Isolation and Screening of Pectinase Producing Bacteria

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DECLARATION

I declare that this thesis entitled "Isolation and Screening of Pectinase Producing Bacteria" is the result of my own research except as cited in the references.

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PEMENCILAN DAN PENYARING BAKTERIA PENGHASIL PEKTINASE

ABSTRAK

Enzim memainkan peranan penting dalam pelbagai proses industri, dengan pectinase menjadi penting kerana aplikasinya dalam pengekstrakan jus buah dan rawatan sisa. Walau bagaimanapun, sumber semasa enzim pectinase sering terhad dalam hasil dan spesifisiti, menyorot keperluan untuk meneroka sumber mikrob yang baru. Objektif kajian merangkumi penyaringan dan mengasingkan bakteria pectinolytic, mengukur aktiviti pectinase, dan mengkarakterisasikan strain yang diasingkan melalui kajian morfologi dan molekul. Tanah, sebagai tapak pelbagai komuniti mikrob, menawarkan potensi besar untuk aplikasi bioteknologi. Dalam tesis ini, kami mengkaji kepelbagaian mikrob dalam tanah dan cuba mengenal pasti strain yang mampu menghasilkan enzim pectinase. Teknik pencairan berurutan digunakan untuk mengurangkan kandungan mikrob, diikuti dengan penyaringan pada agar nutrien dan pemilihan koloni positif pada agar pectin. Analisis morfologi mengenal pasti enam strain berbeza, tiga gram positif dan tiga gram negatif, yang kemudiannya dikarakterisasikan. Pengenalaksanaan molekul melibatkan fermentasi semalaman dalam calon Luberia Bertani, ekstraksi DNA, dan elektroforesis gel, yang mendedahkan kepelbagaian genetik strain yang dipilih.

Spektrofotometri NanoDrop digunakan untuk menilai keaslian dan kepekatan DNA. Hasilnya menunjukkan variasi dalam kepekatan DNA dan nisbah keaslian di kalangan isolat. Sebagai contoh, ST3 menunjukkan kepekatan DNA tertinggi dan nisbah keaslian tertinggi, menunjukkan jumlah DNA tulen yang tinggi dalam sampel. Sebaliknya, ST10 menunjukkan kepekatan DNA terendah dan nisbah keaslian negatif, menunjukkan kemungkinan pencemaran atau masalah dengan sampel tersebut. Aktiviti pectinase diukur menggunakan kaedah DNS, yang menunjukkan potensi enzimatik strain yang diasingkan. Ujian aktiviti enzim mendedahkan kepelbagaian di antara strain, dengan ST3 dan ST13 menunjukkan aktiviti pectinase tertinggi. Kajian ini menekankan kepentingan mikrob tanah dalam proses bioteknologi dan menyediakan pandangan berharga tentang isolasi dan pencirian strain yang menghasilkan pectinase, dan hasil

ini menyumbang kepada pengenalpastian sumber-sumber baru enzim pectinase, dengan demikian memajukan pembangunan proses enzimatik yang cekap dalam pelbagai industri.

Kata kunci: Bakteria penghasil pectinase, Aplikasi industri, Enzim, bakteria Pectinolytic, agar Pectin, ujian Pectinase.

ISOLATION AND SCREENING OF PECTINASE PRODUCING BACTERIA

ABSTRACT

Enzymes play a crucial role in various industrial processes, with pectinases being of particular significance due to their application in fruit juice extraction and waste treatment. However, the current sources of pectinase enzymes are often limited in yield and specificity, highlighting the need for exploration of novel microbial sources. The study's objectives encompass screening and isolating pectinolytic bacteria, measuring pectinase activity, and characterizing isolated strains through morphological and molecular studies. Soil, as a reservoir of diverse microbial communities, offers immense potential for biotechnological applications. In this thesis, we investigated soil microbial diversity and sought to identify strains capable of producing pectinase enzymes. Serial dilution techniques were employed to reduce microbial content, followed by screening on nutrient agar and subsequent selection of positive colonies on pectin agar. Morphological analysis identified six distinct strains, three gram-positive and three gram-negative, which were further characterized.

