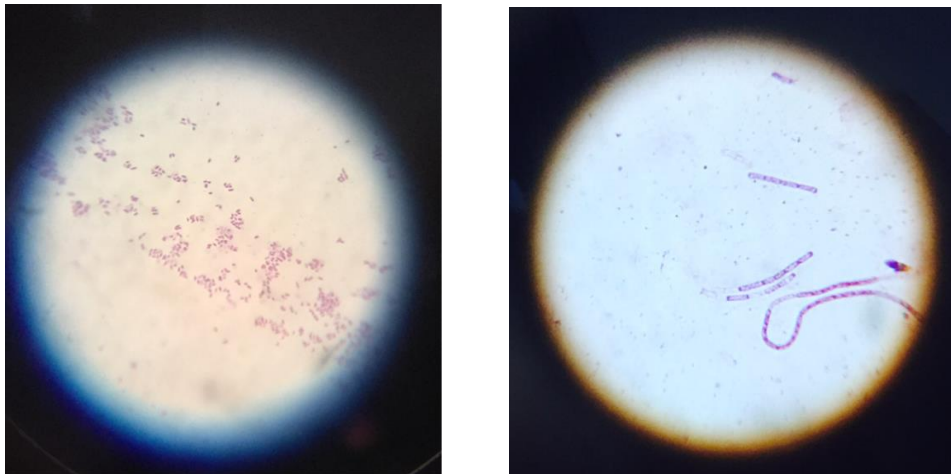


Figure 11: Gram Staining (a) Strain PE1 and (b) Strain PE2

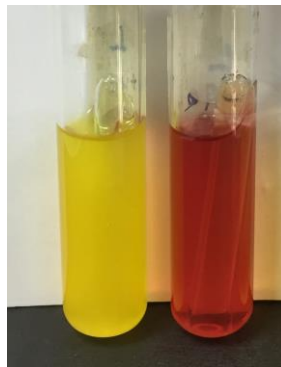


Figure 12: Phenol Red Carbohydrate Broth (a) positive result and (b) negative result

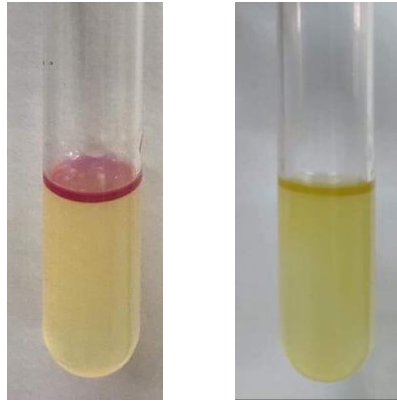


Figure 13: Indole Test (a) positive result and (b) negative result

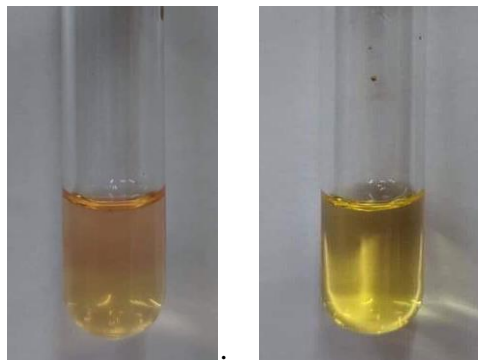


Figure 14: Vogues- Proskauer Test (a) positive result and (b) negative result

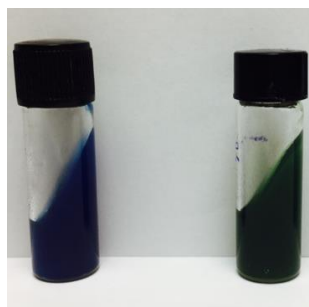


Figure 15: Citrate Test (a) positive result and (b) negative result



Figure 16: Catalase Test (a) positive control, (b) positive result and (c) negative result

