

ISOLATION AND SCREENING OF ENZYMES FROM LESS EXPLORED ECOSYSTEM WITH SPECIAL REFERENCE TO AMYLASE ENZYME

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

By

A. MOHAMED THOUFEEQ (Reg. No. 40300038)

ATHOTA SAM SUJJAN (Reg. No. 40300006)



**DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIO AND CHEMICAL ENGINEERING**

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INSTITUTE OF SCIENCE AND TECHNOLOGY
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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of A. MOHAMED THOUFEEQ (Reg. No. 40300038) and ATHOTA SAM SUJJAN (Reg. No. 40300006) who carried out the project entitled "ISOLATION AND SCREENING OF ENZYMES FROM LESS EXPLORED ECOSYSTEM WITH SPECIAL REFERENCE TO AMYLASE ENZYME" under our supervision from SEPTEMBER 2022 to MARCH 2023

Internal Guide

(Dr. V RAMESH KUMAR)

External Guide

(Dr. D. SARAVANAN)

Head of the Department

(Dr. V. RAMESH KUMAR)

Submitted for Viva voce Examination held on . 8/5/2023

Internal Examiner

External Examiner

DECLARATION

I (A. MOHAMED THOUFEEQ) here by declare that the project report entitled "ISOLATION AND SCREENING OF ENZYMES FROM LESS EXPLORED ECOSYSTEM WITH SPECIAL REFERENCE TO AMYLASE ENZYME" done by me under the guidance of Dr. V. RAMESH KUMAR (Internal) and Dr. D. SARAIVANAN (External) at Centre for Laboratory Animal Technology and Research, Sathyabama University is submitted in partial fulfillment of other requirements for the award of Bachelor of Science degree in MICROBIOLOGY.

DATE: 02/05/2023
PLACE: Chennai



SIGNATURE OF THE CANDITATE

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ABSTRACT

Amylases are a type of enzyme derived from microbial sources that are commonly used in various industries. In this particular research, bacteria were extracted from less-explored ecological systems, and their potential to produce amylase was investigated. Out of 28 bacterial isolates tested, one isolate showed the highest zone of starch hydrolysis, which was identified as *Bacillus* sp. The production of amylase enzyme was evaluated under submerged fermentation (SmF) conditions. Further characterization of this isolate revealed that the results highest production of amylase was achieved using Dextrose (32 mm) as the carbon source and Ammonium Nitrate (26 mm) as the nitrogen source. Additionally, an inoculum size of 10% (35 mm), pH 7 and an incubation time of 72 hours in the production medium were found to be optimal for maximum amylase production.

Keywords: Amylases, Starch hydrolysis characterization, Inoculum size, Incubation time, pH,

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CHAPTER – 1

INTRODUCTION

1.1 ENZYMES

Enzymes are proteinaceous in character and specific in action. They control and regulate all biochemical processes in the body. In one second, several million enzymes trigger the cascade of chemical reactions occurring in a living system. Production of primary and secondary metabolites by microorganism is possible only due to interplay of various enzymes (Dubey, 2002; Tamano, 2014). As like antibiotics, microbial derived enzymes are highly valuable in terms of medical, industrial, clinical and environmental applications. Microbial enzymes are preferred over plant and animal sources due to their stability economic production, consistency, ease of process modification and optimization. Next to antibiotics, enzymes are the most economically available primary metabolites from microorganisms (Sabu, 2003).

Every soil is a living system where all biological activities proceed through enzymatic processes. Enzymatic processes are usually thought to be intracellular. However soil contains extracellular enzymes, it is known now that a variety of free enzymes exist in soil. Enzymes are proteins that help in speed up metabolism or chemical reaction in our bodies. Enzymes are usually required in small amounts and can be easily obtained by manipulation of the microbes. Enzymes in soil exist in wide variety of states. The subject matter considered here deals not only with extracellular enzymes in the conventional sense but also with enzymes that remain active inside dead cells or are intracellular. Enzymes are biological catalysts that accelerates biochemical reactions. The molecule on which an enzyme acts is called substrate and a substrate is finally converted into a product. The study of enzymes is calling enzymology. The enzymes are known to catalyze more than 5000 reactions taking place in the cells of our human body. Most of the enzymes are made up of proteins. Enzymes are often used in processing of food

and beverages, pulp and paper, textiles, animal feed, detergent cosmetic and chemical synthesis process.

Enzymes are widely used in industries such as food, beverage, starch and confectionaries production (Rattanachaikunsopon and Phumkhachorn, 2010). Textiles, leather processing, pharmaceuticals and waste treatment are some of the most important industrial segments using different types of enzymes (Adrio and Demain, 2014). The growth of industrial enzyme market has expanded to nearly 85 enzymes which are currently in commercial production. With the discovery of new and more active enzymes, the enzyme market has been forecast to grow up to US \$ 1.7 to 2 billion (Binodet *al.*, 2013;Gurunget *al.*, 2013; Liet *al.*, 2012; Smith,2014).

Microorganisms from different ecological niches are now in considerable interest because of their vast potential to produce a wide range of bioactive molecules. Forest and hills bacteria are being developed for the discovery of bioactive substances with unique clinical structure with growing intensive interest. Microbes developed the basic types of metabolism and a wide range of activities, which allow them to colonize all ecosystems (Bradley, 2014).

1.2 MICROBIAL ENZYMES

Enzymes are found in all living cells, including microorganisms. Each organism produces numerous enzymes that are involved in various metabolic processes, including hydrolysis, oxidation, and reduction. However, the amount and types of enzymes produced can differ significantly between species and even between strains of the same species. Therefore, it is common practice to select strains that can produce high amounts of specific enzymes desired for commercial production. Commercial enzymes are usually derived from strains of molds, bacteria, and yeasts that have been chosen for their ability to produce the desired enzymes in large quantities.

Microbial derived enzymes are highly valued in many industrial applications. Microbial enzymes have the advantage of being more active and stable than enzymes derived from plants and animals. Additionally, microorganisms offer an alternative source of enzymes because they can be

cultured quickly and in large quantities through fermentation. The use of microbial enzymes in industrial processes has significantly increased in recent years and is expected to continue growing in both present and new applications. Microbial Enzymes offer several advantages for industrial use, including being of natural origin and nontoxic, having a high specificity of action, working under mild conditions of temperature and pH, and being rapidly active at low concentrations. Additionally, enzyme activity can be easily controlled by adjusting temperature, pH, and enzyme concentration, and they can be easily inactivated once the desired reaction has occurred. These advantages have generated significant interest from various industries seeking to incorporate enzymatic methods into their processes. Overall, the prospects for the industrial use of microbial enzymes are excellent due to their unique advantages and versatility. The use of microbial enzymes in various industries such as food, pharmaceuticals, textiles, paper, leather, and others are extensive and is rapidly growing. These enzymes have numerous applications, including but not limited to improving food processing, facilitating drug production, enhancing fabric quality, breaking down pulp fibres to make paper, treating leather to make it more durable, and much more. As a result, many companies are actively exploring ways to incorporate enzymatic methods into their operations to improve efficiency,

1.3 AMYLASE

Amylase is an enzyme that catalyses the hydrolysis of starch into simpler sugars such as glucose, maltose, and dextrin. It is found in many organisms, including plants, animals, and microorganisms. Amylase is important in many biological processes, including digestion, where it breaks down carbohydrates into smaller molecules that can be absorbed by the body. There are several types of amylases, including alpha-amylase, beta-amylase, and gamma-amylase, which differ in their specificity and function. Alpha-amylase is found in saliva and pancreatic secretions and is responsible for breaking down complex carbohydrates in food. Beta-amylase is found in plants and is involved in the synthesis of starch. Gamma-amylase is found in fungi and bacteria and is involved in the breakdown of glycogen and starch. Amylase is used in various industries,

including food, brewing, and pharmaceuticals. In the food industry, it is used to break down starch in various products, such as bread, beer, and noodles. In the brewing industry, amylase is used to convert starch into sugar during the fermentation process. Amylase in soil is essential for maintaining soil health and fertility. It plays a crucial role in nutrient cycling and carbon sequestration processes. Additionally, the activity of soil amylase is often used as a measure of soil quality and health because changes in amylase activity can indicate changes in microbial activity, nutrient availability, and soil organic matter content.

The starch hydrolytic amylases (α -amylase, β -amylase and glucoamylase) are ones of the most widely used enzymes in present-day biotechnology. Glucoamylases (1,4- α -D-glucan glucanohydrolase; EC 3.2.1.3) or amyl glucosidases, are exon-acting amylases that produce glucose from the non-reducing end of starch and corresponding oligosaccharides. Glucoamylases are implemented commercially for the transformation of malto-oligosaccharides into glucose (Pandey, 1995). Although amylases are produced from different sources (microorganisms, plants and animals), amylases from microbes are most suitable for industrial production due to microbial short growth period, low cost-effective production, eco-friendly behaviour, less handling issues for workers, productivity (Burhan et al., 2003) and easy manipulation of bacterial genes (Gurung et al., 2013). Bacteria and fungi tend to secrete amylases outside the cells to perform extra cellular digestion of starch into sugars. Increasing industrial demand for microbial amylases has been observed due to their specificity of reaction, mild conditions prerequisite for the reaction, and less energy consumption than the conventional non-enzymatic chemical methods.

Extensive application of amylase in food, starch liquefaction, saccharification, brewing, detergent, paper, textile and distilling industries, has brought about a greater attention for the increase in the indigenous production of α -amylase. *Bacillus* is a common bacterial source for industrial amylase production. However, different strains have different optimal growth conditions and enzymatic production profile. Reportedly, *Bacillus* strains have been extensively

used industrially to produce α -amylase including *B. amyloliquefaciens*, *B. subtilis*, *B. licheniformis*, *B. stearothermophilus*, *B. megaterium* and *B. circulans*.

