

PRODUCTION AND CHARACTERIZATION OF CELLULASE FROM BACTERIA SPECIES USING LIQUID FERMENTATION

PROJECT WORK

Submitted in partial fulfillment of the requirements for the award of
Bachelor of Science degree in Microbiology

By

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SCHOOL OF BIO AND CHEMICAL ENGINEERING

DEPARTMENT OF BIOTECHNOLOGY

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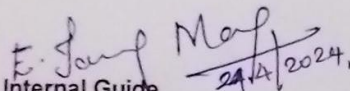
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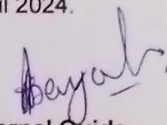
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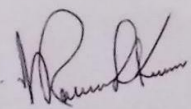
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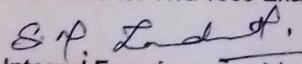
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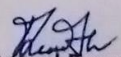

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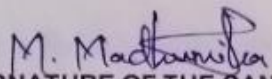

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DECLARATION

I, **Madhumitha. M** (41300021) hereby declare that the Project Report entitled "PRODUCTION AND CHARACTERIZATION OF CELLULASE FROM BACTERIA SPECIES USING LIQUID FERMENTATION" done by me under the guidance of **Mrs. E. Jancy Mary** Assistant Professor Internal guide, Department of Biotechnology and **Dr. A. Dayanandan** Professor (Research) External guide at Centre for Molecular and Nano Medical Science, Sathyabama Institute of Science and Technology, Chennai, is submitted in partial fulfillment of the requirements for the award of Bachelor of Science degree in Microbiology.

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ABSTRACT

Cellulase producing bacteria were isolated from soil and identified as *Bacillus Species*. Optimization of the fermentation medium for maximum cellulase production was carried out. The culture conditions like pH, CMC Concentration, carbon sources, and nitrogen sources were optimized. The optimum conditions found for cellulase production were 37°C at pH5 with lactose as carbon source, ammonium sulfate as nitrogen source, and 1.5% as CMC concentration production of cellulase. The cellulase was produced using the optimized condition. The crude enzymes were purified using ammonium sulfate precipitation and dialysis. Total enzyme activity of in crude enzyme was 4.78 U/ml and 27.456 U/ml of total activity after ammonium sulfate precipitation, 7.758 U/ml of total activity as dialysis. The Characterization conditions found for cellulase production were 37°C at pH4 and Manganese as Metal ions and Triton as Detergents. The purified Cellulase was applied to improve fibre smoothness in cotton fabric and hydrolytic potential of cellulase to hydrolyze cellulase substrate which is useful in paper industry was studied.

KeyWords: Cellulase, Soil, Fermentation, *Bacillus sp*, Carboxymethyl cellulase

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LIST OF ABBREVIATIONS

CMC	Carboxymethyl Cellulase
DNS	3, 5- dinitrosalicylic acid
K_2HPO_4	Dipotassium phosphate
$MgSO_4 \cdot 7H_2O$	Magnesium Sulfate heptahydrate
OD	Optimal Density
P	Product
S	Substrate
SDS	Sodium dodecyl Sulfate

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CHAPTER 1

INTRODUCTION

Enzymes are biological catalysts, sometimes referred to as biocatalyst that quicken biological reactions in living things. They can also be taken out of cells and utilized as catalysts for a variety of significant commercial operations. They are used in cleaning products and washing powders, for example, and are essential parts of analytical tools and tests used in forensics, medical, and environmental fields.

They are also important in the modification of antibiotics and the production of sweeteners. The word "enzyme" was originally used in 1878 by the German scientist Wilhelm Kuhne. (Robinson PK *et al.*, 2015)

1.1 ENZYME ARE POTENT CATALYST

It is believed that the best method to characterize the enormous catalytic activity of enzymes also known as turnover rate, turnover frequency, or turnover number. In a given amount of time usually a minute or a second, this constant indicates the number of substrate molecules that one enzyme molecule may convert into product. (Robinson PK *et al.*, 2015)

1.2 ENZYME ARE SPECIFIC CATALYST

Enzymes catalyze the conversion of a single kind of substrate molecule into a product molecule, or at most a range of related types, demonstrating amazing selectivity in addition to their great potency as catalysts. There are several enzymes that are particular to groups. For example, a common enzyme is alkaline phosphatase. It is capable to eliminating phosphate groups from the substrates. (Robinson PK *et al.*, 2015).

1.3 ENZYME NAME AND CLASSIFICATION

Although some proteolytic enzymes (trypsin, chymotrypsin, papain, etc.) frequently include the suffix -in, popular names for enzymes, also known as "trivial names," typically end relate to the reaction they catalyze. An enzyme's substrate of

choice is often specified by its trivial name, which includes things like glucose oxidase, alcohol dehydrogenase. A few meaningless names are such catalase, and diastase. (Robinson PK *et al.*, 2015).

The Enzyme Commission was established by the International Union of Biochemistry in response due to the increasing complexity and irregularity in enzyme naming. Published in 1961, the first Enzyme Commission Report offered a methodical approach to enzyme naming. Details on around 3,200 distinct enzymes were included in the sixth edition, which was released in 1992. Supplements released on a yearly basis have since increased this number to over 5,000. (Robinson PK *et al.*, 2015).

1.4 ENZYME STRUCTURE AND SUBSTRATE BINDING

It is possible for the amino acids to stack as more polypeptide chains. The active site a little region that is incorporated that are bent and folded to create a particular three-dimensional shape. Binding of the substrate occurs. Less than ten of the component amino acids may be involved in the active site. (Robinson PK *et al.*, 2015).

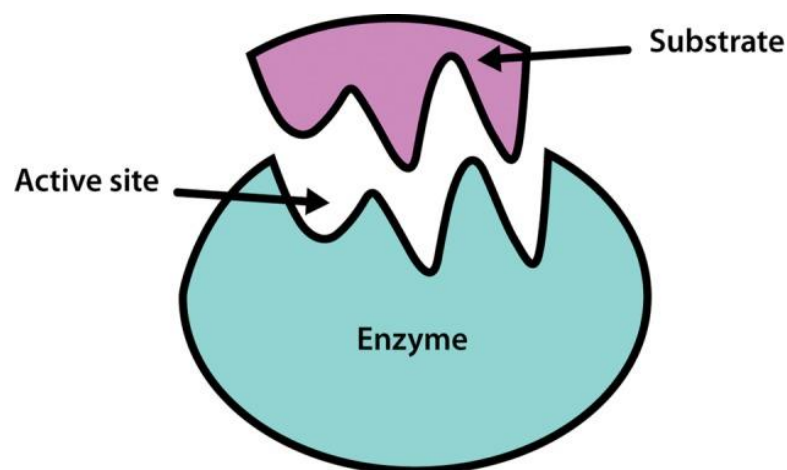


Fig 1.1: Enzyme Structure and Substrate Binding

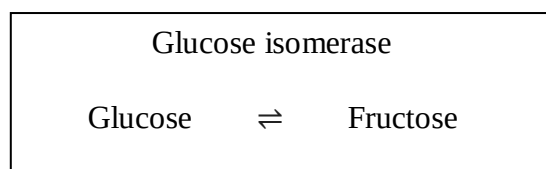
1.5 ENZYME AND REACTION EQUILIBRIUM

Enzymes only speed up the process of establishing reaction equilibrium; they do not essentially alter the energetics or structure of the products or reagents. So let's start by defining what is meant by "chemical equilibrium."