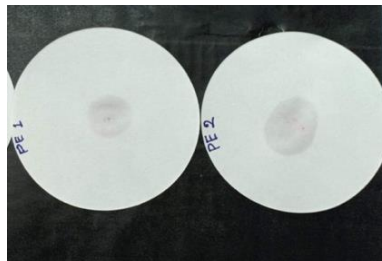


Figure 17: Oxidase Test - (a) positive result and (b) negative result

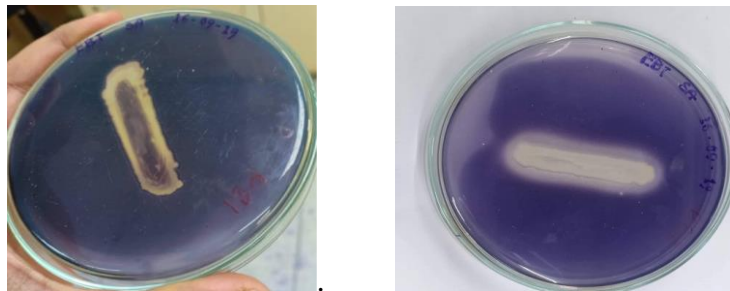


Figure 18: Starch Hydrolysis Test (a) negative result and (b) positive result

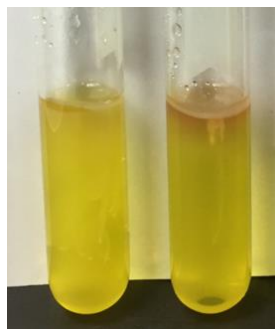


Figure 19: Motility Indole Urease Test (a) motile and (b) non-motile

3.3 Enzyme Assay

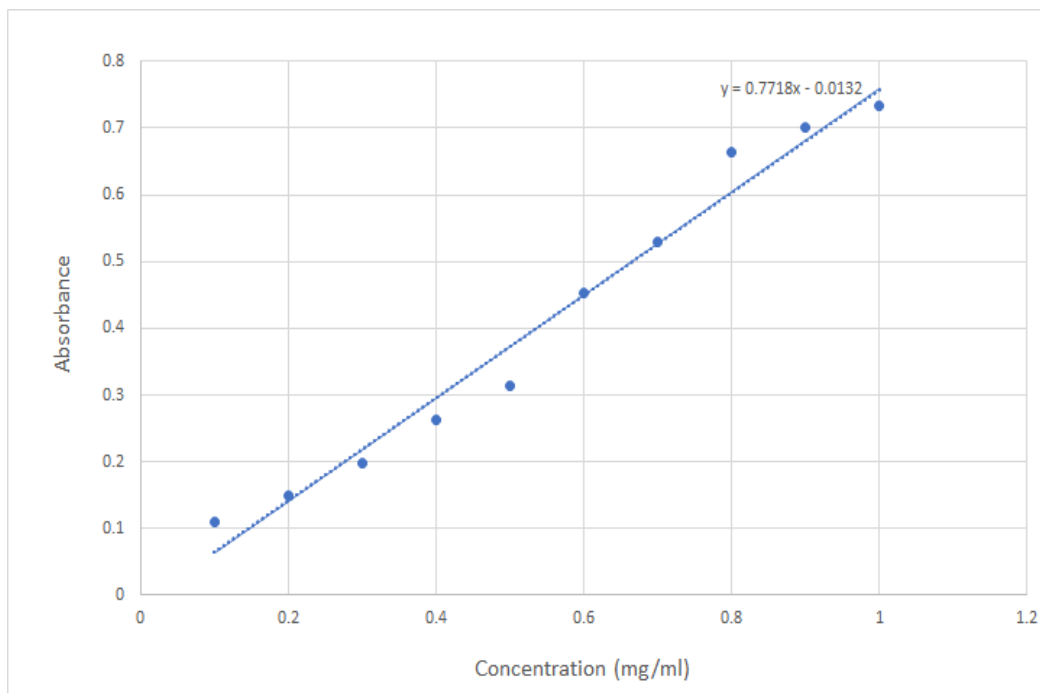


Figure 20: Standard Curve of Glucose Liberation Assay

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 at temperature:	Concentration of Glucose Liberated	Enzyme Activity (U/mL)
33 °C	0.4 mg/mL	0.074 U/mL
37 °C	0.8 mg/mL	0.147 U/mL
43 °C	0.95 mg/mL	0.175 U/mL
46 °C	0.36 mg/mL	0.067 U/mL