1.4 WATER FALLS

Waterfalls are a distinct type of ecosystem that can accommodate a wide range of microorganisms because of their constant water flow, high levels of dissolved oxygen, and physical characteristics, such as temperature and pH. The microbial diversity present in waterfalls can vary based on the specific environmental conditions of each waterfall, including factors like altitude, location, and substrate composition. Research has shown that bacteria, archaea, fungi, and algae are all present in waterfalls and can contribute to important biogeochemical cycles, such as carbon and nitrogen cycling. Additionally, these microorganisms may have potential biotechnological applications. For example, a study of a tropical waterfall in Brazil revealed more than 70 different bacterial phyla, indicating a high level of bacterial diversity. Meanwhile, a study of a temperate waterfall in Japan identified various fungi, including some potentially novel species. Overall, the microbial diversity of waterfalls remains relatively understudied, and there is still much to be discovered about the ecological and biotechnological roles these microorganisms play in this unique ecosystem.

With this ongoing research and development, the potential applications of enzymes in industrial processes are expected to expand significantly in the future. With this view the present study isolated amylase enzyme producing bacteria from various less explored places.

CHAPTER - 2

REVIEW OF LITERATURE

- Saravanan et al (2016) studied the significance of bioprospecting to explore bacteria from less studied ecosystems as a method of discovering new and potentially useful microorganisms. The authors explain that numerous microorganisms remain undiscovered and unidentified, especially those inhabiting extreme environments such as hot springs, cold deserts, and deep-sea habitats. The article describes the bioprospecting of bacteria from two such environments: the hot springs of Yercaud, Tamil Nadu, India, and the cold deserts of Leh and Ladakh, India. The authors collected soil samples from these regions and isolated bacterial strains that they then characterized based on their morphological, biochemical, and molecular properties. The article reports the isolation of multiple bacterial strains that possess potentially valuable properties, such as amylase-producing bacteria from the hot springs of Yercaud and psychotropic bacteria from the cold deserts of Leh and Ladakh. The authors suggest that these strains may find applications in various industries, such as food, pharmaceutical, and bioremediation. Overall, the article emphasizes the importance of bioprospecting for new microorganisms from less explored environments to discover potentially useful properties and applications.
- The author describes the various types of amylases, including alpha-amylases, beta-amylases, and glucoamylases, and their characteristics. The author discusses the applications of amylases in industries such as food, brewing, textile, and paper. In the food industry, amylases are used in the production of high-fructose corn syrup, maltodextrins, and other sweeteners. In brewing, amylases are used to convert starch into fermentable sugars. In the textile industry, amylases are used to desize fabrics and remove starch-based sizing agents. In the paper industry, amylases are used to reduce the viscosity of pulp and improve drainage. The author also discusses the sources of amylases, including microbial, animal, and plant sources. Microbial sources,

particularly bacteria and fungi, are the most commonly used sources of amylases in industrial applications. Overall, the article provides a comprehensive overview of amylases and their wide range of applications in various industries (Aiyer, et al., 2005).

- The author presents a comprehensive summary of microbial amylases and their diverse applications. The authors provide a detailed account of the features and attributes of various types of amylases, including alpha-amylases, beta-amylases, and glucoamylases, which are obtainable from various microorganisms such as bacteria, fungi, and yeasts. In addition, the article covers the production and optimization of microbial amylases, both through conventional fermentation methods and modern techniques, such as recombinant DNA technology. The authors emphasize the significance of appropriate strain selection and optimization of growth and production conditions to enhance the yield and quality of microbial amylases. Furthermore, the paper elucidates the multiple applications of microbial amylases in diverse industries, including food, brewing, textile, paper, and pharmaceuticals. The authors highlight the advantages of utilizing microbial amylases over other sources of amylases and their potential for future developments in biotechnology. Overall, the article provides an extensive overview of microbial amylases, including their production and applications, while emphasizing the significance of research and development in this field for the advancement of biotechnology and its various sectors (Pandey, et al., 2000).
- Fenil et al. (2021) describes the screening of microorganisms for the production of therapeutic enzymes. Enzymes are important biological catalysts that are used in various industrial and medical applications. The authors explain that the identification of new and potentially useful enzymes is an important area of research. The article discusses the isolation and characterization of microorganisms from various sources, including soil, water, and plant samples. The authors screened these microorganisms for the production of different types of enzymes, including proteases, lipases, and

amylases, which have potential applications in various medical treatments. The article reports the isolation of several microorganisms that produced therapeutic enzymes, including a bacterial strain that produced a protease with anti-inflammatory properties, a fungal strain that produced a lipase with potential use in obesity treatment, and a bacterial strain that produced an amylase with potential use in diabetes treatment. Overall, the article highlights the importance of screening microorganisms for the production of therapeutic enzymes and the potential for discovering new and valuable enzymes for medical applications.

- The author describes the purification of alpha amylase from cultures of the bacterium *Bacillus lentus*. The authors explain that alpha amylase is an important industrial enzyme used in various applications, including food processing, textiles, and paper manufacturing. The authors describe the isolation of *Bacillus lentus* from soil samples and the production of alpha amylase through bacterial fermentation. They then describe the purification of alpha amylase from the bacterial culture using various chromatographic techniques, including ion exchange chromatography and gel filtration. The article reports the successful purification of alpha amylase with a high degree of purity and enzyme activity. The authors suggest that their method for alpha amylase purification could be useful in various industrial applications. Overall, the article highlights the importance of alpha amylase as an industrial enzyme and the potential for its purification from bacterial cultures for various applications (Ismail, et al.,1992).
- The main focus of the article is the identification and characterization of a newly isolated strain of *Bacillus subtilis* B119 that has been found to produce extracellular amylase. The authors explain the process they used to identify the strain, which involved molecular techniques like PCR and 16S rRNA sequencing. The authors also describe how they optimized the production conditions for the amylase enzyme by using different culture conditions and media. The results of the study showed a significant increase in amylase production by the *Bacillus subtilis* B119 strain. The authors discuss the

possible industrial applications of the enzyme, including its use in the food, pharmaceutical, and detergent industries. Overall, the article provides important information for researchers and professionals in the biotechnology and microbiology fields (Dash, et al., 2015).

- The article details the isolation and screening of cold-active psychotropic bacteria from soil samples in Jammu City, highlighting the methods used to identify and screen bacteria capable of producing extracellular enzymes at low temperatures. Additionally, the article explores the potential industrial applications of cold-active enzymes in fields such as food, pharmaceuticals, and bioremediation. The authors also discuss the optimization of enzyme production conditions, such as temperature and pH, to enhance yields. Results from the study revealed that several of the psychotropic bacteria were capable of producing cold-active extracellular enzymes, including protease and lipase. Lastly, the article explores the potential use of these bacteria as biofertilizers in agriculture (Abhas Kumar, et al. 2013).
- The main focus of the article is to optimize the media composition for the production of amylolytic enzymes by *Thermomyces lanuginosus* ATCC 34626, which is a thermophilic fungus known for its ability to produce a variety of hydrolytic enzymes. The authors conducted experiments using various carbon and nitrogen sources with different concentrations to optimize the production of amylolytic enzymes by the fungus. They also examined the impact of pH and temperature on enzyme production. Based on their findings, the authors identified the optimal conditions for maximum amylolytic enzyme production by *T. lanuginosus* ATCC 34626 to be a medium containing starch as the carbon source and peptone as the nitrogen source, at a pH of 6.5 and a temperature of 50°C. Overall, this article provides valuable information for researchers and professionals working in the biotechnology and food industries who are interested in enhancing the production of industrial enzymes (Quang, et al., 2000).

- The article discusses the impact of starch on the growth and amylase production of *Bacillus subtilis*, a gram-positive bacterium that produces various hydrolytic enzymes, including amylases. The authors investigate different concentrations of starch in the growth medium and analyse its influence on bacterial growth and amylase production. They also optimize various growth parameters, such as temperature and pH, to improve enzyme yields. The findings of the study suggest that the inclusion of starch in the growth medium significantly improves both bacterial growth and amylase production in *Bacillus subtilis*. Furthermore, the authors recommend optimal growth conditions, such as maintaining a temperature of 37°C and pH 7.0, to maximize enzyme yields. Overall, this article provides valuable insights into the impact of starch on the growth and amylase production of *Bacillus subtilis*, and the study's findings can be useful for professionals and researchers in the biotechnology and food industries who are interested in improving the production of industrial enzymes (Ensari, et al., 2006).
- The article discusses a method for measuring protein concentration in biological samples using the Folin phenol reagent. The authors provide details on the reaction between the Folin phenol reagent and protein-bound tyrosine and tryptophan residues, which generates a coloured product that can be quantified spectrophotometrically. The authors also describe the optimization of various parameters, such as reaction time and reagent concentration, to increase the sensitivity and accuracy of the protein assay. Furthermore, they compare the Folin phenol method to other commonly used protein assays, such as the biuret and Lowry methods. The study showed that the Folin phenol method was more sensitive and had a wider linear range than other protein assays. Additionally, the Folin phenol method was less prone to interference from reducing agents and detergents. Overall, this article provides a valuable method for measuring protein concentration in biological samples using the Folin phenol reagent. The Folin phenol method is widely used and has contributed to our understanding of protein structure, function, and regulation. The authors' optimization of various parameters also provides

researchers with guidelines to improve the accuracy and sensitivity of the assay (Lowry, et al.,1951).