Molecular identification involved overnight fermentation in LB broth, DNA extraction, and gel electrophoresis, revealing the genetic diversity of the selected strains. NanoDrop spectrophotometry was employed to evaluate DNA purity and concentration. The results showed variations in DNA concentration and purity ratios across the isolates. For instance, ST3 exhibited the highest concentration of DNA and the highest purity ratio, indicating a high amount of pure DNA in the sample. In contrast, ST10 displayed the lowest concentration of DNA and a negative purity ratio, suggesting potential contamination or issues with the sample. Pectinase activity was quantified using the DNS method, demonstrating the enzymatic potential of the isolated strains. Enzyme activity assays revealed variability among strains, with ST3 and ST13 demonstrating the highest pectinase activities. This study underscores the importance of soil microbes in biotechnological processes and provides valuable insights into the isolation and characterization

of pectinase-producing strains, and these results contribute to the identification of novel sources of pectinase enzymes, thereby advancing the development of efficient enzymatic processes in various industries.

Keywords: Pectinase-producing bacteria, Industrial applications, Enzymes, Pectinolytic bacteria, Pectin agar, Pectinase assay

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LIST OF ABBREVIATIONS

µmol	micromole
µl	microliter
ng	nanogram
PCR	Polymerase chain reaction
kDa	Kilodalton
HG	Homogalacturonan
GalA	galacturonic acid
HM	high-methoxy
LM	low-methoxy
RG-I	rhamnogalacturonan I
Rha	rhamnose
Ara	arabinose
Gal	galactose
Xyl	xylose
RG-II	rhamnogalacturonan II
PE	pectineterase
PPases	proteopectinase
SmF	Submerged fermentation.
SSF	Solid-state fermentation
spp	species plural
DNS	dinitrosalicylic acid
UV	ultraviolet
LB	Luria bertani
EDTA	Ethylenediaminetetraacetic acid
GTE	Glucose-Tris-EDTA
TAE	tris-acetate-EDTA
DNA	Deoxyribonucleic acid
ml	milliliter
g	gram
℃	celcius
mm	millimeter
OD	optical density
M	molar
nm	nanometer
ST	strain
bp	basepair
rpm	Revolutions Per Minute

LIST OF SYMBOLS

%	percentage
U	Unit
V	Volt
α	Alpha

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Enzymes are tiny atoms found in the cells of living beings that can speed up chemical events without changing the outcome. Microorganisms represent most of the industrial demand for enzymes. Microorganisms are commonly used in industry for enzyme synthesis due to their high growth capabilities, short life period, and ease of genetic manipulation. Pectinases, also known as pectinolytic enzymes, are a diverse group of enzymes that target pectin and depolymerize it by hydrolysis processes . Pectinolytic enzyme or pectinase enzymes is one of the enzymes in industrial demand. Henri Braconnot was the one who first isolated and described pectinase in 1825 (Shahapurkar, 2023). The first commercial use was in 1930 for extracting juices. In the 1960s, it started to be used in wine production. Pectinases are a type of complex enzyme found in higher plant bacteria that uses pectin as a substrate (Abdollahzadeh and Pazhang et al., 2020).

Pectin is a component of the primary cell wall found mostly in the middle lamella area of dicotyledonous plants (Van Buren, 1991 ; Mahto & Yadav et al., 2023). Pectin acts as a binding material between cells, alongside cellulose and hemicellulose. Pectin is classified as a homogalacturonan, consisting of a series of galacturonic acid units connected by α-1,4 glycosidic bonds (Artur & Pieczywek et al., 2021) These pectin substances are vital components of plant cells, possessing a high molecular weight, carrying a negative charge, and exhibiting acidic properties. They play a crucial role in providing structural integrity to the stems and non-woody parts of plants. During the ripening of fruits and other edible produce, pectin is enzymatically degraded by pectinase enzymes (Prado & Melfi et al., 2016 ; Shi & Liu et al., 2022).

Pectinolytic bacteria comprise a diverse type of enzymes that effectively hydrolyze the pectin regions of homogalacturonan and rhamnogalacturonan, which are complex polymers (Kim & Healey et al., 2019). Pectinase itself is a heteropolysaccharide with a high molecular weight, composed of galacturonic acid residues linkage by 1,4 glycosidic bonds (Samanta, 2019). These residues possess carboxyl groups, which can undergo methylation to form methoxyl groups. The pectin molecule contains additional side chains such as L-rhamnose, arabinose, galactose, and xylose (Thakur & Singh et al., 1997 ; de Souza and Kawaguti, 2021).