Table 3: Difference in Enzyme Activity based on Incubation Temperature

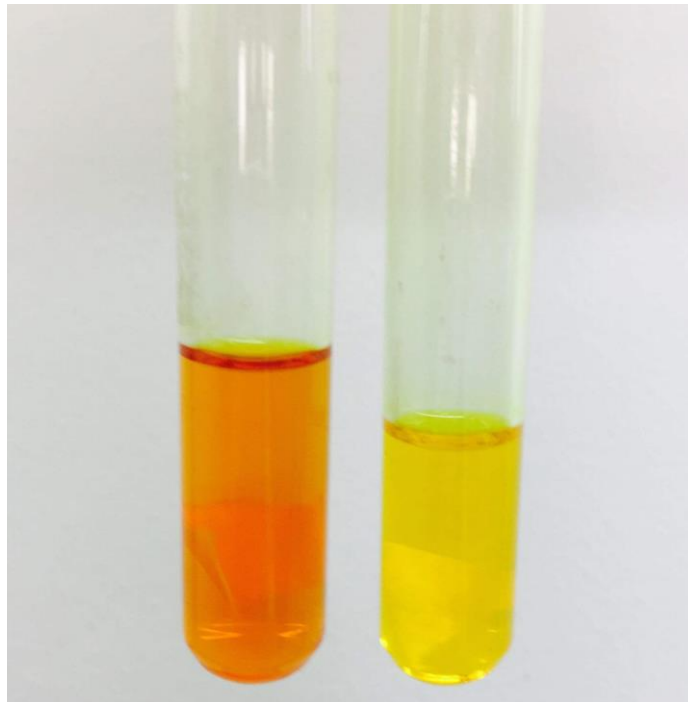


Figure 21: Liberation of glucose by Bacterial Enzyme: Left: Positive result; Right: Blank