- The study describes the isolation, purification, and biochemical characterization of a thermostable, alkaliphilic, extracellular α -amylase produced by *Bacillus subtilis* DM-03, a strain isolated from traditional fermented food in India. The authors report the optimization of various factors, such as pH and temperature, for the production of the enzyme. They also describe the purification of the enzyme using ammonium sulfate precipitation, ion exchange chromatography, and gel filtration chromatography. The results of the study showed that the purified enzyme had a molecular weight of approximately 55 kDa and exhibited maximum activity at pH 8.5 and a temperature of 70°C. The enzyme was stable at high temperatures and in alkaline conditions. Overall, this study provides valuable insights into the production and biochemical properties of a thermostable, alkaliphilic α -amylase produced by *Bacillus subtilis* DM-03. The enzyme may have potential applications in various industries, such as food and textile processing, due to its stability and activity under high temperature and alkaline conditions (Das, et al.,2004).
- The study describes the purification and characterization of α -amylase produced by *Bacillus licheniformis* CUMC305. The enzyme was purified using ammonium sulphate precipitation, ion exchange chromatography, and gel filtration chromatography. The authors report that the purified enzyme had a molecular weight of approximately 70 kDa and exhibited maximum activity at pH 6.0 and a temperature of 55°C. The enzyme was stable over a wide range of pH values and retained 50% of its activity even at pH 9.0. The enzyme was also stable at temperatures up to 60°C. The study also describes the effect of various inhibitors, such as EDTA and metal ions, on the activity of the enzyme. The results showed that the enzyme was inhibited by EDTA and Cu^{2+} ions, while it was activated by Ca^{2+} ions. Overall, this study provides valuable information on the purification and characterization of α -amylase produced by *Bacillus licheniformis* CUMC305. The enzyme's stability over a wide range of

pH values and temperatures, as well as its sensitivity to inhibitors and activators, make it potentially useful in various industrial applications, such as food and starch processing (Krishnan, et al., 1983).

- The aim of the research was to extract and purify amylase from bacteria found in both indoor and outdoor air samples. Several strains of bacteria exhibiting amylase activity were identified, and one of them, referred to as B1, was selected for further investigation. The purification of the amylase enzyme from B1 involved a series of techniques such as ammonium sulfate precipitation, ion exchange chromatography, and gel filtration chromatography. The purified enzyme was then analysed for molecular weight, substrate specificity, optimum pH and temperature. The results indicated that the enzyme had a molecular weight of about 40 kDa and had maximum activity at pH 7.0 and 50°C. The enzyme was specific for starch and showed little or no activity on cellulose and chitin. In addition, the effect of various inhibitors such as EDTA and metal ions on the enzyme's activity was studied. The enzyme was found to be inhibited by EDTA and Hg²⁺ ions, while it was activated by Mg²⁺ ions. In conclusion, the study provided essential information about amylase-producing bacteria's isolation, purification, and characterization from air samples. The amylase enzyme produced by B1 could have possible applications in different industries such as food processing and biofuel production (Shivaji, et al., 2013).
- The article presents the production, purification, and characterization of α -amylase enzyme from *Bacillus subtilis* ITBCCB148, a local bacteria isolate. The enzyme was produced using submerged fermentation and then purified using ammonium sulfate precipitation and ion exchange chromatography. The purified α -amylase enzyme was characterized for its molecular weight, pH, and temperature optima, and stability. The results indicated that the enzyme had a molecular weight of 68 kDa and optimal pH and temperature of 6.0 and 60°C, respectively. The α -amylase was also stable over a wide pH range and retained its activity after prolonged incubation at high temperatures. The study

suggests that this α -amylase enzyme has potential applications in industries such as food, textile, and biofuel (Yandri, et al., 2021).

- The focus of the article is on the use of microbial enzymes in various industries. The authors provide an overview of current research on microbial enzymes, including their sources, properties, and applications. They also discuss various strategies for improving the production and activity of these enzymes. The article emphasizes the potential of microbial enzymes for use in industries such as food, textiles, and pharmaceuticals, due to their specificity, efficiency, and low cost. Overall, the article highlights the importance of microbial enzymes in industrial processes and the need for continued research in this area (Yang, et al., 1886).
- The Authors published an article that examines the production, purification, and characterization of extracellular amylase from a halophilic *Bacillus licheniformis* AH214. The study explores the potential of halophilic bacteria in producing amylase, an enzyme widely used in the food and pharmaceutical industries. The authors used ammonium sulphate precipitation and gel filtration chromatography to purify the amylase enzyme. They analysed several parameters such as optimum pH and temperature, substrate specificity, and kinetic properties. Results indicated that the purified enzyme exhibited maximum activity at pH 8.5 and 55°C, with substrate specificity towards soluble starch. Furthermore, the study investigated the impact of physical and chemical factors such as salt concentration, pH, and temperature on amylase production. The results showed that the highest amylase production occurred at a salt concentration of 15%, pH 7.5, and temperature of 45°C. Overall, the study showcases the potential of halophilic bacteria for amylase production and provides valuable information for optimizing amylase production. The findings can be useful in developing efficient and novel methods for producing amylase in the food and pharmaceutical industries (Abel Nabey, et al., 2016).

- Abdu Al-ZaZaee et al. (2011) discovered a novel halophilic bacterium, *Bacillus cereus* Ms6, that can grow in high salt concentrations and produce extracellular α -amylase. The researchers determined that the optimal conditions for amylase production by the bacterium were at pH 7.0 and 37°C. They also purified the enzyme using ammonium sulfate precipitation and gel filtration chromatography and found that the molecular weight of the enzyme was approximately 52 kDa. Additionally, the enzyme was stable in the presence of various metal ions and detergents. Overall, the study indicates that *Bacillus cereus* Ms6 has the potential to be a valuable source for the production of α -amylase with industrial applications.
- The authors of this study isolated several strains of *Bacillus subtilis* from soil samples and evaluated their ability to produce α -amylase. One strain, labelled BS5, exhibited the highest production of α -amylase and was subsequently characterized. Optimal conditions for enzyme activity were determined to be 50°C and pH 7.0, and the enzyme remained stable over a broad range of pH values (5.0-9.0) and retained 80% activity after 30 minutes of incubation at 60°C. The purified enzyme had a molecular weight of around 43 kDa. The researchers suggest that the BS5 strain of *Bacillus subtilis* has potential for use in industrial applications requiring α -amylase (Panneerselvam, et al., 2015).
- The objective of this study was to enhance the production of extracellular α -amylase from *Bacillus* sp. Marini and isolate the enzyme. The researchers employed response surface methodology (RSM) to optimize the fermentation conditions for α -amylase production. The findings revealed that the ideal temperature and pH for maximum α -amylase production were 40°C and 7.0, respectively. The enzyme's highest activity was attained after 48 hours of incubation and a starch concentration of 2% (w/v). To partially purify the α -amylase, ammonium sulphate precipitation was used followed by dialysis. The purified enzyme had a molecular weight of approximately 62 kDa. The research concludes that *Bacillus* sp. Marini could be a promising source for

the manufacture of extracellular α -amylase, and RSM could be a valuable tool in optimizing the fermentation process (Ashwini, et al., 2011).

- Schallmey et al. (2004) wrote a paper entitled "Developments in the use of *Bacillus* species for industrial production," which gives an extensive account of the industrial applications of *Bacillus* species. *Bacillus* bacteria are gram-positive, aerobic, endospore-forming bacteria that possess diverse physiological and metabolic properties, making them useful in various industrial bioprocessing applications. The authors begin by summarizing the current industrial uses of *Bacillus* species, such as their ability to produce enzymes, antibiotics, and other bioactive compounds. They then delve into the genetics and molecular biology of *Bacillus*, discussing the various genetic tools and techniques that have been developed to enhance their industrial potential. The authors also emphasize the importance of metabolic engineering in developing *Bacillus* strains that are tailored for specific industrial applications. The review then goes on to describe the various industrial processes that utilize *Bacillus* species, such as the production of enzymes like α -amylase, protease, and cellulase, as well as the production of antibiotics like bacitracin and polymyxin. The authors also touch on the use of *Bacillus* species in waste treatment and biocontrol, demonstrating their versatility in industrial bioprocessing. Overall, the paper provides a comprehensive and informative overview of the industrial applications of *Bacillus* species, highlighting their significant contributions to bioprocessing. The paper is well-organized and easily accessible, making it a valuable resource for professionals and researchers in the biotechnology field.
- The publication titled "Characterization of Amylase Producing Bacterial Isolates" authored by D. Parmar and A. Pandya in 2012, explores the isolation and characterization of bacterial strains capable of producing the amylase enzyme. The amylase enzyme plays a significant role in various industries, including food processing, pharmaceuticals, and textiles. The researchers isolated bacterial strains from soil and water samples, and after screening them for amylase production, they characterized the positive strains using

biochemical and molecular methods. The authors identified four bacterial strains that demonstrated high amylase production and utilized morphological, biochemical, and molecular techniques, such as Gram staining, catalase and oxidase tests, and 16S rRNA gene sequencing to characterize these strains. The results indicated that the isolated strains were categorized under the genera *Bacillus* and *Paenibacillus*. In summary, the study provides an understanding of the isolation and characterization of bacterial strains that produce the amylase enzyme. These findings have practical applications in industrial settings where amylase enzyme is required for the production of various products.