This is oversimplifying the situation because it is always more accurate to express this response as follows: $S \rightleftharpoons P$

This suggests that there is an equilibrium. It can be most beneficial to examine a response. The equilibrium point is quite central in order to comprehend this idea. (Robinson PK *et al.*, 2015).

For example:



1.6 CELLULASE

Cellulases are one of the widely used industrial enzymes which are commercially available for more than 30 years. (Gupta R., *et al.*, 2011). Cellulose is commonly degraded by an enzyme called cellulase. Numerous microorganisms, primarily bacteria and fungi, generated this enzyme. For the production of cellulase, bacteria that grow more quickly than fungi can be used. It is uncommon to use microorganisms that generate cellulase. Some bacterial taxa possess the capacity to degrade cell walls, such as *Bacillus*, *Micrococcus*, *Cellulomonas*, *Cellvibrio*, and *Pseudomonas sp.* (Nakamura K, *et al.*, 1982).

Enzyme production in microorganisms is strictly regulated, and this regulation can be tightened to boost the amount of enzymes produced. There seems to be a complicated relationship between cellulase yields and a number of factors, including inoculum size, temperature, pH level, inducer presence, medium additions, (Palavesam A. *et al.*, 2006).

1.7 GLOBAL DEMAND FOR CELLULASE

Enzymes are highly beneficial for numerous industrial operations. The sector has witnessed a discernible increase in demand in recent years due to their numerous applications. The high level of consumer demand for enzymes has been discovered to be caused by a number of important reasons, according to market data from 2017 on the demand for enzymes worldwide. For instance, rising per capita wealth in developing nations contributes significantly to an increase in industrial applications relating to consumers. (Lee Y.*et al.*, 2008).

1.8 MOLECULAR STRUCTURE OF CELLULASE

Fungus, bacteria, protozoa, and actinomycetes are some of its sources. Both catalytic and non-catalytic modules make into the structure of cellulase. Cellulase catalytic modules have been categorized into multiple families based on crystal structures and amino acid sequences. The non-catalytic carbohydrate binding modules.

Both aerobic and anaerobic bacteria manufacture these enzymes. There are two types of cellulase systems: complicated and non-complex. A non-complex cellulase system, which is a conglomeration of extracellular collaborating enzymes, was produced by aerobic cellulolytic microbes. Anaerobic microbes create complex cellulase systems called cellulosomes. (Ratnayake, *et al.*, 2019).

1.9 BREAKDOWN OF CELLULOSE BY CELLULASE

The glycosidic connections that hold the β -Dglucosyl residues of cellulose together are also dissolved when the enzyme cellulase hydrolyzes cellulose. The general structure of cellulase comprises two distinct globular domains: a cellulose-binding domain that enhances the enzyme's ability to attach to insoluble macromolecular cellulose, despite lacking catalytic activity, and a catalytic region that is in charge of the actual hydrolysis reaction. (Sukumaran *et al.*, 2005).

1.10 FERMENTATION PROCESS FOR PRODUCTION

1.10.1 Submerged Fermentation

For the purpose of producing cellulase enzymes, cellulolytic species were evaluated using a basic salt medium that contained carboxymethyl cellulose and had been sterilized. Yeast and sterile production medium were placed in flasks, and fermentation took place. Common inoculums. A revolving shaker was used to cultivate the microorganisms in the flasks (Lokhande, *et al.*, 2017)

1.10.2 Solid State Fermentation

This process was done in a flask with distilled water and coir waste in it. After being cleaned, the flasks were allowed to reach room temperature. After the inoculum was added and well blended, the mixture was incubated in a humidified incubator. The flasks were periodically given a light shaking. (Mrudula, *et al.*, 2011).

1.11 APPLICATION

Cellulases have been significant biocatalysts for a long time. Many other industries have demonstrated how well they may be used. Many sectors, including textiles, paper and pulp, laundry and detergent, agriculture, medicine, and food and feed, rely heavily on the utilization of microbial cellulases. Enzymes find their main uses in the food and beverage, textile, animal feed, and biofuel industries. (Kuhad *et al.*, 2010)

1.11.1 Cellulase in Paper and Pulp Industry

The paper and pulp industry has employed cellulase for a number of uses. Significant energy savings were achieved through the enzymatic modification of coarse mechanical pulp employing cellulase. Additionally cellulases have been employed to modify the characteristics of fibers in order to enhance the paper industry's beat ability, run ability, and drainage. It has been found that cellulases are the most efficient at recycling used paper from books, periodicals, and newspapers.

This recycling could add value by repurposing the fiber by deinking it and using it to make new newspapers or ethanol. (Kuhad *et al.*, 2010)

1.11.2 Cellulase in Textile and Laundry Industry

Bio polishing and bio finishing procedures have in common is similarity. The growth of fuzz on the surface of cellulosic fiber materials, such as cotton and linen, causes these materials to constantly lose their look. The short strands sticking out from the fabric's surface caused fuzz. Sometimes fuzz is haphazardly sewn to the fabric, giving it a ball-like appearance that detracts from the fabric's attractiveness. We refer to this as pilling. The main goal of the biopolishing procedure is to eliminate cotton microfibrils. It adds a glossy, smooth look that improves the color, hand feel, and appearance of the fabric. Additionally, this is improving the fabric's hydrophilicity, moisture absorbance, and color brightness. (Anish. R *et al.*, 2007).

CHAPTER 2

LITERATURE SURVEY

The author reported by many industrial and biotechnological applications will benefit from the high activity and stability of cellulase enzymes at high temperatures and neutral to alkaline pH levels. (Sonia Sethi, *et al.*, 2013). Cellulase can be produced by this bacterium in a submerged culture. The excellent option would be the *Bacillus* isolate. Source of cellulase if additional research is done on medium optimization and genetic improvement. (Md Arafat, *et al.*, 2018). Worldwide, a number of companies produce cellulases for commercial use. These sectors use the enzymes in a wide range of applications, including food, agriculture, animal feed, fermentation, pulp and paper, and textiles. Modern biotechnology methods will make novel enzymes and new enzyme applications for the many industries available, particularly in the field of microbial genetics. There may be further areas where cellulase research needs to progress, such as enhancing cellulase activity or using protein engineering to give desirable characteristics to enzymes. (Kuhad RC, *et al.*, 2011).

We concentrated our research efforts primarily on screening from natural habitat for microbe isolation in order to establish an economically viable technology. This generates a significant number of cellulases. Bacteria that produce cellulase were isolated from soil. (Rasul *et al.*, 2015). Technologies for pre-treating cellulosic biomass for a better microbial attack, ways to produce cellulases economically, and, lastly, organism development strategies to improve the properties of enzymes to increase their specific activities, process tolerance, and thermal stability are the future challenges for producing cellulases. (Sudha H. *et al.*, 2022). Furthermore, the ability of the crude enzyme to degrade filter paper has been used to characterize its CMCase activity. (Islam K, *et al.*, 2016). Bacteria *B. licheniformis* strain PANG L is a thermo-acidic cellulase-producing bacteria. Its optimal activity was observed at pH 5 and 60°C, it exhibited high stability at pH ranges of 3–5. (Shyaula M, *et al.*, 2023). The biotechnology of cellulases and related enzymes has advanced dramatically, garnering interest from all over the world. Cellulases, hemicellulases, and pectinases are currently widely employed in research and development as well as the food, beverage, textile industries, as well as the paper and pulp sectors. (Bhat

et al., 2000). The goal of the current experiment was to improve cellulase production by the cellulolytic bacteria by optimizing nutritional and environmental conditions. A variety of staining techniques, biochemical analysis, and partial purification were used to identify the cellulase-producing *Bacillus* species that was isolated from cow dung. (Shanmugapriya. K et al., 2012). It is possible that bacteria in this belt that produce cellulase will be useful in creating the crucial commercial product cellulase. The additional usage for municipal solid waste biodegradation. The Vidarbha region's saline belt is a rich potential source of bacteria that produce cellulase, which is beneficial in a variety of industries including agriculture and pharmaceuticals (Shilpa Lokhande et al., 2017).