3.4 Protein Estimation

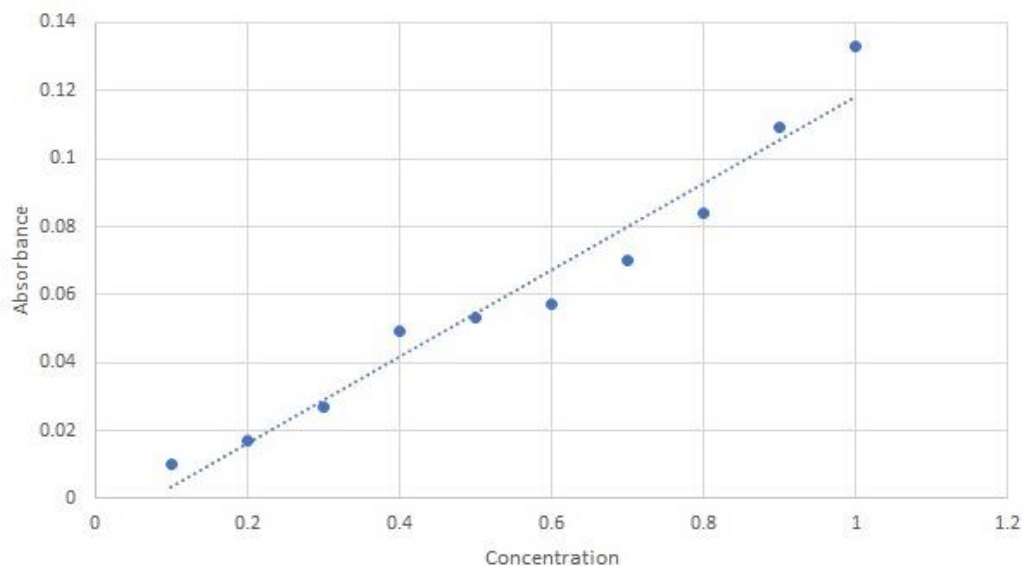


Figure 22: Standard Curve of Biuret's Assay

Regression coefficient, $r = 0.978$

Hence, $R^2 = 0.956$

Estimating protein concentration is necessary to find out specific activity of the enzyme. Using the above graph as a standard curve, the protein concentration was estimated to be 0.09 mg/mL.

Hence, specific activity of the enzyme was found to be 1.8 U/mL

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

Carbon Source of Enzyme:	Enzyme Activity (U/mL)	Specific Enzyme Activity (U/mL)
1% Carboxymethylcellulose	0.175 U/mL	1.795 U/mL
2% Carboxymethylcellulose	0.246 U/mL	1.805 U/mL
3% Carboxymethylcellulose	0.324 U/ mL	1.825 U/mL

Table 4: Difference in Enzyme Activity based on Concentration of Protein



Figure 23: Protein Concentration by Biuret's Assay: Left: Blank; Right: Positive result

3.5 Enzyme Optimization

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

Bacterial source of Enzyme	Concentration of Glucose Liberated	Enzyme Activity (U/mL)
<i>Strain PE1</i>	0.18 mg/mL	0.026 U/mL
<i>Strain PE2</i>	0.21 mg/mL	0.037 U/mL
<i>Strain PE1 + Strain PE2</i>	0.95 mg/mL	0.175 U/ml

Table 5: Difference in Enzyme Activity based on Organism

The enzyme activity was tested at intervals of 8 hours, 16 hours, 24 hours and 48 hours. The best activity was obtained after a duration of 24 hours.

Duration	Concentration of Glucose Liberated	Enzyme Activity (U/mL)
8 hours	0.4 mg/mL	0.074 U/mL
16 hours	0.4 mg/mL	0.074 U/mL
24 hours	0.95 mg/mL	0.175 U/mL
48 hours	0.6 mg/mL	0.111 U/mL

Table 6: 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	0.074 U/mL
35 °C	0.76 mg/mL	0.147 U/mL
37 °C	0.95 mg/mL	0.175 U/mL

Table 7: 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
7.0	0.895mg/mL	0.175 U/mL
9.0	0.35 mg/mL	0.064 U/mL
11.0	0.2 mg/mL	0.037 U/mL

Table 8: 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	0.95 mg/mL	0.175U/mL
25 °C	0.85 mg/mL	0.157 U/mL

Table 9: 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	0.25 mg/mL	0.046 U/mL
Carboxymethylcellulose	0.95 mg/mL	0.175 U/mL
Filter Paper	0.38 mg/mL	0.070 U/mL
Vegetable Waste	0.72 mg/mL	0.133 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	0.95 mg/mL	0.175 U/mL
2% Carboxymethylcellulose	0.133 mg/mL	0.246 U/mL
3% Carboxymethylcellulose	0.176 mg/mL	0.324 U/mL

Table 11: Difference in Enzyme Activity based on Concentration of Carbon Source

Chapter 4

Discussion

Enzymes have been found to be more efficient than chemicals in every way. Enzymes are required in very small amounts and they do not get used up in the reactions. Thus, enzymes can reduce costs due to having the ability to be reused. Furthermore, they are safer for the environment since enzymes are biochemicals and they can easily be degraded in the environment. Unlike chemicals, neither do they pile up in the environment nor do they pollute the environment or surrounding flora and fauna.

Cellulolytic bacteria were found to be abounding in various sources such as municipal wastes, bovine refuse, soil samples, etc. On culturing the extracts from various samples, numerous colonies could be found. These colonies were cultured separately and observed for cellulolytic activity. 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 obtained.