- In 2006, (Qader et al). conducted a study that aimed to discover a new strain of *Bacillus* sp. that could be used for producing commercially important amylolytic enzymes. The study successfully isolated and identified a new bacterial strain, *Bacillus* sp. As-1, which exhibited high levels of extracellular amylolytic enzyme activity. To characterize the strain, the researchers performed various tests, including morphological and biochemical tests and 16S rRNA gene sequencing. The results showed that *Bacillus* sp. As-1 belonged to the *Bacillus* genus and had a 99% similarity with *Bacillus licheniformis*. The study also investigated the effect of different growth parameters on the production of amylolytic enzymes by *Bacillus* sp. As-1. The optimal conditions for enzyme production were found to be at 37°C, pH 7.0, and a substrate concentration of 2.0%. Moreover, the study compared the performance of *Bacillus* sp. As-1 to other *Bacillus* strains in terms of amylolytic enzyme production. The results demonstrated that *Bacillus* sp. As-1 produced higher levels of amylolytic enzymes than the other strains tested, suggesting its potential for commercial enzyme production. Overall, the study conducted by Qader et al. (2006) provides significant insights into the isolation and characterization of a new *Bacillus* strain that could be used for producing commercially important amylolytic enzymes. The findings of this study have implications for the biotechnology industry, as the enzymes produced by *Bacillus* sp. As-1 have various industrial applications.

- The research conducted by Fisher et al. (1961) and published in *Biochemical Preparations* is a noteworthy contribution to the fields of enzymology and human physiology. The article offers a thorough account of the process used to purify α -amylase from human saliva, which involved several techniques such as fractionation, dialysis, and ion exchange chromatography. The authors also present a comprehensive analysis of the physical and chemical characteristics of the purified enzyme, including its molecular weight, pH and temperature optima, and substrate specificity. One of the key findings of the study was that the α -amylase from human saliva was distinguishable from the enzyme present in pancreatic tissue, which had already been extensively studied. The authors observed that the human saliva enzyme had a higher molecular weight and exhibited variations in its substrate specificity and response to inhibitors. The article also explores the potential biological functions of α -amylase in the oral cavity and highlights its potential application as a diagnostic tool for certain medical conditions. In summary, Fisher and Stein's work on α -amylase from human saliva serves as a crucial foundation for future studies on the enzyme and contributes to our comprehension of human physiology and enzymology. Although the research may be considered outdated by contemporary standards, it remains a valuable resource for researchers investigating the history of enzymology.
- It is common practice to screen soil microorganisms for their ability to produce amylase, an enzyme that breaks down starch into simpler sugars. In this study, the researchers from the Department of Microbiology at the Federal University of Technology in Minna, Niger State, Nigeria aimed to isolate and identify amylase-producing microorganisms from soil samples. The researchers collected soil samples from various locations in Minna and incubated them in nutrient broth containing starch as the sole carbon source. After incubation, the samples were screened for amylase production using the iodine-potassium iodide test. The microorganisms that showed positive results were further characterized and identified based on morphological and biochemical tests. The study found that out of the 20 soil samples collected,

14 contained amylase-producing microorganisms. The identified microorganisms included *Bacillus subtilis*, *Bacillus poilus*, *Bacillus licheniformis*, and *Pseudomonas aeruginosa*. The researchers noted that *Bacillus subtilis* was the most dominant amylase-producing microorganism among the isolates. Overall, this study highlights the importance of screening soil microorganisms for their ability to produce valuable enzymes such as amylase. Identifying and characterizing such microorganisms can have important applications in industries such as food, pharmaceutical, and biotechnology (Bala, et al., 2002).

- The authors of the article first discuss the importance of producing amylase and the need for an efficient process. They also review previous studies that have employed various techniques to isolate microorganisms that can produce amylase from sources like soil, water, and plants. These studies have utilized screening methods such as the DNS assay, Congo Red assay, and iodine-potassium iodide test. Next, the authors outline their own methodology for identifying and characterizing amylase-producing microorganisms. They collected soil samples from different parts of India and incubated them in nutrient broth with starch as the only carbon source. The samples were then screened for amylase production using the iodine-potassium iodide test, and those that tested positive were further characterized using morphological and biochemical tests. The authors identified several microorganisms capable of producing amylase, including *Bacillus subtilis*, *Bacillus licheniformis*, and *Pseudomonas aeruginosa*. They also optimized the enzyme production process by adjusting different parameters such as substrate concentration, temperature, and pH. Finally, the authors concluded that their study successfully isolated and characterized several microorganisms capable of producing amylase and optimized the enzyme production process. The findings of this study have important implications for various industries such as food, pharmaceutical, and biotechnology. The authors suggest further research to identify more amylase-producing microorganisms and refine the enzyme production process. Overall, the article provides a detailed overview

of previous research and a valuable methodology for isolating and characterizing amylase producers (Murali, et al., 2017).

- The article discusses the authors' methodology for isolating and characterizing α -amylase produced by *Bacillus subtilis*. They cultured the bacteria in a nutrient medium with starch as the only carbon source and screened for α -amylase production using the iodine-potassium iodide test. The authors also characterized the enzyme using various biochemical tests to assess the impact of factors such as pH, temperature, and substrate concentration on its activity. The authors discovered that *Bacillus subtilis* produced α -amylase optimally at a pH of 7.5 and a temperature of 50°C. They also observed that the enzyme's activity depended on the substrate concentration, with an optimal concentration of 2% starch. As a result, the authors concluded that α -amylase produced by *Bacillus subtilis* had the potential for use in a variety of industries due to its optimal activity conditions. In summary, the article provides a comprehensive review of past studies on α -amylase production and a practical approach for isolating and characterizing the enzyme. The authors' findings have the potential to benefit a range of industries, including food, brewing, and paper. The article's clear and concise presentation makes it a valuable resource for researchers and students studying α -amylase production and its applications (Nadia Riaz, et al., 2009).
- The article by Teodoro et al. (2000) focuses on the culture conditions required for the production of thermostable amylase by *Bacillus* sp. The authors aimed to investigate the optimal conditions for the production of the enzyme by manipulating cultural conditions such as temperature, pH, and substrate concentration. The research involved growing *Bacillus* sp. in a basal medium containing starch as the sole carbon source, and varying cultural conditions to determine the optimal conditions for enzyme production. The results showed that the maximum amylase activity occurred at 60°C, pH 7.0, and a starch concentration of 2.0%, and the enzyme was found to be thermostable, retaining 100% of its activity after being heated at 60°C for 1 hour. The research has significant implications for the biotechnology industry, as

thermostable amylases have several industrial applications, including in the food and pharmaceutical industries. The study's results on the optimal culture conditions for enzyme production could also be used to optimize the production of other industrial enzymes. The article is well-presented and provides a valuable contribution to the study of thermostable amylases and the production of industrial enzymes. The methodology is clear and sound, and the results are significant and useful for researchers and students in the field. The article is concise and easy to follow, making it a valuable resource for those interested in the subject matter.

- Norman's patent (1982) highlights the importance of optimizing the conditions under which the enzymes are used. For example, the patent recommends controlling the pH and temperature during the saccharification process to ensure maximum enzyme activity and stability. This attention to detail and emphasis on optimal conditions is crucial for achieving the desired end product. Furthermore, the patent also mentions the use of activated carbon as a purification step to remove impurities from the glucose syrup. This is an important consideration as impurities can affect the taste, colour, and shelf-life of the final product. In conclusion, Norman's patent presents a comprehensive method for the saccharification of starch hydrolysates to produce glucose syrup. The use of multiple enzymes and optimization of conditions is key to achieving a high yield of glucose syrup with a high glucose content. This patent is a valuable resource for those in the food and beverage industries interested in the production of glucose syrup.
- Miller's (1959) paper on "Use of Dinitro salicylic Acid Reagent for Determination of Reducing Sugar" is a significant work that describes a simple and effective method to quantify reducing sugars. Quantification of reducing sugars is crucial in many fields such as biochemistry, food science, and microbiology as it provides valuable information about sugar amounts in a sample. Miller presented a colorimetric method that involved using the DNS reagent to react with the reducing sugar in the presence of heat, resulting in a coloured product proportional to the amount of reducing sugar present in the

sample. The coloured product was then measured using a spectrophotometer at 540 nm. Miller demonstrated the applicability of the DNS method to a wide range of reducing sugars, including monosaccharides, disaccharides, and polysaccharides. Miller also compared the DNS method with other commonly used methods such as Fehling's and Benedict's and showed that the DNS method was more accurate and sensitive in quantifying reducing sugars. Miller's paper has become a classic reference in biochemistry, and the DNS method has become a standard method for quantifying reducing sugars in many research and industrial applications due to its simplicity, speed, and accuracy. In summary, Miller's paper demonstrates the impact a simple yet effective method can have on a field's progress.

- The article "SDS-PAGE of Proteins" published in CSH Protocols in 2006 and authored by (Richard, et al.,2004) provides an overview of the standard procedure for separating proteins using SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis). The author explains the principles of SDS-PAGE and the factors that affect protein migration in the gel matrix. The article begins with an introduction to SDS-PAGE, highlighting the importance of this technique in protein analysis and identification. The author then describes the composition and function of the different components of the SDS-PAGE system, including the sample buffer, gel matrix, electrophoresis apparatus, and staining and visualization methods. The article also covers the key steps involved in SDS-PAGE, including sample preparation, gel casting, electrophoresis, staining, and destaining. The author emphasizes the importance of carefully controlling experimental conditions, such as pH and temperature, to ensure accurate and reproducible results. In addition to describing the standard SDS-PAGE procedure, the article also discusses variations and modifications of the technique, such as 2D gel electrophoresis and Western blotting. The author provides practical advice and troubleshooting tips for each step of the protocol, making the article a valuable resource for both novice and experienced researchers. Overall, the article "SDS-PAGE of Proteins" provides a comprehensive overview of the principles

and practical applications of SDS-PAGE in protein analysis. It is a well-written and informative resource that will be of interest to researchers in the fields of proteomics, biochemistry, and molecular biology.