Isolated and identified the cellulase responsible for soil based bacterial production in our work. The 16S rRNA sequencing was verified, and the bacterial isolates were characterized using morphological and biochemical testing. They were identified as *Klebsiella oxytoca* strain Z63, *Bacillus cereus* strain Z9, and *Bacillus licheniformis* strain Z7. Bacteria that produce cellulose can be isolated, screened, and identified to provide a great foundation for the discovery of these useful enzymes in Egypt's industrial and agricultural sectors (Abdel-Aziz S. H. et al., 2021). There are many different kinds of cellulolytic bacteria in soil, some of which have not yet been identified. This study aimed to identify putative cellulose producing soil sample isolates of bacteria. The ability to break down cellulose and other cellulolytic properties led to the selection of particular isolates (Md. Arju Hossain, et al., 2021).

Soil, landfills, and waste material surfaces are home to a diverse array of cellulolytic bacteria, some of which are still awaiting isolation. Soil samples from the dump area were used in this study primarily to isolate putative cellulase-producing bacterial isolates. To improve the quality and quantity of cellulase enzyme, more extensive research based on molecular techniques must be conducted. This could have advantages for a number of industries, including healthcare and the textile sector (Indra Pratap Singh, et al., 2023). The purified cellulase was found to have great substrate selectivity on CMC, indicating that it is an endo- β -1, 4-glucanase. There may be various industrial uses for the refined cellulase. (Islam, F. et al., 2018). One of the most often utilized enzymes needed to prepare fermented foods is cellulase. In addition to the food and starch sectors, where there is a constant need for them, they are also utilized in a number of other industries, including paper, pulp,

textiles, and more. (Sandeep Sharma, *et al.*, 2014). There is much work to be done on the use of advantageous for food, pharmaceutical, and detergent sectors. Numerous commercial and biotechnological uses will benefit from the high activity, stability, and neutral to alkaline pH of cellulase enzymes. (Chaudhary R. K *et al.*, 2023).

Aspergillus fumigatus, *Aspergillus terreus*, and *Aspergillus flavus* are strong cellulase producers, according to this study, and they can contribute to cellulase synthesis in optimal temperature and pH levels. By adjusting for these variables, it is possible to investigate how temperature and pH affect the creation of the cellulase enzyme. For fungi to develop significantly and exhibit the synthesis of cellulase enzymes, which can be used for a variety of industrial uses, they require the ideal pH and temperature (Sheetal Barapatre *et al.*, 2020). It was discovered that 32°C was the ideal temperature. The organism must expend more energy for upkeep at higher temperatures, whereas nutrient transfer is hampered at lower temperatures (Pirt, 1975). *Aspergillus fumigatus*'s cellulase activity from mangrove soil is demonstrated in the study. Furthermore, sawdust may be utilized as a substrate to maximize the synthesis of cellulase. (Khaleel K.M *et al.*, 2011)

CHAPTER 3

AIM AND OBJECTIVES

3.1 AIM

- ❖ To effectively produce & characterize cellulase from bacterial species using liquid fermentation

3.2 OBJECTIVES

- ❖ To isolate bacteria from soil sample
- ❖ To screen & identify cellulase producing bacterial strains
- ❖ To optimize and produce cellulase using liquid fermentation and estimate the enzyme activity
- ❖ To characterize cellulase for enhancing the stability & activity
- ❖ To apply the purified cellulase to improve fibre smoothness in fabric and paper industry

CHAPTER 4

MATERIALS AND METHODS

4.1 MATERIALS

Nutrient agar and CMC, LB broth for culturing bacterial sample. Petridishes was used for plating of culture. Plate reader, pH meter and dry bath, Centrifuge, Magnesium, zinc, Manganese, potassium, Ferrous are used for effect of metal ions. SDS, Tween (80), Triton are used for effect of detergents.



Fig 4.1 Centrifuge



Fig 4.2 Spectrophotometer with Microtiter plate Reader

4.2 METHODOLOGY

4.2.1 Collection of soil sample

The soil sample was collected in the sterile & transported carefully to the laboratory.

4.2.2 Isolation of Microorganisms

Using Nutrient agar medium and the serial dilution procedure, cellulase producing bacteria have been isolated from soil. On the nutrient agar, each colony was streaked separately. The pure culture was kept at 4°C after being streaked in a slant.

4.2.3 Screening for cellulase producing Bacteria

The cellulolytic activity of each pure bacterial strain was assessed on the medium. The bacterial culture was streaked on 1% CMC agar that was incubated at 37°C / 72 as part of the method. 1.0% peptone, 1.0% carboxymethyl cellulose (CMC), 0.2% K₂HPO₄, 1% agar, 0.03 % MgSO₄ · 7H₂O, 0.25 % (NH₄)₂SO₄, and 0.2% gelatin at PH 7 are the ingredients of the medium used for cellulolytic bacteria. The plate was incubated for twenty-four hours at 37°C.

After 15 minutes of flooding the plates with an aqueous solution containing 0.1% Congo red, they were cleaned with 1 M NaCl. The diameter of the clear zone surrounding colonies on CMC agar was measured to show the cellulose activity of the organisms.

4.2.4 Cellulase producing on liquid medium

The effective bacterial isolate was used to create cellulase by cellulose synthesis on liquid medium. First, 100 µl of LB broth (LURIA BERTANI) bacterial culture was cultured for 48 hours at 37°C with 1% CMC as the substrate. Centrifugation was used on the cultures (5000 rpm/10min). For subsequence analysis, the crude enzyme-containing supernatant was kept at 4°C.

4.2.5 Evaluation of cellulase activity

By measuring the amount of reducing sugar released, the 3, 5 DINITROSALICYLIC acid (DNS) technique was used to determine CMC activity. Two milliliters of supernatant and one milliliter of acetate buffer (50 mM, pH 4) containing one percent CMC were combined to create the reaction mixture.

Following a 30-minute incubation period at 37°C, the reaction was halted by adding 2 milliliters of (DNS) reagent, boiling for five minutes, and then cooling to stabilize the color. At 575 nm, the sample's optical density (OD) was measured. Using glucose as a benchmark, 1 unit (U) of enzyme activity was defined as the amount of enzyme releasing 1 μmol of reducing the sugar every minute.

4.2.6 Purification of enzyme

4.2.6.1 Ammonium sulfate precipitation

The crude enzyme that was generated was treated with solid ammonium sulfate until it achieved 70% saturation. The mixture was kept overnight at 4 °C in a magnetic stirrer. This was followed by centrifuging the mixture and further purifying the pellet by dissolving it in 50 mM sodium phosphate buffer saline at pH 7.0. A dialyzer was used to test the partly purified enzyme against the phosphate buffer.

4.2.6.2 Dialysis

After three hours in a beaker against 47.5 ml of distilled water, the crude supernatant from the ammonium sulfate stage was transferred to 2.5 ml of phosphate buffer (pH 7). It was then stored in the dialysis bag. The cellulase enzyme-containing dialysis bag was then concentrated by placing it in a beaker next to sucrose crystals and refrigerated at 4 °C for additional purification.