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. 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. 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 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 four to five folds. Thus, the bacteria were then used together during later stages of enzyme production.

The source of 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.

It was found that an incubation time of 24 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 binding to the substrates in the media and thus, not floating to the top along with the supernatant. However, in case of using filter paper or avicel as carbon sources, the enzyme activity was found to be the most after extracting enzyme after a 72 hour 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 7.0. Reducing or increasing the pH lowered the activity of the enzyme significantly. This might have been due to a part of the enzymes getting denatured under conditions of unfavourable pH. Thus, the optimum activity of the extracted enzyme was noted to be at a pH of 7.0.

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 enzyme must have been produced by the microorganisms.

The temperature at which the enzyme and substrate were incubated during DNS assay²⁹ also proved to be a factor for enzyme activity. Every enzyme has their own optimum temperature at which they show best efficiency. The enzyme activity showed a proportional increase while the temperature was increased steadily. Maximum activity was obtained at a temperature of 43°C. However, increasing the incubation temperature beyond 43°C affected the enzyme activity inversely. Thus, the optimum temperature for the extracted cellulase was found to be 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 colors upon being heated. The intensity of color 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 color 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 color 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

²⁹ Miller, G. (1959, 3 1). Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Analytical Chemistry*, 31(3), 426-428

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.

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 enzyme industrially profitable.

Chapter 5

Conclusion

For further studies, the genes responsible for cellulase production in the microbial strains might be identified. These days, due to the advent of CRISPR-Cas9, gene editing is no longer a dream. If this technology is used to increase the expression of the bacterial genes that produce the enzyme cellulase, the results might be more commercially viable. It might then be further studied and improved so as to make the environment safer and to let the industries in our country be more self-sufficient³⁰.

The polypeptide structure of the enzyme might also be studied to see if the synergistic 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.

The protein can be further studied to check if changing an amino acid or two causes any increase in enzyme activity. In case such a possibility is found, the genes of the microbial strains may then be edited such that the newer version of enzyme is synthesised.

Transportation of any enzyme is tough since correct temperature and environment have to be maintained. Cellulase is no exception. Importing cellulase to our country is expensive as well and raises the price of the commodities. Utilizing bacteria to produce cellulase in our country on a large scale can certainly reduce the cost incurred. The enzyme would not need to be transported extreme distances since it would be produced within the country. Multiple production units can be set up across the various large

³⁰ Panchapakesan, A., & Shankar, N. (2016). Fungal Cellulases: An Overview. In A. Panchapakesan, & N. Shankar, *New and Future Developments in Microbial Biotechnology and Bioengineering: Microbial Cellulase System Properties and Applications* (pp. 9-18). Elsevier Inc

cities as well. This will ensure the cost of enzyme remains low and thus the commodities, especially of the textile industry since Bangladesh has a sizeable sector in the garments industry, also become cheaper and more readily available³¹. Furthermore, if production exceeds demand, Bangladesh can then export enzymes in the global market. This would also further the country's economic development.

Moreover, using vegetable and paper wastes to produce cellulase can yield a two-fold benefit. 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 in the environment by utilizing 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 safer environment for future generations. This can certainly improve the living standards of the country and promote national development as a whole.

³¹ L. R. de Souza Moreira, D. Lo Sciuto and E. X. F. Filho, "An Overview of Cellulose-Degrading Enzymes and Their Applications in Textile Industry," in *New and Future Developments in Microbial Biotechnology and Bioengineering: Microbial Cellulase System Properties and Applications*, Elsevier Inc., 2016, pp. 165-175

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Appendix A.