CHAPTER - 3

AIM AND OBJECTIVES

AIM

To isolate and screen extracellular enzyme producing bacteria from less explored ecosystem

OBJECTIVE:

- Collection of soil samples from less explored ecosystem
- Isolation of morphologically different bacteria from soil samples
- Selection of potent amylase producing bacteria
- Screening for various extracellular enzyme from bacterial isolates.
- Production and Purification of amylase enzyme from potent bacteria
- Optimization of amylase enzyme production from potent bacteria
- Characterization of potent bacteria

CHAPTER - 4

MATERIALS AND METHODS

4.1. COLLECTION OF SOIL SAMPLES

Soil samples were collected from various area such as Tada (lat. 79.84'28° or long 79° 50' 34" E), VittaiahPalem Forest (lat: 13.5993°N; long: 79.9948°E), andGummidipoondi (lat.13.4466 N, long.80.12095 E) and Thiruporur (lat79.8428°N; long. 79° 50' 34" "E). In all location, few grams of soil samples were transferred to sterile polythene bags with sterile spatula and transport to laboratory for process. (Saravanan *et al.*, 2012) (Table 4.1) (Fig 4.1)

Table 4.1 Collection of soil samples

S. No.	Sampling Area	Sample Code
1	Tada Falls	TF
2	VittaiahPalem Forest	VP
3	Gummidipoondi Forest	GF
4	Thiruporur Forest	TP



Fig 4.1 Soil samples from different areas

4.2. ISOLATION, CHARACTERIZATION AND GENERIC IDENTIFICATION OF BACTERIA

Soil samples were serially diluted using each 9 ml of sterile distilled water. Culturable bacterial population from the collected soil samples were isolated and enumerated by adopting standard spread plate method using nutrient agar medium. One gram of soil sample was serially diluted in 9ml of sterilized distilled water from 10^1 to 10^7 . 0.1 ml of diluted sample was transferred aseptically into respective nutrient Agar plates. The sample was spread uniformly by sterile L rod. The plates were incubated at room temperature for 48 - 72 hrs. The bacterial isolates were further sub cultured to obtain pure culture (Saravanan *et al.*, 2012). Morphologically different bacterial colonies were selected from isolation plate and purified using nutrient agar. All the selected isolates were sub cultured and maintained on nutrient agar slants and preserved at 4°C.

4.3. DETECTION OF VARIOUS ENZYMATIC ACTIVITIES OF SOIL BACTERIA

All the bacterial isolates were screened for various enzymatic activities such as amylase, lipase, protease, glutaminase, asparaginase, cellulase, urease and pectinase by agar plate method (Table 4.2). All the bacterial isolates were spot inoculated on respective enzyme screening media and incubated at 28° C for 48 hours. Enzymatic activity was determined by observing the zone of clearance or zone of colour change by visual inspection or after the addition of developing reagents (Gayathri *al.*, 2010). Further work was directed towards to production of amylase enzyme, from a potential bacterial strain by submerged fermentation.

Table 4.2 Screening mediums and interpretation of extracellular enzymes

Enzyme	Media	Method of Detection	Reagents used
Amylase	Starch Agar	Zone of clearance	1% Iodine
Protease	Skim Milk Agar	Zone of clearance	-
Cellulase	CMC Agar	Zone of clearance	Congo red
Pectinase	Minimal agar + 1%Pectin	Growth	-
Urease	Christensons Urea Agar	Pink Colour	-
Agarase	Artificial Agar Medium	Zone of clearance	1% Iodine
Glutaminase	Nutrient agar + 1% glutamine	Pink colour	-

4.4. SELECTION OF POTENT AMYLASE PRODUCING BACTERIA

Morphological different Bacterial isolates were screened for amylolytic activity by starch hydrolysis test on starch agar plate. The bacterial isolates were spot inoculated on the starch agar plate and incubated at 27°C for 24 -48 hrs. After incubation iodine solution was flooded in plate with dropper for 60 seconds. A clear zone of hydrolysis around the growth indicates positive for amylase production.

4.5. PRODUCTION OF AMYLASE ENZYME FROM POTENT ISOLATE

4.5.1. Inoculum preparation

For the preparation of inoculum, 10 ml of nutrient broth was prepared and inoculated with potent bacterial culture and incubated at 28°C for 18 hours.

4.5.2. Amylase production from bacteria by submerged fermentation:

For production of amylase enzyme, the methodology recommended by Gebreselema (2015) was followed. Each 100 ml of MBM (Mineral Broth Medium) medium at pH 7 was prepared in 500 ml Erlen Meyer flask and used for the production of amylase enzyme. After sterilization, 5% of potent bacterial inoculum was transferred in to MBM production medium and incubated at 28°C in rotary shaker for 120 hours. After incubation, fermentation medium was removed from shaker and centrifuged by using cooling centrifuge at 10,000 rpm for 30 minutes at 4°C. The clear supernatant was collected in screw cap tube and stored at 4°C to use as a crude enzyme

4.6 QUALITATIVE ASSAY OF AMYLASE:

For this study, starch agar was prepared and a well with 5 mm diameter was made at the centre of the agar medium by using well puncher. About 50 µl of crude enzyme was added in to the well and plates were incubated at 27°C for 24 hours. One uninoculated plate was kept as control (Balagurunathan *et al.*, 2010). Bacterial strain with its crude amylase enzyme showed maximum zone of colour change by adding of 1% iodine on starch agar medium.

4.7. SEPARATION AND ACTIVITY OF AMYLASE FROM POTENTIAL STRAIN:

Fine powdered ammonium sulphate was slowly added to sterile distilled water so as to reach 80% saturation. Sterile distilled water added in to 80% saturation so as to reach 40% saturation. The crude enzyme was slowly added to saturated ammonium sulphate to form precipitation. The mixture was kept at 4°C for overnight for precipitation. The precipitated protein was removed by centrifugation at 10,000 rpm at 4°C for 20 minutes. (Sabuet *et al.*, 2005).

4.8. ESTIMATION OF PROTEIN BY LOWRY'S METHOD

The crude protein present in the precipitation content was estimated by the method of Lowry *et al.*, (1951).

Reagents:

Alkaline copper reagent:

Solution A: 2% sodium carbonate in 0.1N sodium hydroxide.

Solution B: 1% copper sulphate in water

Solution C: 2% Sodium potassium tartarate in water.

Above solution was mixed in the proportion of 100:1:1(A: B:C, by volume) immediately before use.

Standard bovine serum albumin:

BSA was used as standard solution to quantify the amount of protein released. BSA stock was prepared by taking 30 mg of BSA in 30 ml of sterile double distilled water. A series of BSA standard was prepared from the stock solution in different concentrations like 0.1, 0.2, 0.3... 1.0 mg/ml.

Extraction of protein

The extraction of the protein can be done by using sonication. The culture was taken in clean autoclaved falcon tubes and centrifuged at 4000 rpm for 5 minutes. After centrifugation add 2 ml of phosphate buffer solution to the

supernatant and kept in ice. Sonicate the sample at 0.7 amp for five minutes. After sonication the samples were sealed and kept in the centrifuge at 10000 rpm for 15 minutes at 4°C to extract the protein contain in the supernatant.

Procedure

To 0.1 ml of sample or standard, add 0.1 ml of 2 N NaOH and hydrolyse at 100°C for 10 min in a heating block or boiling water bath. Cool the hydrolysate to room temperature and added 1 ml of freshly mixed complex-forming reagent. Let the solution stand at room temperature for 10 min. Added 0.1 ml of Folin reagent, using a vortex mixer, and let the mixture stand at room temperature for 30-60 min (do not exceed 60 min). Read the absorbance at 660nm. Plot a standard curve of absorbance as a function of initial protein concentration and use it to determine the unknown protein concentrations.

4.9. QUANTITATIVE ASSAY OF AMYLASE ENZYME

Amylase activity were assayed by measuring the amount of reducing sugars released from starch using dinitro salicylic acid method (Bernfeld 1995)

4.9.1 Estimation of Maltose by DNS method

A standard maltose curve is prepared using standard as 1mg/ml. d maltose solution is taken and made up to mark by adding distill H₂O. Then add 1ml of D.N.S reagent to each tube and incubated it in boiling Different aliquots of standard water bath for 15 min. After cooling 8 ml of distilled water was added and mix it thoroughly and read the absorbance at 540 nm in a UV spectrophotometer

4.9.2 Enzyme activity

Enzyme activity is determined by DNS method. 0.5ml of isolated enzyme is incubating for 10 min at 370 C with 0.5 ml of substrate (ET) & 0.5 ml of substrate for (EC). After 10 min arrest the reaction by adding 1 ml of DNS to both the tube followed by 0.5 ml of enzyme to control tube only. Then kept the tubes in boiling water bath for 15 min. The solution is then dilute with 8 ml of distilled water & read the absorbance at 540 nm

4.10. OPTIMIZATION OF AMYLASE PRODUCTION

Bacterial inoculum was prepared by inoculating potential bacteria culture on nutrient broth and kept in rotary shaker for 18 hours at 28°C. Each 5 ml of nutrient broth culture was used as inoculum.

4.10.1. Effect of carbon source:

To study the effect of carbon source about 5 ml of 18hours potential bacterial culture inoculum was inoculated into series of flasks containing 100 ml of minimal broth medium (replacing with carbon source) with 1% different carbon sources (glucose, fructose, sucrose, mannitol and starch). All the flasks were incubated at 28°C in rotary shaker with 120 rpm for 120 hours.

4.10.2. Effect of nitrogen source:

To study the effect of nitrogen source about 5 ml of 18hours potential bacterial strain inoculum was inoculated into series of flasks containing 100 ml of Minimal broth medium (replacing with nitrogen source) with 1% different nitrogen sources (yeast extract, malt extract, beef extract, peptone, urea). All the flasks were incubated at 28°C in rotary shaker with 120 rpm for 120 hours.