4.2.7 Optimization of cellulase production

4.2.7.1 pH

CMC liquid medium was used to examine the effect of culture medium pH change on bacterial growth and cellulase production capability. The modified pH values are 5, 8, and 11. The culture was incubated for twenty-four hours at 37°C. A calculation was made of the enzyme activity.

4.2.7.2 Carbon source

The role of different sources, including lactose, glucose, and starch, in the medium's production is investigated. The culture was incubated for twenty-four hours at 37°C. A calculation was made of the enzyme activity.

4.2.7.3 Nitrogen source

In the process of creating the medium, the effects of several nitrogen sources, including yeast extract, peptone, urea, and ammonium sulfate at a concentration of 0.5%, were investigated. For twenty-four hours, the culture was incubated at 37°C. It was computed what the enzyme activity was.

4.2.7.4 CMC concentration

A range of CMC concentrations, including 0.5%, 1%, 1.5%, and 2%, were investigated for the medium's manufacture. The culture was incubated for twenty-four hours at 37°C. A calculation was made of the enzyme activity.

4.2.8 Characterization of cellulase production

4.2.8.1 Effect of pH

Incubating the enzyme combination with 1% CMC in the buffer allowed for the determination of the cellulase ideal pH. pH4, 7, 12, phosphate buffer, 0.1 M. 20 minutes were spent incubating the reaction mixtures at 37°C.

4.2.8.2 Effect of Metal ions

The presence of magnesium chloride, manganese and potassium chloride, zinc sulfate, and ferrous sulfate metal ions are allowed researchers to assess how different metal ions affected the activity of the enzyme. Each metal ion's concentration is 10 mM in 0.1 M phosphate buffer. For 20 minutes, the reaction mixtures were incubated at 37°C.

4.2.8.3 Effect of detergents

The presence of SDS (Sodium dodecyl Sulphate), Tween (80), and Triton detergents allowed researchers to assess how different detergents affected the enzyme activity. Each detergent has a 50 mM concentration in 0.1M phosphate buffer. For twenty minutes, the reaction mixtures were incubated at 37°C.

4.2.9 Cellulase application

4.2.9.1 Cotton fabric

A sample of about the size of a circle was added to the beaker holding the cellulase in the sodium phosphate buffer and stirred. A concentration of 2% cellulase was employed. The treatment lasted for 60 minutes at 55°C in temperature. Finally, a 10-minute hot treatment at 85 °C ended the bio polishing process. Analysis was done on the alteration. The control was kept in the same treatment without enzyme.

4.2.9.2 Filter paper

The partially purified cellulase was analysed against the cellulosic substrate to understand the degradation capacity. The substrate used in the study was filter paper. The filter paper was cut in the equal size. Three test tube was filled in with sterile distilled water. The filter paper was added to the test tube. The test tube 1 was kept as control. Test tube 2 had the partially purified enzyme (4 ml). Test tube 3 had the partially purified enzyme (6 ml). Test tubes were incubated at 37°C for 24 hrs. The change in the filter paper was analysed.

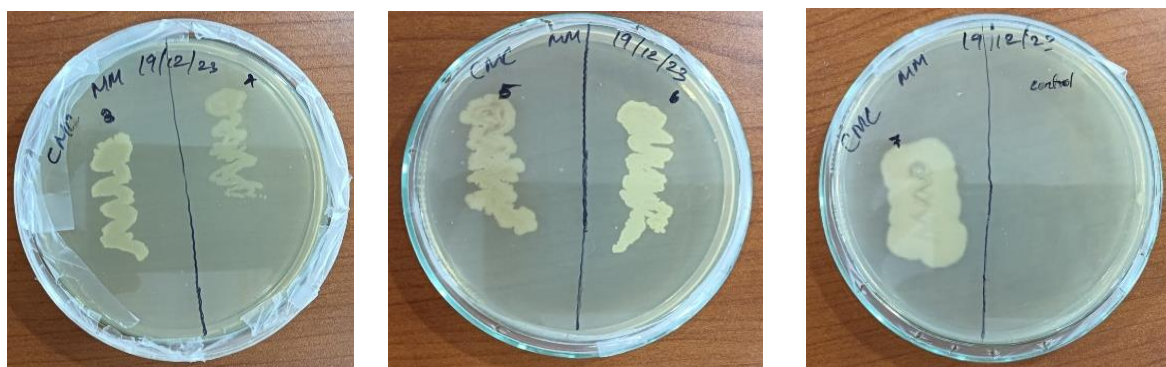
CHAPTER 5

RESULTS AND DISCUSSION

5.1 ISOLATION OF BACTERIA FROM SOIL SAMPLE

The individual colonies serial diluted plate were streaked on the specific carboxymethyl cellulose (CMC) agar medium.

Billion microorganisms, including bacteria, are known to exist in the soil. In the same way, it was stated that the different sediments abundant source of cellulosic carbon in the form of litter, which serves as a substrate for bacterial degradation, making it ideal for cellulase-producing bacteria. (Asha *et al.*, 2012).



Sample 3

Sample 6

Sample 7

Fig 5.1: Isolation of Bacterial colonies from soil sample

5.2 SCREENING OF CELLULASE FROM BACTERIA

It was possible for three isolates to degraded CMC. When the Congo red technique is used to screen the isolation plates. Sample 3 plate had a 1.5 mm clear zone, Sample 6 had 3 mm clear zone, and Sample 7 had 1 mm clear zone.



Fig 5.2: Screening of Cellulase from Bacteria

Table 5.2.1: Diameter of clear zone

SI. NO	ISOLATE	DIAMETER OF CLEAR ZONE
1	Sample 6	3mm
2	Sample 7	1mm
3	Sample 3	1.5mm

5.3 IDENTIFICATION OF BACTERIA

Since Sample 6 had the maximum zone of clearing, it was examined under a microscope for identification and for Gram staining. The bacterial strains were rod-shaped, Gram-positive *Bacillus species*.

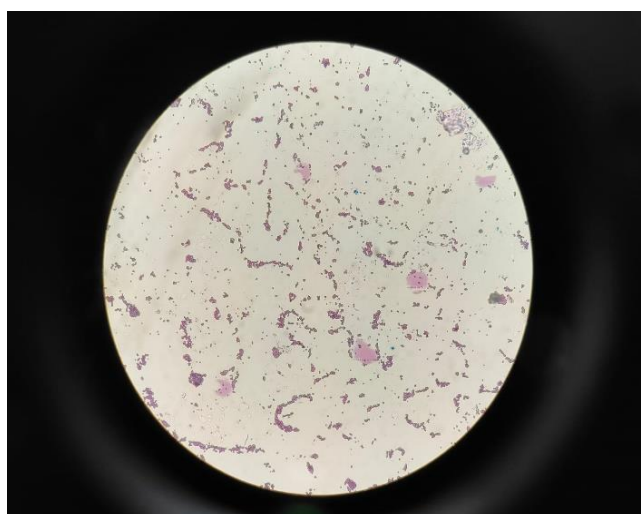


Fig 5.3: Gram Staining of Bacteria

5.4 GLUCOSE STANDARD CURVE

The standard curve was obtained using glucose. The straight line equation formula of $y = 0.1443x + 0.4931$. The Regretting coefficient formula of $R^2 = 0.9883$. These was used to calibrate enzyme activity.