1. Preparation of Carboxymethylcellulose Agar Medium³²

Reagents required:

- | | |
|---------------------------------|-------|
| • Ammonium Dihydrogen Phosphate | 0.1% |
| • Magnesium Sulphate | 0.1% |
| • Potassium Chloride | 0.02% |
| • Yeast Extract | 0.1% |
| • Carboxymethylcellulose | 2.6% |
| • Agar | 0.3% |
| • Distilled Water | |

Procedure:

- The salts were measured and added into distilled water.
- The mixture was heated until the salts were thoroughly mixed.
- Carboxymethylcellulose and agar was added into the mixture and heated while stirring occasionally.
- The mixture was heated until the components were completely and evenly dissolved.
- The media was then autoclaved and poured into sterile petri dishes.

³² "CMCA (Carboxymethylcellulose agar) - Bugwoodwiki," [Online]. Available: [https://wiki.bugwood.org/CMCA_\(Carboxymethylcellulose_agar\)](https://wiki.bugwood.org/CMCA_(Carboxymethylcellulose_agar))

2. Preparation of Carboxymethylcellulose Broth

Reagents required:

- Ammonium Dihydrogen Phosphate 0.1%
- Magnesium Sulphate 0.1%
- Potassium Chloride 0.02%
- Yeast Extract 0.1%
- Carboxymethylcellulose 2.6%
- Distilled Water

Procedure:

- The salts were measured and added into distilled water.
- The mixture was heated until the salts were thoroughly mixed.
- Carboxymethylcellulose was added into the mixture and heated while stirring occasionally.
- The mixture was heated until the components were completely and evenly dissolved.
- The media was then poured into a number of Erlenmeyer flasks and autoclaved

3. Preparation of Filter Paper Broth

Reagents required:

- Ammonium Dihydrogen Phosphate 0.1%
- Magnesium Sulphate 0.1%
- Potassium Chloride 0.02%
- Yeast Extract 0.1%
- Filter Paper
- Distilled Water

Procedure:

- The salts were all measured and added into distilled water.
- The mixture was heated lightly in order to evenly dissolve the salts.
- The mixture was cooled and distributed into a number of Erlenmeyer flasks.
- A sheet of 90mm filter paper was then added to every flask.
- The flasks were then autoclaved.

4. Preparation of Dinitrosalicylic Acid

Reagents required:

- Dinitrosalicylic Acid Powder 1 gram
- Potassium-Sodium Tartarate 30 grams
- Sodium Hydroxide (2N) 20 mL
- Distilled Water

Procedure:

- 1 gram Dinitrosalicylic powder was added to 50 mL distilled water and mixed with a glass stirrer.
- 30 grams of Potassium-Sodium Tartarate was slowly added into the mixture and stirred.
- 20 mL of 2N Sodium Hydroxide was added into the mixture and stirred continuously.
- The rest of the distilled water was added and the final volume was brought up to 100 mL.
- After the reagents were thoroughly mixed, the mixture was transferred to an amber reagent bottle and stored in the refrigerator.

5. Preparation of Biuret's Reagent

Reagents required:

- | | |
|------------------------------|-----------|
| • Sodium Hydroxide | 9 grams |
| • Copper Sulphate | 0.45 gram |
| • Potassium-Sodium Tartarate | 1.8 grams |
| • Distilled Water | |

Procedure:

- 9 grams of Sodium Hydroxide was added to distilled water to make the volume 90 mL.
- 0.45 gram of Copper Sulphate and 1.8 grams of Potassium-Sodium Tartarate were added into a separate flask of distilled water and the volume was brought up to 150 mL.
- Both the solutions were mixed thoroughly.
- Distilled water was added to bring the mixture up to 300 mL.

Appendix B.

Biochemical Tests³³:

1. Gram Staining

This test was carried out to find out the gram sensitivity of the obtained microbial strains as well as to observe cell morphology, size and arrangement. Smears were made on clean glass slides from fresh cultures of the microorganisms. The smears were heat fixed. Crystal violet solution was poured on the slides and allowed to stay for a minute. After washing with distilled water, the slide was flooded with Gram's Iodine solution and kept for a minute. The slides were washed off with 90% ethanol by holding it at an angle of 45 degrees to ensure that the bacteria was not washed off. The slides were then flooded with Safranin solution and kept for 45 seconds. The slides were then washed off and air dried. After drying they were observed under a microscope.