Pectinase was the first enzyme to find application in household settings. Its primary function is the breakdown of pectin into smaller molecules such as oligosaccharides and monosaccharides (Bonnin & Garnier et al., 2014 ; Ropartz and Ralet, 2020). The biotechnological applications of pectinase are extensive, particularly in the extraction, clarification, and liquefaction of fruit juices and wines within the industry (Reddy and Sreeramulu, 2012). Pectinolytic enzymes also play an important role in plant pathogenicity and the spoilage of fruits and vegetables. Thus, the present study focuses on isolating and identifying the pectinase-producing bacteria from the soil, understanding their phylogenetic relationship with each other, and studying their characterization.

1.2 Problem Statement

The industrial demand for pectinase enzymes, pivotal in processes such as fruit juice extraction and waste treatment, necessitates a continual exploration for novel microbial sources. Despite the known applications of pectinases, the current sources are often associated with limitations in terms of yield and specificity. This research addresses the existing gap in our understanding by focusing on the isolation and screening of pectinase-producing bacteria. The scarcity of diverse and efficient sources poses a challenge in meeting the increasing demand for these enzymes, urging the need for systematic exploration and identification of novel strains capable of robust pectinase production.

The conventional methods for isolating pectinolytic bacteria have been limited, and there is a need for a more targeted approach. The utilization of pectin agar as a selective medium offers a promising avenue for the isolation of bacterial strains with high pectinolytic potential. However, the systematic screening and isolation of such strains from diverse environmental sources remain an unexplored domain. Consequently, this research aims to bridge this gap by employing pectin agar for the systematic screening and isolation of pectinase-producing bacteria, setting the stage for the subsequent assessment of their enzymatic activity.

In addition to the isolation and screening process, the lack of comprehensive identification and characterization of the isolated strains hinders their potential application in industrial settings. Existing studies often lack a detailed morphological and molecular analysis of pectinolytic bacteria. This research aims to address this limitation by not only isolating and measuring pectinase activity but also by conducting morphological studies and molecular identification. This holistic approach is vital for understanding the structural and genetic attributes of the isolated strains, contributing to the broader goals of optimizing pectinase production and promoting sustainable industrial practices.

1.3 Objectives

Based on the problem statement as described above, the main objective of this research is to isolate and screen pectinase producing bacteria. The objective to be achieved in this research are as follows:

a) To isolate and screen the pectinolytic bacteria using pectin agar.
b) To measure pectinase activity of selective bacteria using pectinase assay method.
c) To characterize isolated strain pectinolytic bacteria by morphological study and molecular identification.

1.4 Scope of Study

The study is about isolating and screening pectinolytic producing bacteria from soil using pectin agar. The soil sample needs to find potential pectinase producing bacteria using pectin agar as a selective medium because it favors the growth of pectinolytic microorganisms while inhibiting the growth of non-pectinolytic organisms. Pectinolytic bacteria can utilize pectin as a carbon source in the isolation process from soil. Bacterial strains with high pectinolytic activity can be found and chosen using pectin agar. The clear zones observed around the colonies can be an indicator of the potential of the strain to produce pectinolytic enzymes, aiding in the identification of promising isolates for further study and application.

Using the pectinase assay method, measure the pectinase activity of selective bacteria to identify a special enzyme and to prove its presence or absence in a distinct specimen, like an organism or a tissue. A pectinase assay was carried out to determine the amount of the enzyme in the sample.

Finally, identify the characterization of isolated strains of pectinolytic bacteria by morphological study and molecular method to identify uncommon pectinolytic bacteria and directly analyst the genetic material of the organism, providing a higher level of accuracy compared to phenotypic or biochemical identification methods, which can be subjective and prone to errors.

1.5 Signification of study

The focus of the study is to produce valuable consumption by isolating pectinase producing bacteria from waste. This may aid in the discovery of new bacterial strains that can produce pectinase enzymes. By increasing the variety of potential sources for pectinase production, it becomes possible to find enzymes with unique properties or increased activity that may be useful in a variety of industrial applications. By isolating and screening pectinase producing bacteria, the study contributes to the development of efficient and eco-friendly enzymatic processes in these industries, leading to improved product quality, increased efficiency, and reduced environmental impact.

CHAPTER 2

LITERATURE REVIEW

2.1 Enzyme

Enzymes play an important role in the survival of all forms of life. These catalysts, predominantly composed of proteins (excluding ribozymes), facilitate, and accelerate biological reactions (Millan-Linares & Montserrat-de la Paz et al., 2021). Enzymes are protein molecules with a high molecular weight, consisting of chains linked together of amino acids by peptide bonds. Their primary function is to lower the activation energy required for chemical processes to occur within living organisms. In comparison of chemical catalysts, enzymes offer numerous advantages, including high specificity, remarkable catalytic efficiency, and adjustable activity. These characteristics make enzymes highly valuable in industries such as pharmaceuticals, chemicals, and food production (Oumer and Abate, 2018). Their presence and functionality are crucial for the efficient execution of these biological processes.