4.10.3. Effect of pH:

To study the effect of pH about 5 ml of 18hours inoculum was inoculated into series of flasks containing 100 ml of minimal broth medium with different pH (5, 6, 7, 8 and 9). All the flasks were incubated at 28°C in rotary shaker with 120 rpm for 120 hours.

4.10.4 Effect of temperature:

To study the effect of temperature about 5 ml 18hours potential bacterial strain inoculum was inoculated into series of flasks containing 100 ml of minimal broth medium with different temperature (25°C, 30, 35, 40 and 45°C). All the flasks were incubated at 28°C in rotary shaker with 120 rpm for 120 hours.

4.10.5 Effect of incubation:

To study the effect of incubation period, about 5 ml 18hours potential bacterial strain inoculum was inoculated into series of flasks containing 100 ml of minimal

broth medium with incubation duration such as 24, 48, 72, 96 and 120 hours. All the flasks were incubated at 28°C in rotary shaker with 120 rpm for 120 hours.

4.11. CHARACTERIZATION OF POTENT ISOLATE

Microscopic, cultural and biochemical characteristics of potential strain were studied by adopting standard procedure and the potential strain was identified with the help of Bergey's manual of Systematic Bacteriology.

4.11.1 Gram staining: Gram staining was performed for bacteria stain as indicated by the normal technique. A smear of bacterial cells was ready on a spotless glass slide by a delicate intensity obsession. The intensity fixed smear was overflowed with gem violet arrangement briefly. Smear was washed with water followed by adding severe Gram's iodine. The smear was decolorized with 95% ethyl liquor and flushed with water. At long last safranin was utilized as counter stains for 60-80 sec and washed with water. Cells were then inspected under microscope. (Kohinoor Begum et al.,2017).

4.11.2 Motility test [*Hanging drop method*]: soak the four edges of the coverslip with water to keep the coverslip solidly in place. A ring of petroleum jelly might be utilized instead of water to keep the coverslip immovably set up on a magnifying instrument slide on the off chance that magnifying instrument slides with focal gloom are not accessible. The Vaseline-fixed gloom or some of the time utilizing plasticise likewise dials back the drying-out process, so the living beings can be noticed for longer periods. place a little drop of fluid bacterial culture in the focal point of a coverslip rearrange a slide with a focal despondency over the coverslip the coverslip will adhere to the slide and when the slide is rearranged the drop of bacterial culture will be suspended in the well inspect minutely (x400).

Positive outcome: A shooting, crisscross, tumbling or other coordinated development.

Negative outcome: No development or Brownian movement as it were

4.11.3 Catalase test: It was performed by taking a drop of 3% hydrogen peroxide was added to 48 hr old bacterial province on a perfect glass slide and blended utilizing a sterile tooth-pick. The foam shown catalase action.

4.11.4 Ooxidase test: It was performed with 1% arrangement of Tetramethyl-pphenyldiamine-dihydrochloride which was soaked in a piece of filter paper. A culture was placed on filter paper by using sterile glass rod furthermore, addressed to the paper with impregnated Reagent. A dim purple variety improvement inside 5-10 second was viewed as positive and no difference as negative.

CHAPTER - 5

RESULTS AND DISCUSSION

5.1. ISOLATION AND CHARACTERIZATION OF BACTERIA FROM FOREST SOIL

Totally 28 morphologically different bacterial colonies were isolated from the soil sample in which 10 isolates were from Thiruporur forest (TP), 7 isolates from Vittaiiah Palem (VP) forest, 05 from Gummidipoondi (GA)forest, 06 isolates from Tada falls (TF). The bacterial population in the collected soil samples was ranged between 1.3×10^3 and 1.9×10^5 cfu/gm of soil. Majority of the bacterial isolates produced non pigmented colonies on nutrient agar. Of the 28 isolates 5 were pigmented and 23 (68.8%) were non-pigmented isolates. Pigments include yellow, orange and white. (Fig 5.1) (Table 5.1). In Previous study Saravanan et al, (2015) isolated bacteria from soil samples collected from kodaikannal forest.

Table 5.1 Isolation of bacteria from different soil samples

S. No.	Sample Name	No. of isolates
1	TP	10
2	VP	7
3	GA	5
4	TH	6
Total		28

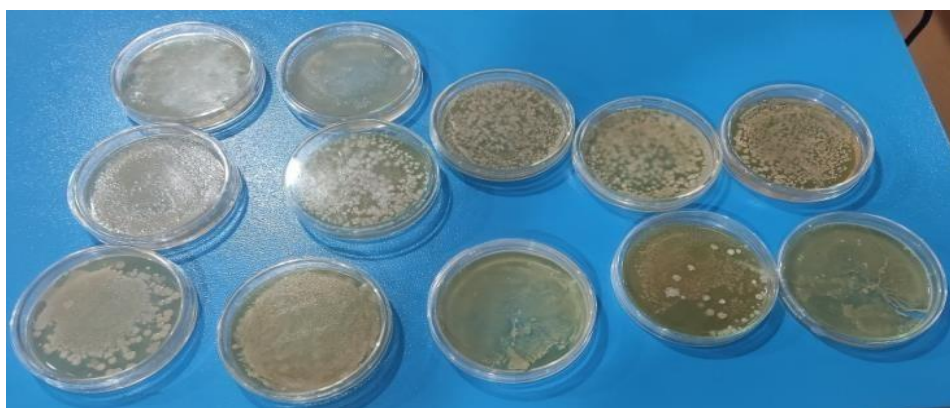


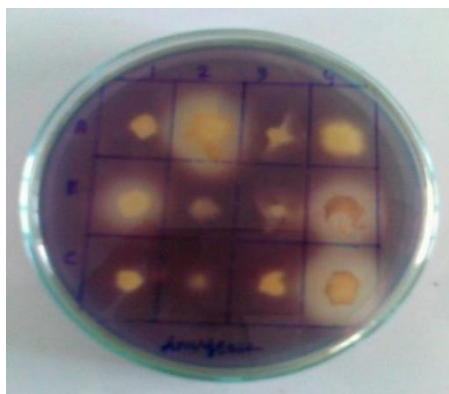
Fig 5.1 Isolation of bacterial isolates from soil samples

5.2. DETECTION OF VARIOUS ENZYMATIC ACTIVITIES OF FOREST BACTERIA

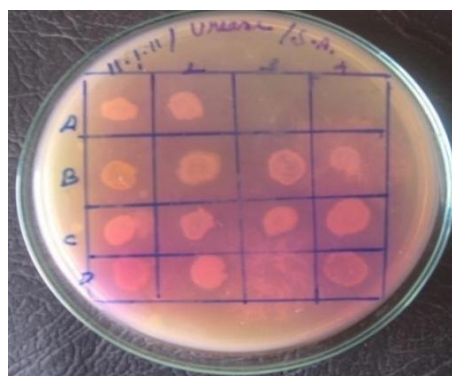
In enzyme screening, all isolates exhibited one/more enzymatic activities. In this present study out of 28 bacterial isolates 13 isolates exhibited pectinase activity followed by protease activity by 10 isolates. Apart from this, 24 isolates exhibited urease, amylase activity by 16 isolates, cellulase 01 isolates, agarase activity by 01 isolates and there is no glutamine producing bacteria (Fig 5.2). (Table 5.2). In previous study by Praveen Kumar et al, (2015) screened various enzymatic activities in soil collected from Northern Tamil Nadu

Table 5.2 Number of isolates showing positive for various enzymes

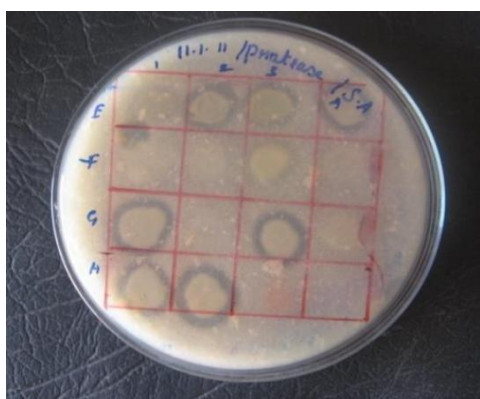
S. No.	Enzymes	No. of Positive Isolates
1	Amylase	16
2	Protease	10
3	Cellulase	01
4	Pectinase	13
5	Urease	24
6	Agarase	01
7	Glutaminase	0



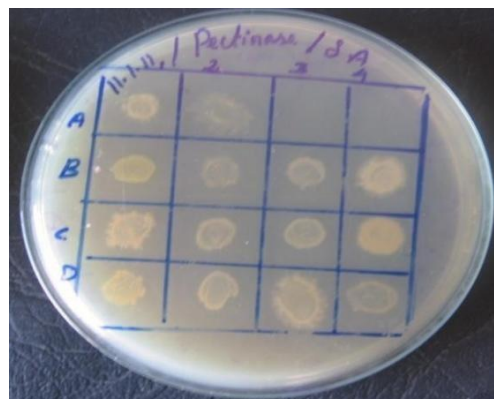
(a) Amylase



(b) Urease



(c) Protease



(d) Pectinase

Fig 5.2 (a-d) Enzyme activity by bacterial isolates

5.3. SELECTION OF POTENTIAL AMYLASE PRODUCING BACTERIA

Among the 15 amylase producing isolates, bacterial isolate TF1 showed maximum size of 20 mm in diameter of zone of clearance in starch agar after flooded with iodine. Hence the strain TF1 was selected as potential isolate for further studies (Table 5.3) (Figure 5.3).