A deep violet/purple colour indicates the organism to be Gram positive, whereas, Gram negative organisms are stained pink. The difference in staining pattern is determined by the cell wall structure of the microorganisms. In case of Gram positive organisms, the presence of a large number of peptidoglycan layers keep the primary stain and mordant complex from coming out of the cell after decolourisation by alcohol washing. On the other hand, Gram negative organisms do not have a thick peptidoglycan layer. Their cell walls are composed of 1 - 2 layers of peptidoglycan and a layer of lipopolysaccharide. On washing with alcohol, the lipopolysaccharide layer dissolves and the thin peptidoglycan layer is perforated. Thus, the Crystal Violet - Iodine complex

³³ Microbiology Lab: MOLB 2210. (n.d.). Retrieved from https://www.uwyo.edu/molb2210_lab/info/biochemical_tests.htm?fbclid=IwAR1rf9GTh1gXq4-823iTQy_3XrB5frc2NzWKOq_YN1hBzFIB4iUWqiYLyrE#sulfur

comes out of the cell. The counterstain, safranin, can now penetrate the cell. The cell thus gets stained a pink colour.

2. Phenol Red Carbohydrate Broth

This test is used to observe whether or not a microorganism can utilise a particular carbohydrate as an energy source. The ability of the microorganism to breakdown pyruvate into Hydrogen and Carbon dioxide is also tested.

In order to perform this test, the carbohydrate of choice is added to a solution of phenol red broth. In case the microorganism can utilise the provided carbohydrate, acid is released as a by-product. Phenol red acts as an indicator and turns yellow in the presence of acid. Thus, carbohydrate fermentation can be observed. Furthermore, whether or not the microorganism produced gas as a by-product can also be observed with the help of a Durham tube. If the microorganism is able to ferment pyruvate - the end product of glycolysis - into Hydrogen and Carbon dioxide, the gas will be trapped inside the Durham tube. A bubble that forms in the Durham tube will be an indicator of gaseous by-product formation.

In this experiment, four different carbohydrate sources - namely, Dextrose, Lactose, Sucrose and Mannitol - were used for testing fermentation capabilities of the microorganisms.

3. Mannitol Salt Agar

This test can determine whether or not the organism can utilize mannitol as a carbon source. This media acts as a differential media as well as a selective media. The principles of this media are the same as that of phenol carbohydrate broth. Thus, microorganisms that can utilize Mannitol as a carbon source turn the media yellow upon

growth. The media remains red in case of growth of microorganisms that are unable to ferment mannitol.

4. Indole test

This test is used to detect bacteria that can produce the enzyme tryptophanase. This enzyme helps the bacteria to utilise the amino acid tryptophan. Indole is released during this reaction. Using Kovac's reagent, one can identify the presence of indole. In the presence of indole, the reaction with Kovac's reagent forms a red dye called rosindole. This dye is what gives the characteristic red colour to positive indole tests.

5. Methyl red test

This test, developed in 1915 by Clark and Lubs, deduces whether or not a microorganism utilises mixed acid pathway while fermenting glucose. Different organic acids (acetic acid, formic acid and lactic acid) are formed if an organism utilises this pathway. The combined acids lower the pH well below pH 4.4.

Adding Methyl Red as an indicator gives red colour if the pH is below 4.4. In case the mixed acid pathway has not been utilised, the pH will not be lowered to a large extent. The pH in that case usually remains above 6.0. Methyl Red gives a yellow colour in that situation, indicating the absence of enough acids to overcome the phosphate buffer wall.

6. Vogues-Proskauer test

Whether an organism can utilise glucose to form acetoin, a precursor of 2,3 butanediol, is deduced from this test. If the results are positive, it means that the organism utilises the 2,3 butanediol pathway for glucose fermentation.