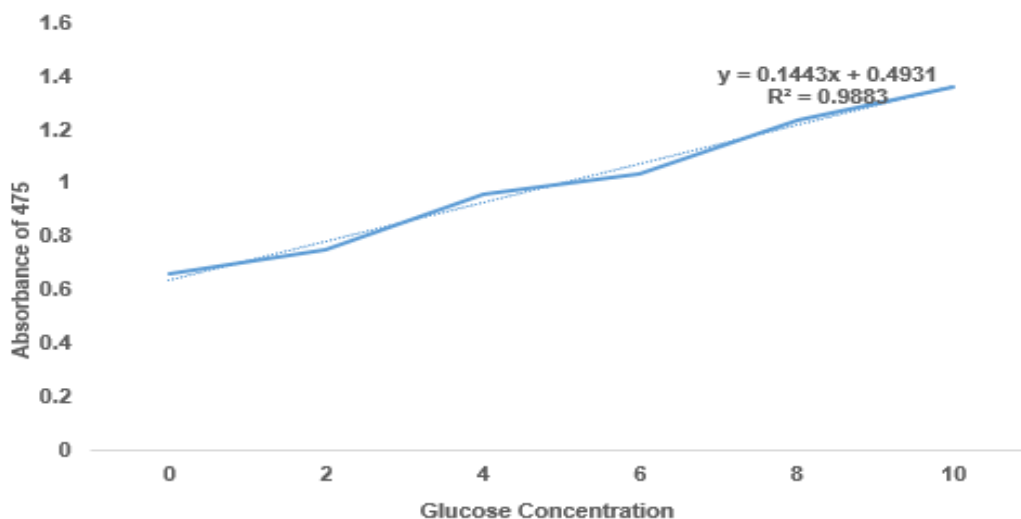


Fig 5.4: Glucose standard curve

5.5 OPTIMIZATION OF CELLULASE PRODUCTION

5.5.1pH

The enzyme activity was evaluated at three different beginning pH values 5.0, 8.0, and 11.0 while maintaining the other conditions unchanged. The ideal starting pH for the fermentation medium was 5.0, and while the amount of enzyme remained consistent within the pH 5 range, the ability to produce enzyme was greatly reduced at the initial pH of 8, 11.

The four isolates were each given varying pH media to develop in, with the highest enzyme activity being noted in the pH media for *Pseudomonas fluorescens*, *Bacillus subtilis*, and *Serratia marscens*. (Sethi et al., 2013)

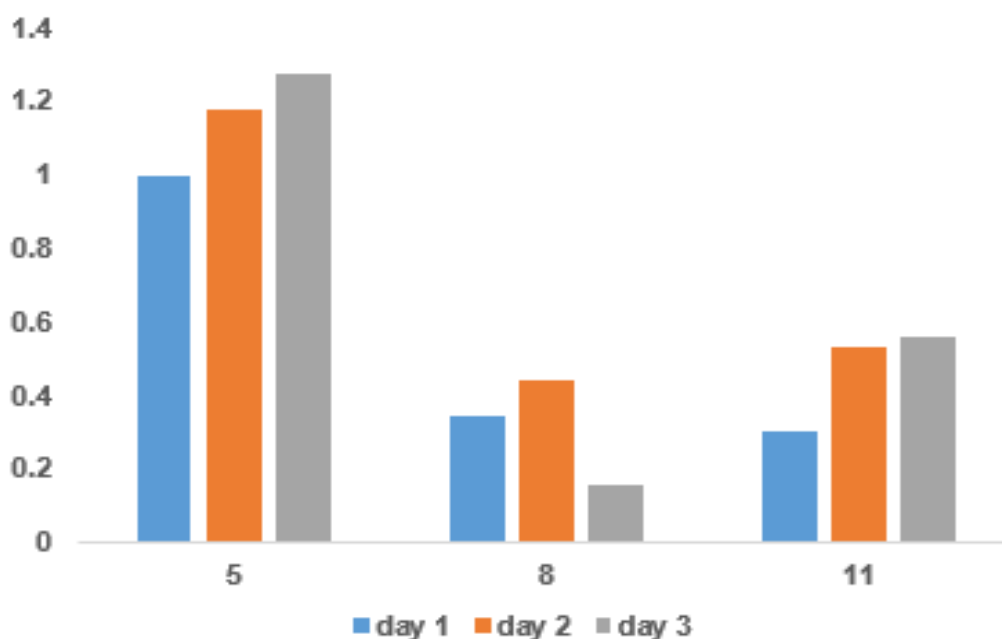


Fig 5.5: Optimization of pH in Cellulase Production

5.5.2 Carbon source

Carbon sources are essential for the growth and reproduction of bacteria. Since they are nutrients for bacterial growth. A variety of carbon sources, including glucose, lactose, and starch—the original carbon source in growth media. The greatest amount of cellulase was produced after 24 hours of incubation, according to the results, when compared to other carbon sources. After 24 hours of incubation, lactose also shown strong cellulase synthesis. Thus, it was discovered that the ideal supply for cellulase synthesis was glucose.

Glucose was the original carbon source in growth media. According to the results, after a 24-hour incubation period, glucose produced the most cellulase when compared to other carbon sources. At 24 hours of incubation, fructose and lactose likewise shown substantial cellulase synthesis. Thus, it was discovered that the optimal supply for cellulase synthesis was glucose. (Sethi *et al.*, 2013)

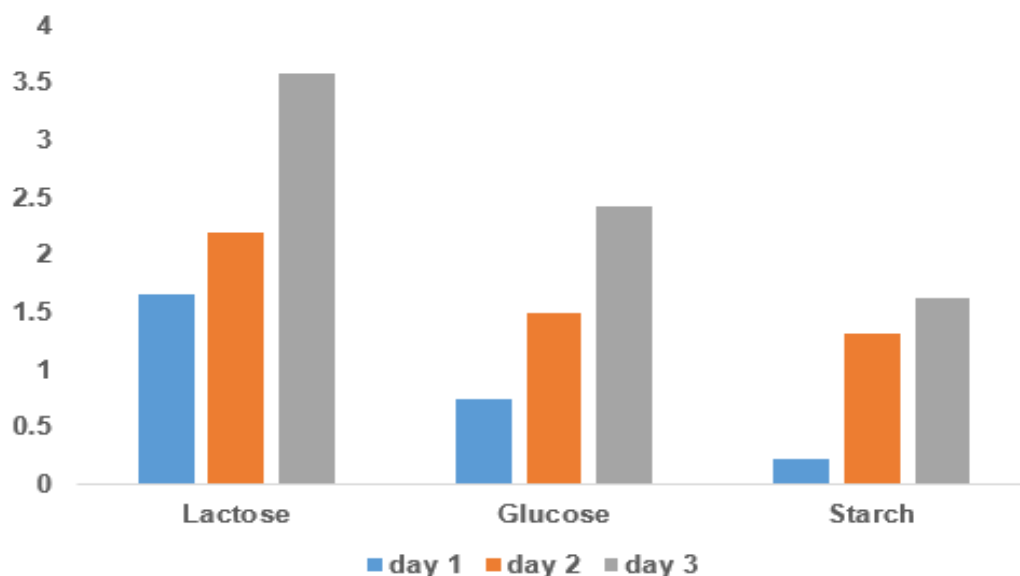


Fig 5.6: Optimization of Carbon source in Cellulase Production

5.5.3 Nitrogen source

It has been demonstrated that the synthesis of cellulase is susceptible to inhibition by various nitrogen sources. Ammonium sulphate, urea, and yeast extract were used in place of peptone in the growth medium to examine the impact of different nitrogen sources. Ammonium sulphate was determined to be the most effective nitrogen source for cellulase synthesis out of all the nitrogen sources that were examined.

It has been demonstrated that certain sources of nitrogen and carbohydrates can suppress the production of extracellular cellulase. Ammonium sulphate was out of all the nitrogen sources that were investigated. (Sethi *et al.*, 2013).