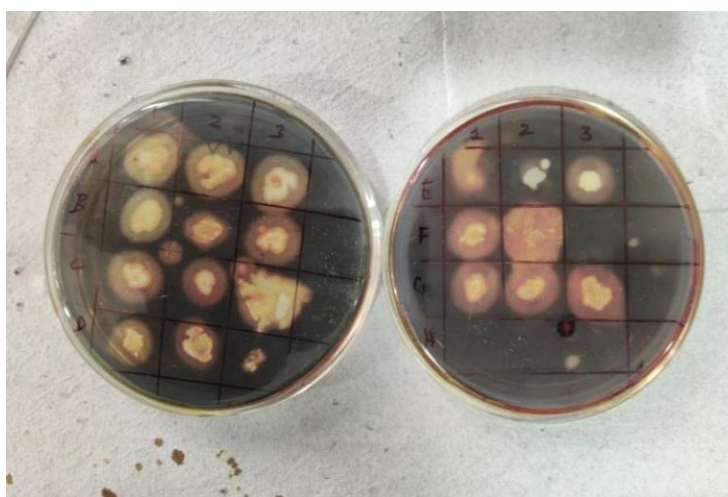


Figure 5.3 Screening of amylase enzyme producing bacteria

Table 5.3 Screening of amylase producing bacterial isolates

Bacterial isolates	Zone of clearance in mm in diameter
TP - 1	16
TP - 4	18
TP - 5	16
TP - 5*	19
TP - 6	10
TP - 6*	14
TP - 7	14
TP - 7*	12
TP - 8	10
TH - 1	10
VP - 7	12
VP - 10	14
TF - 1	20
GA - 3	14
GA - 4	16

5.4. PRODUCTION AND PRECIPITATION OF AMYLASE ENZYME

During fermentation, after 72 hours there was no characteristic colour change was observed in the medium. The culture broth was collected, centrifuged and the supernatant is used as crude enzyme for semi quantitative assay (Figure 5.4). In the Previous study Das et al, (2017) done production and precipitation of amylase enzyme from soil



Fig 5.4 Production of amylase enzyme by shake flask method

5.5. QUALITATIVE ASSAY

In qualitative assay, the crude enzyme and the precipitated protein prepared from the strain TF1 showed 24 mm in diameter of zone of clearance in starch agar

5.6. PROTEIN ESTIMATION

Based on the optical density of purified enzyme by TF1 and standard protein Bovine serum albumin (BSA), the protein content present in the purified enzyme was estimated as 0.35mg/ml. (Figure 5.5) (Table 5.4) in the previous study Shivaji Bole et al (2013) has done Lowrys method for the estimation of protein in amylase enzyme

Table 5.4 Procedure and values of Lowry's Method

Test tube	Std BSA sol.(ml)	dH2O (ml)	Alkaline Cu reagent(ml)	Incubation (min)	F.C. Reagent (ml)	Incubation (min)	O.D at 660nm
Blank	0.0	3	5	10	0.5	30	0.201
S1	0.5	2.5	5	10	0.5	30	0.333
S2	1	2	5	10	0.5	30	0.445
S3	1.5	1.5	5	10	0.5	30	0.587
S4	2	1	5	10	0.5	30	0.663
S5	2.5	0.5	5	10	0.5	30	0.757
T1	1	2	5	10	0.5	30	0.511

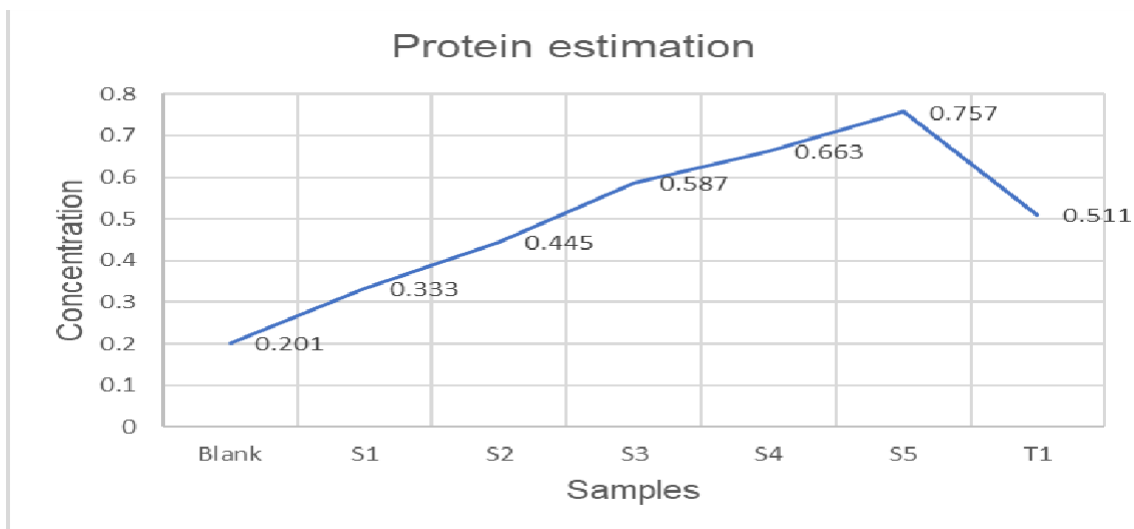


Fig 5.5 Graph showing protein estimation in crude enzyme

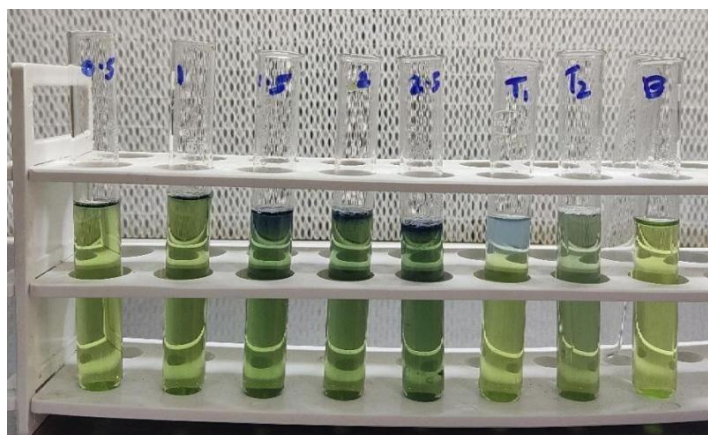


Figure 5.6 Experimental results of protein estimation in crude enzyme

5.7. QUANTITATIVE ASSAY OF AMYLASE ENZYME

Based on the optical density of purified enzyme by TF1 and standard Maltose curve graph represents the amylase content present in the purified enzyme was estimated

Table 5.5 Maltose content absorbed in UV at 540nm

S. No.	Std Maltose . (ml)	dH2O (ml)	Conc of maltose (µg /ml)	DNS (ml)	Incubation (min)	dH2O (ml)	OD at 540 nm
BLANK	0.0	1.0	0	1.0	10	8.0	0.0
S1	0.2	0.8	200	1.0	10	8.0	0.07
S2	0.4	0.6	400	1.0	10	8.0	0.14
S3	0.6	0.4	600	1.0	10	8.0	0.21
S4	0.8	0.2	800	1.0	10	8.0	0.28
S5	1.0	0.0	1000	1.0	10	8.0	0.35

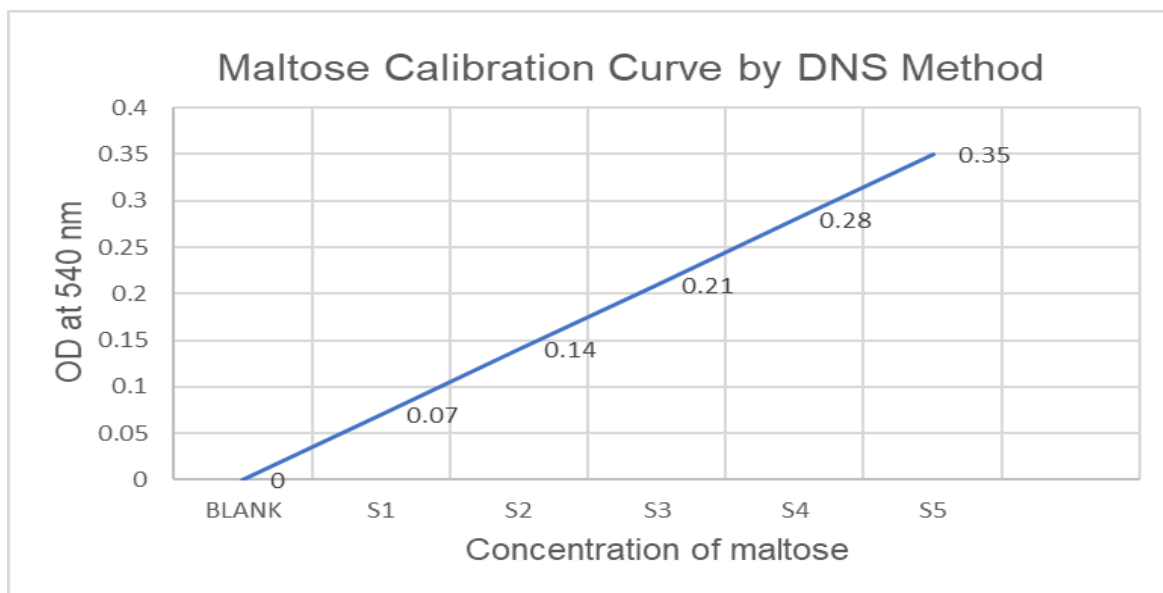


Fig 5.7 Graph showing Maltose estimation by DNS method

Table 5.6 Enzyme activity by maltose

S. No.	Std enzyme (ml)	Substrate	Incubation (min)	DNS (ml)	dH2O (ml)	OD at 540 nm
BLANK	0	0.5	30	1	8	0.0
T1	1	0.5	30	1	8	0.10