Adding α -naphthol and Potassium hydroxide to a tube of microorganisms cultured in MR-VP broth can help to detect the presence or absence of acetoin in the tube. If a pinkish-brownish colour is noted, the test result is positive. However, brownish-yellow colour indicates a negative result.

An organism can usually only utilise one of the pathways to ferment glucose. Hence, Methyl Red and Vogues-Proskauer tests usually give results opposite to each other. If an organism is positive for Methyl Red test, it is likely to be negative for Vogues-Proskauer test and vice versa. However, it is to be noted that this is not always the case.

7. Citrate test

This test utilises Simmons Citrate agar media in order to check for citrate utilisation by the microbes. If the bacteria can produce the enzyme Citrase, citrate is hydrolysed into oxaloacetic acid and acetic acid. Oxaloacetic acid then gets hydrolysed into pyruvic acid and carbon dioxide. In case carbon dioxide is produced, it reacts with the components of the medium and gives an alkaline compound. Bromothymol blue, which is used as an indicator in the media, turns blue in alkaline pH. Thus, in the event that citrate is utilised by the microbe, the media turns blue instead of the initial green colour. If the microbe is unable to utilise citrate, the media will remain green as it was prior to inoculation.

8. Catalase Test

Organisms with the ability to produce the enzyme catalase can be identified using this test. Catalase enzyme can break down Hydrogen Peroxide (H_2O_2) into water and oxygen gas. The reaction that takes place during the breakdown is as follows:



Hydrogen peroxide can be extremely harmful and toxic to the microorganism. Hence, production of catalase enzyme is like a detoxifying procedure.

Presence of catalase enzyme can be detected by using a 3% solution of Hydrogen peroxide. The Hydrogen Peroxide solution is placed on a glass slide and a small amount of microbial fresh culture of the test organism is added. Production of oxygen gas would lead to bubbling, a proof that the organism produces catalase. In case no catalase is produced, no bubbling occurs.

9.Oxidase Test

This is a test to determine whether or not the organism being tested produces the enzyme Oxidase or not. Oxygen is toxic to a lot of micro-organisms. The effect can be extremely lethal. In order to tackle this issue, oxidase acts as part of the electron transport chain and transfers electrons to Oxygen, thus turning it into water.

N,N-dimethyl-p-phenylenediamine is the compound used to test the presence of oxidase. A small amount of fresh microbial culture is smeared on a filter paper. Two to three drops of the reagent is then poured on the smear. After 10 - 30 seconds, the colour of the filter paper changes in case oxidase is produced. In case oxidase is not produced, no colour change will be observed.

The readings are not to be taken after more than 30 seconds have passed. This is to avoid false positives upon reaction of the reagent with atmospheric oxygen.

10.Starch Hydrolysis Test

Some bacteria are able to hydrolyse amylose and amylopectin present in starch. Hence, they are able to utilise starch as an energy source. This is possible due to the production of enzymes oligo-1,6-glucosidase and α -amylase. The huge size of amylopectin and

amylose molecules make it difficult for them to pass through the bacterial cell wall. Thus, unless they are broken down into glucose, they cannot be utilized. Hence, organisms with the ability to produce oligo-1,6-glucosidase and α -amylase can utilise starch as a source of energy.

Flooding the culture plates with Gram's iodine gives us the results. If the microorganism is able to hydrolyse starch, clear zones can be observed after flooding with Gram's Iodine. A clear zone formed around the colonies is an indicator that starch hydrolysis has taken place.

11. Motility test

This test is used to check whether the microorganism has any locomotive organs such as flagella, and thus are capable of motility. A soft agar such as Motility Indole Urease agar can be used for the test. The organism is inoculated by stabbing the media once with a needle.

In case the organism is motile, the organism grows and spreads out. The entire media then turns turbid due to the growth. However, if the microorganism is non-motile, they cannot grow away from the stab line. Thus, growth will only be observed along the stab line and the rest of the media will remain clear.