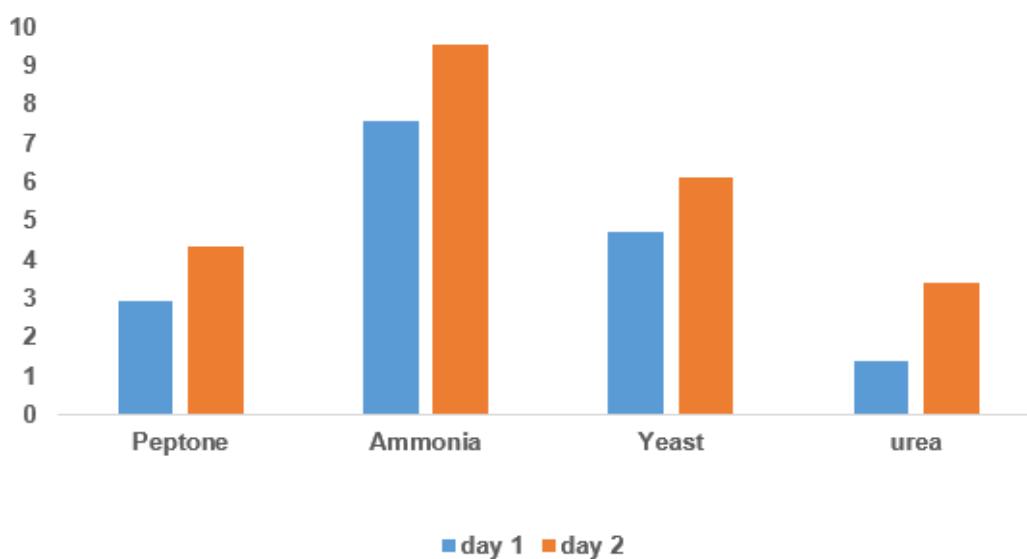


Fig 5.7: Optimization of Nitrogen source in cellulase production

5.5.4 CMC Concentration

It has been demonstrated that varying CMC Concentrations can suppress the production of cellulase. After testing several concentrations of CMC (0.5%), 1%, 1.5%, and 2% in the growth medium, it was determined that 1.5% was the optimal concentration for the generation of cellulase.

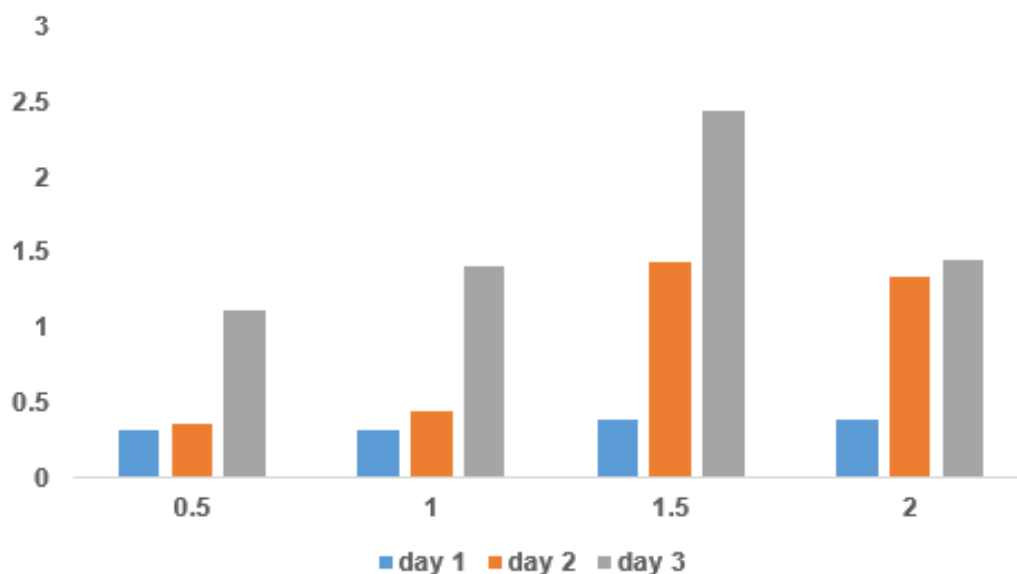


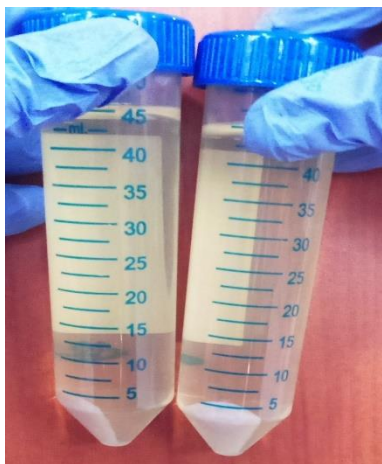
Fig 5.8: Optimization of CMC Concentration in Cellulase Production

5.6 PURIFICATION OF ENZYME

The crude enzyme's precipitation with ammonium sulfate was standardized, and 70% saturation was shown to be the point of maximum activity. There was no additional concentration employed; only 70% ammonium sulfate. The overall activity of the crude enzyme was 4.78 U/ml, while the ammonium sulfate precipitate enzyme displayed 27.456 U/ml. 7.758 U/ml of total activity was shown by the dialyzed enzyme.

Table 5.6.1: Purification of enzyme

Purification	Enzyme volume (ml)	Unit activity (u/ml)	Total activity(u) (u/ml × u/ml)
Crude enzyme	100	0.0478	4.78
Precipitate	20	1.3728	27.456
Dialysis	5	1.5516	7.758



Crude Enzyme



Precipitate Enzyme



Dialysis

Fig 5.9: Purification of enzymes

5.7 CHARACTERIZATION OF CELLULASE PRODUCTION

5.7.1 Effect of PH

The maximal cellulase activity was kept at pH 4 in 0.1 M sodium phosphate buffer. The activity was decreased at pH 7 and pH 12. Since the enzyme is acidophilic, pH 4 was shown to be the ideal value at which it functions at its best.

The *Bacillus* strain yielded the same tendency as well. (Sharma. A *et al.*, 2009). Found that an alkaline pH was ideal for the best activity of *Bacillus sp.* In acidic pH ranges of 3 to 6, the enzyme demonstrated good stability. (Bischoff K. M *et al.*, 2006).

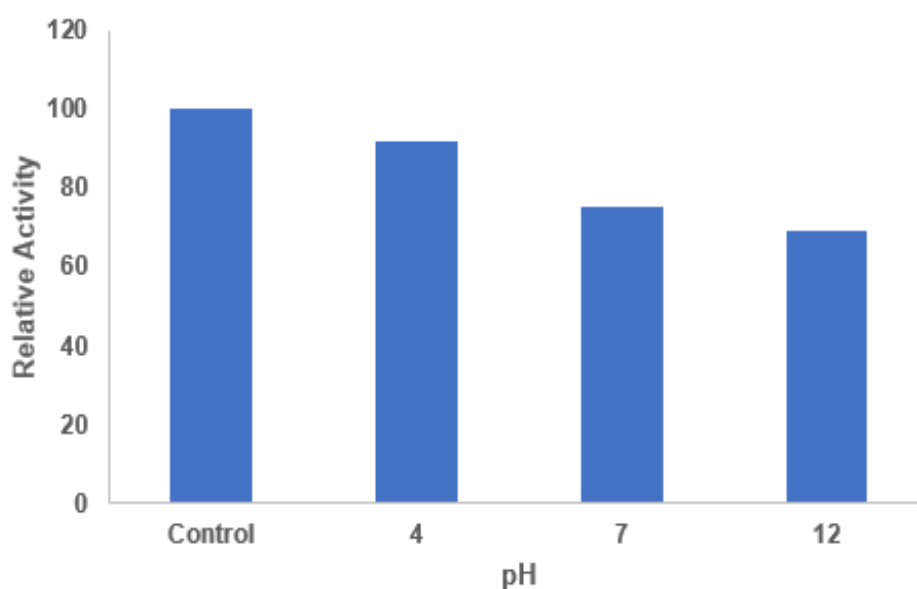


Fig 5.10: Effect of pH

5.7.2 Effect of Metal ions

The enzyme activity was increased in the present of Manganese and the present of Magnesium Chloride, Zinc Sulphate and Ferrous Sulphate, Potassium Chloride ions are decreased in the activity of the enzyme.