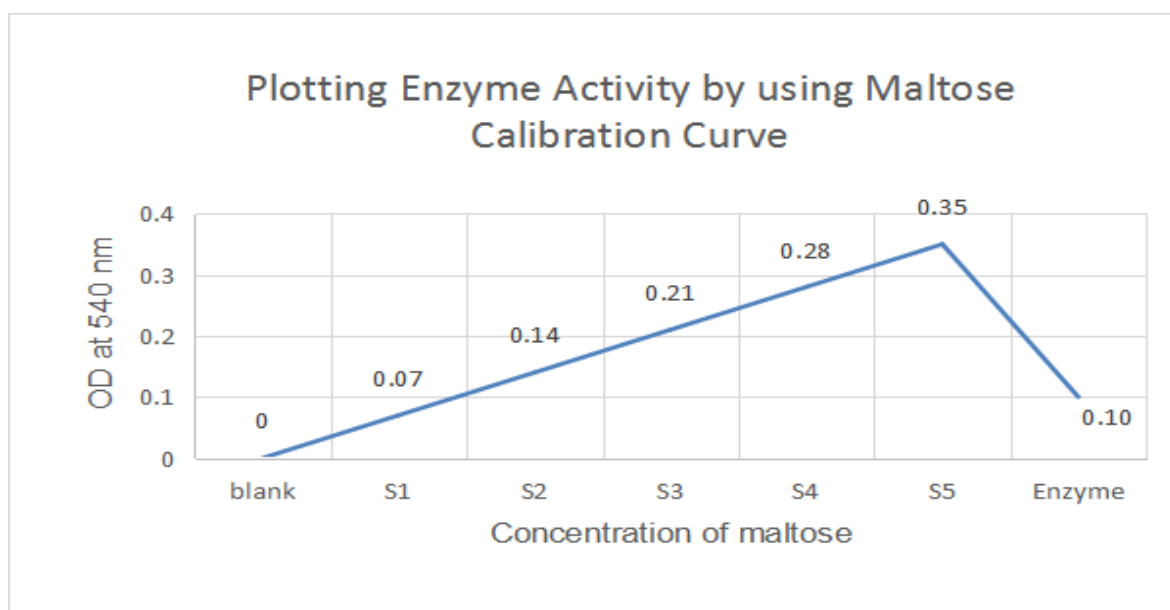


Fig 5.8 Graph showing enzyme activity

5.8. OPTIMIZATION OF AMYLASE PRODUCTION

In optimization studies, variables such as dextrose (carbon source), ammonium nitrate (nitrogen source) and pH 7 at 72 hours of inoculum size 10% was found to influence the enzyme production (Figure 5.9). In the previous study Indira et al (2012) has done optimization for effect of pH, Temperature, Carbon source, Nitrogen source and Inoculum size.

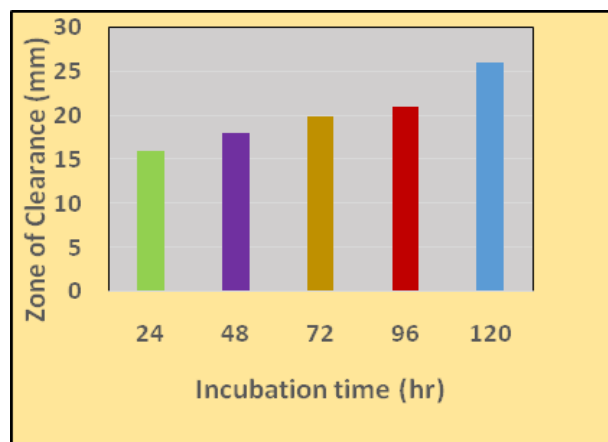
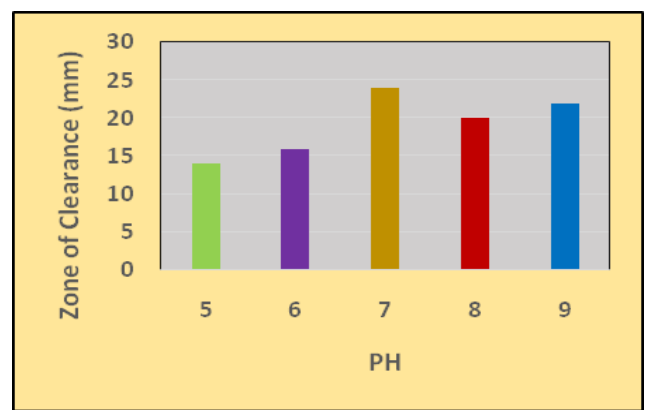
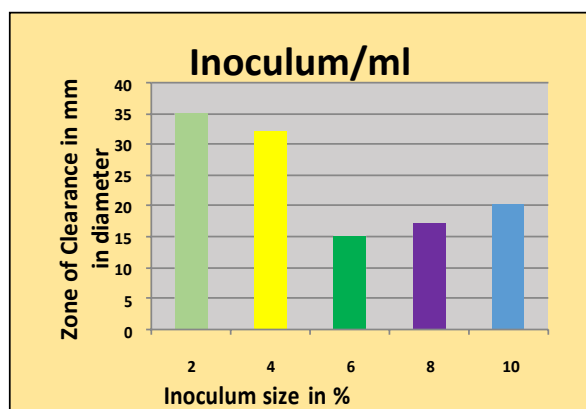
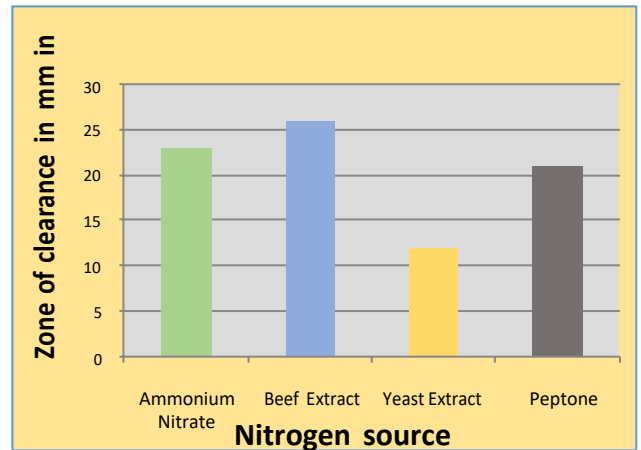
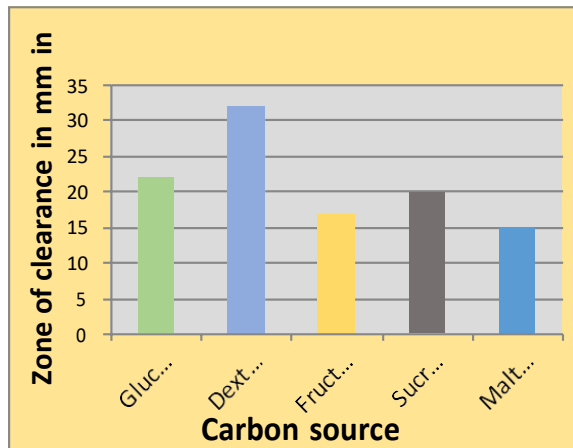


Fig 5.9 Optimization of amylase enzyme by potent isolates

5.9 CHARACTERIZATION OF PURE BACTERIA ISOLATE

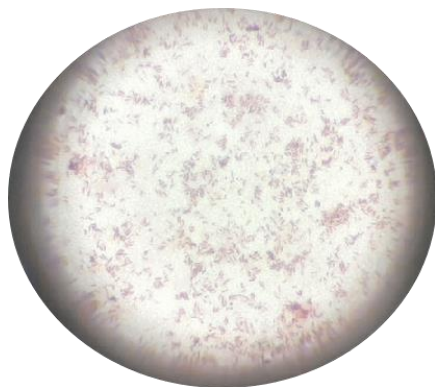


Fig 5.10 Microscopic view of potent isolate

In this study, gram staining and biochemical tests were used to analyse the characteristics of bacterial isolates. In contrast, the bacteria isolates were gram positive Bacilli

Table 5.5 Characterization of potent isolate

Characterization	Result
Gram staining	Gram positive (Bacilli)
Motility	Motile bacteria
Catalase	Positive
Oxidase	Negative
Hydrolysis of starch	Positive
Citrate utilization	Positive
Indole production	Negative

CHAPTER - 6

SUMMARY & CONCLUSION

SUMMARY:

Enzymatic potential of less explored soil bacteria was investigated. Totally 28 morphologically different bacterial cultures including were isolated from five different regions of less explored ecosystem, around Chennai.

In the screening for enzyme activity, maximum of 13 isolates exhibited pectinase activity followed by protease activity by 10 isolates. Apart from this, 24 isolates exhibited urease, amylase activity by 21 bisolates, glutaminase activity by 0 isolates, cellulase 1 isolates, phosphatase by 8 isolates

Bacterial strain TF1 showed maximum size of 20 mm in diameter of clear zone around colony in starch agar plate. Hence the isolate TF1 was selected as potential isolate for further studies.

Amylase enzyme was partially purified from the cell free supernatant by precipitation, followed estimation of protein analysis. Variables such as Dextrose (carbon source), Ammonium Nitrate (nitrogen source) and pH 7 at 96 hours of incubation period at 37°C was found to influence the enzyme production.

The amylase enzyme producing isolate TF1 isolated from Tada falls was identified as *Bacillus species*.

CONCLUSION:

Microorganisms from less explored ecosystems are the promising source for potent enzyme for various applications. In the present study, diverse pattern of various enzymatic activities is exhibited by bacteria isolated from different soil samples. Bacterial isolates TF1 showed the promising source for amylase enzyme. Finding of the present work evidenced that the less explored ecosystems investigated in this study deserves the potential for amylase enzyme resources for industrial applications.

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