Metal ions like Pb^{2+} , Cd^{2+} , and Ba^{2+} were found to enhance the cellulase activity. In Ni^{2+} and Zn^{2+} , there was a partial suppression. According to the research that Gaur and Tiwari carried out (Gaur *et al.*, 2015) In the presence of Ni^{2+} and Zn^{2+} , a partial inhibition of cellulase was found. On the other hand, Mn^{2+} and Ca^{2+} significantly reduced the activity in the *Bacillus* strain investigation. (Sadhu .S *et al.*, 2013).

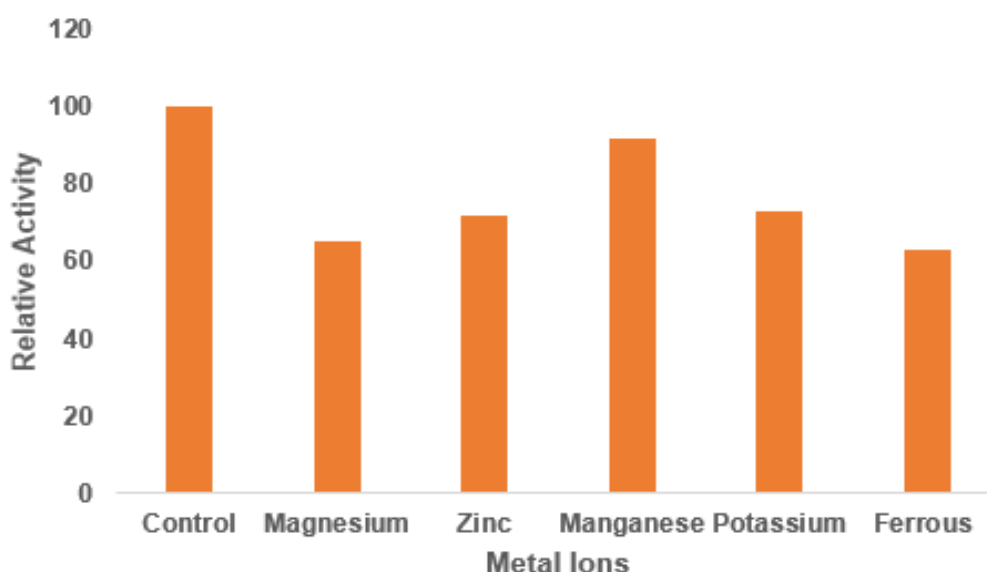


Fig 5.11: Effect of Metal ions

5.7.3 Effect of detergents

The enzyme's activity increased when Triton. SDS, Tween (80) are the enzyme activity reduced when cellulase was introduced to the detergent.

They work their way through the textile's inter fibril space, remove stains and preserving the fabric's qualities in characterization. (Ladeira S.A *et al.*, 2015)

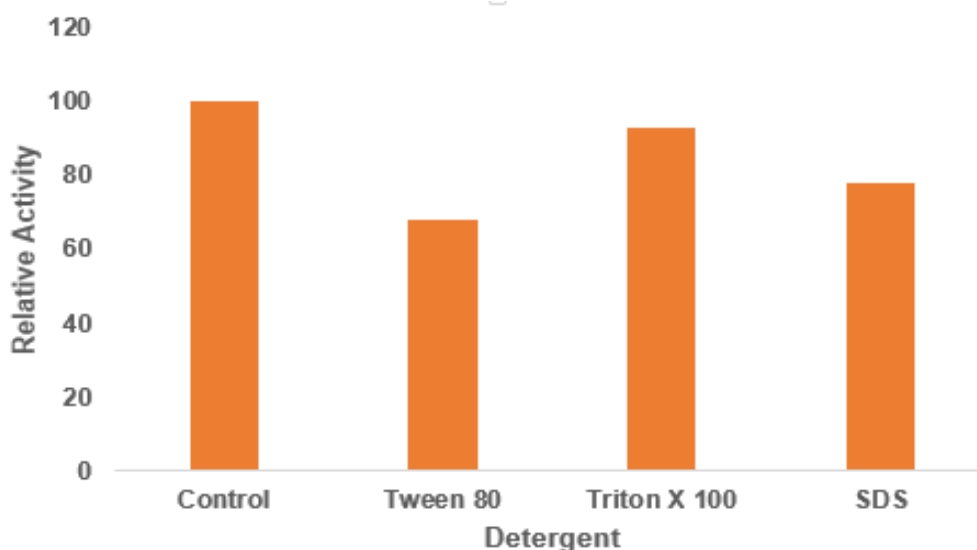


Fig 5.12: Effect of Detergents

5.8 CELLULASE APPLICATION

5.8.1 Cotton fabric

The fabric was tested with cellulase for the Smoothness & to check the quality of the fabrics.

The fabric treated with cellulase & fabric treated without cellulase (Control) was kept at 55°C for 1hr.

The fabric treated with cellulase was smooth & hard no change in colour & texture of the fabric.

Thus the cellulase enzyme produced by *Bacillus sp.* was effective in maintaining the smoothness of the fabric. The produced cellulase can be applied for the detergents formulation with further study condition.



Control

Sample

Fig 5.13: Cotton Fabric

5.8.2 Filter paper

Thus the cellulase enzyme produced by *Bacillus sp.* It has degraded the filter paper was good.

The main component in the creation of high-grade pulp and paper is cellulase. It is employed to extract the lignocellulosic portion and debark the biomass. This enzyme is also employed in biobleaching and deink recycling processes for used paper. (Basak *et al.*, 2021)

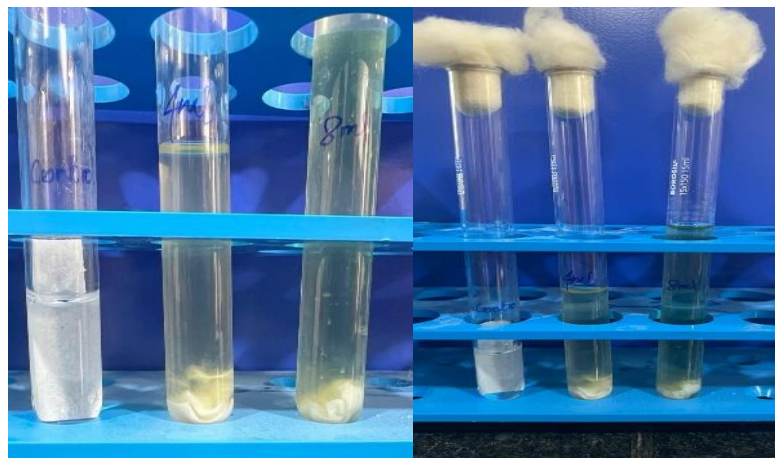


Fig 5.14: Filter paper

CHAPTER 6

SUMMARY AND CONCLUSION

The aim of the present work was effectively produce & characterize cellulase from bacterial species using liquid fermentation from soil. The most cellulases were produced by *Bacillus sp.* The best carbon and nitrogen sources were lactose and ammonium sulfate, while the optimal CMC concentration was 1.5%. The ideal pH was found to be 37°C/24 hours pH5. The crude enzymes were purified using ammonium sulfate precipitation and dialysis. Total enzyme activity of in crude enzyme was 4.78 U/ml and 27.456 U/ml of total activity after ammonium sulfate precipitation, 7.758 U/ml of total activity as dialysis. The characterization were determined as 37°C/20min best pH4 and best Metal ions and Detergents were Manganese and Triton. The application of cellulase is used to improve fibre smoothness in cotton fabric and hydrolytic potential of cellulase to hydrolyze cellulase substrate which is useful in paper industry was studied.

REFERENCES

- Abdel-Aziz, S. H., Ibrahim, A. M., Guirgis, A. A., Dawwam, G. E., & Elsabayty, Z. E. Isolation and screening of cellulase producing bacteria isolated from soil. *Benha Journal of Applied Sciences*, 6(3), (2021):207-213.
- Basavaraj I. Patagundi, C.T. Shivasharan and Kaliwal. B. B, Isolation and Characterization of Cellulase producing bacteria from Soil, *International Journal of Current Microbiology Applied Science*.3.5 (2014): 59-69
- Behera. B. C, Sethi. B. K, Mishra. R. R, Dutta. S. K, Thatoi. H. N, Microbial Cellulases Diversity & biotechnology with reference to mangrove environment, *Journal of Genetic Engineering and Technology*, 15(1). (2017), 197-210
- Bhagat, Shweta Ashok, and Seema Sambhaji Kokitkar. "Isolation and identification of bacteria with cellulose-degrading potential from soil and optimization of cellulase production." *Journal of Applied Biology and Biotechnology* 9.6 (2021): 154-161.
- Bhat. M. k. "Cellulases and related enzymes in biotechnology" *Biotechnology*,(5): (2000), 355-83
- Chavda, Nikita R., Priti H. Patel, and Roshani K. Chaudhary. "Isolation and Production of Cellulase from Bacteria Using Agro Waste." *Biosciences Biotechnology Research Asia* 20.4 (2023): 1467-1479.
- Faiz Rasul, Amber Afroz, Umer Rashid, Sajid Mehmood, Kalsoom Sughra, Nadia Zeeshan, Screening and characterization of cellulase producing bacteria from soil and waste molasses of sugar industry. 2015. 230-238
- Gilna. V. V and Khaleel. K. M, Cellulase enzyme Activity of *Aspergillus fumigatus* From Mangrove Soil on Lignocellulosic Substrate. *Recent Research in Science and Technology*, 3(1) (2011): 132-134

Hiral Shah, Ankita Patel, Rinku Valand, Payal Rathod, Nidhi Gondaliya. "Isolation, Production and Optimization of Cellulase by *Bacillus Pumilus* under Submerged Fermentation using Laboratory Medium by Smf Method." *Towards Excellence*. 13(1) (2021) 717-733

Hossain, M., Ahammed, M. A., Sobuj, S., Shifat, S., & Somadder, P. (2021). Cellulase producing bacteria isolation, screening and media optimization from local soil sample. *American Journal of Microbiological Research*, 9(3), (2021):62-74.

Indra Pratap Singh, Swapna Kumar Srivastava, Screening of Cellulase Production Bacteria from Soil and Optimization of Medium for enhancing Cellulase activity. *Europe Chemical*. 12 (2023). 173 – 185

Islam, Farjana, and Narayan Roy. "Screening, purification and characterization of cellulase from cellulase producing bacteria in molasses." *BMC research notes* 11 (2018): 1-6.

Islam, M., Sarkar, P. K., Mohiuddin, A. K. M., & Suzaudulla, M. Optimization of fermentation condition for cellulase enzyme production from *Bacillus* sp. *Malaysian Journal of Halal Research*, 2(2), (2019):19-24.

Jayasekara, Sandhya, and Renuka Ratnayake. "Microbial cellulases: an overview and applications." *Cellulose* 22.92 (2019): 10-5772.

Khatewada P, Ahmed J, Sohag MH, Islam K, Azad AK, Isolation, Screening and Characterization of Cellulase Producing Bacterial Isolates from Municipal Solid Wastes and Rice Straw Wastes. *J Bioprocess Biotech*, 6(4) (2016):280

Manita Shyaula, Sunil Regmi, Deegendra Khadka, Ram Chandra Poudel, Agni Dhakal, Devesh Koirala, Jaishree Sijipati, Anjana Singh, Jyoti Maharjan, "Characterization of Thermostable Cellulase from *Bacillus licheniformis* PANGL Isolated from the Himalayan Soil." *International Journal of Microbiology*, (2023), 12

Manzum, Amika Ahmed, and Md Arafat Al Mamun. "Isolation of Bacillus spp. bacteria from soil for production of cellulase." *Nepal Journal of Biotechnology* 6.1 (2018): 57-61.

Miller, Gail Lorenz. "Use of dinitrosalicylic acid reagent for determination of reducing sugar." *Analytical chemistry* 31.3 (1959): 426-428.

Muhammad Irfan, Asma Safdar, Quratulain Syed, Muhammad Nadeem, Isolation and screening of cellulolytic bacteria from soil and optimization of cellulase production and activity. *Turkish Journal of Biochemistry*, 37 (3) (2012).

Ram, Lekh, Kuldeep Kaur, and Sandeep Sharma. "Screening isolation and characterization of cellulase producing microorganisms from soil." *International journal of pharmaceutical science invention* 3.3 (2014): 12-18.

Ramesh Chander Kuhad, Rishi Gupta, Ajay Singh. "Microbial Cellulases and Their Industrial Application." *Enzyme Research*. (2011), 10

Sadhu, Sangrila, and Tushar Kanti Maiti. "Cellulase production by bacteria: a review." *Microbiology Research Journal International*, 2013, 3(3)

Sethi, S., Datta, A., Gupta, B. L., & Gupta, S. (2013). Optimization of cellulase production from bacteria isolated from soil. *ISRN Biotechnology*, 2013. (3)4.

Shanmugapriya. K, Saravana. P. S and Manoharan. M, "Screening and partial purification of cellulase from cellulase producing bacteria," *International Journal of Advanced Biotechnology Research*, 13(1) (2012):509-514

Sheetal Barapatre, Isolation of Fungi and Optimization of pH and Temperature for Cellulase Production. *Nature Environment and Pollution Technology*. 19.4 (2020), 1729-1735

Shilpa Lokhande and Pethe AS, "Isolation and screening of cellulolytic bacteria from soil and optimization of cellulase production" *International Journal of Life Sciences*, 5(2) (2017): 277-282

Singhania, R. R., Sukumaran, R. K., Patel, A. K., Larroche, C., & Pandey, A. Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases. *Enzyme Microbial Technology*, 46 (2010):541-549

Sudha H. Vishwakarmaa, Trupti J. Pandyab, Microbial Production of Cellulase Enzyme and its Application. *International Journal of Research*, 8 (2022). 161-169

Tandon, D., and N. Sharma. "Isolation of potential novel cellulolytic and xylanolytic bacteria and optimization of their cultural conditions for enhanced production of cellulase and xylanase." *J Agroaliment Proc Technology*, 20 (2014): 231-240.