2.1.1 Pectinolytic enzyme

Pectinolytic enzymes are a group of enzymes that specifically target and break down substrates containing pectin. Through hydrolysis, these enzymes cleave the linkages present in pectin, leading to the degradation of pectin into smaller molecules such as oligosaccharides and monosaccharides (Patidar & Nighojkar et al., 2018). In the realm of plants, pectinolytic enzymes hold significant importance as they are involved in processes like cellular growth, elongation, and fruit ripening. Bacterial pectolytic activity is particularly relevant in the context of plant pathogenesis, as these enzymes are among the first to initiate tissue degradation (Winkelmann, 1992 ; Collmer & Berman et al., 2012).While fungal sources are known to be potent producers of pectic enzymes, it is worth noting that bacteria also have the capacity to produce pectinolytic

enzymes if appropriate methods are employed. The application of these enzymes spans across a wide range of industries, showcasing their versatility and potential for use in various sectors (A Collmer and Keen, 1986).

2.1.2 Bacterial pectinolytic enzyme

Pectinolytic bacteria are a class of bacteria capable of producing and secreting pectinolytic enzymes, enabling them to utilize pectin as a carbon source. These bacteria have evolved this ability to access nutrients and energy present in plant materials. Pectinolytic bacteria can be found in diverse environments, including soil, water, and various plant surfaces. They encompass a large scale of bacterial genera, such as *Bacillus*, *Pseudomonas, Erwinia, Xanthomonas*, and *Klebsiella*, among others (Bhatt & Suyal et al., 2022). The pectinolytic enzymes produced by these bacteria are highly valuable in numerous industries, including food processing, textiles, and the generation of biofuels. Notably, bacterial strains are often preferred over fungal strains due to their ease of fermentation and the potential for strain improvement to enhance enzyme production (Tapre and Jain, 2014). This advantage makes bacterial pectinolytic enzymes more desirable for industrial applications.

2.2 Structure of pectin

The structure of pectin provides the foundational understanding for the action of pectinase enzymes. Pectin is a heteropolysaccharide with a molecular weight ranging from 30 to 300 kDa, primarily composed of homogalacturonan (Noor & Shah et al., 2021). This main component consists of repeating units of galacturonic acid linked together through glycosidic linkages, forming a linear chain with side chains attached (Sharma & Gautam et al., 2021). These side chains, which include sugars like rhamnose, arabinose, and galactose, vary in arrangement and distribution along the pectin backbone, resulting in diverse types and structures of pectin. (Moore & Farrant et al., 2008). Pectic polysaccharides are broadly classified into three major groups: homogalacturonan, rhamnogalacturonan-I, and rhamnogalacturonan-II (Mutter & Beldman et al., 1994). Understanding the composition and arrangement of pectin molecules lays the groundwork for elucidating the mechanisms by which pectinolytic enzymes, including pectinase, break down these complex compounds.

2.2.1 Homogalacturonan (HG)

Homogalacturonan (HG) is a major component of pectin and is found in the majority of pectic compounds. It is a linear polymer made up of repeated galacturonic acid (GalA) units connected by -(1,4) glycosidic linkages (Pieczywek & Cieśla et al., 2021). The galacturonic acid units of homogalacturonan can be modified in a variety of ways, including methylation and acetylation. Methylation is the addition of methyl groups to galacturonic acid units. This influences how soluble and gelling the pectin is. More methylation makes pectin more hydrophobic and prone to gel formation, while less methylation makes it more water soluble (Luzio and Cameron, 2008). Acetylation is the addition of acetyl groups to galacturonic acid units. This influences pectin's stability and interactions, as well as its capacity to create gels. When exposed to enzymes, acetylated pectin is more stable and less prone to degrade. The quantity and structure of methylation and acetylation in HG might vary, resulting in various forms of pectin with distinct characteristics. High-methoxy pectin (HM pectin) has high methylation and low acetylation,

whereas low-methoxy pectin (LM pectin) has low methylation and high acetylation (Ropartz and Ralet, 2020).

2.2.2 Rhamnogalacturonan I (RG-I)

Along with homogalacturonan, rhamnogalacturonan I (RG-I) is a significant component of pectin. RG-I is a branched polysaccharide with a more complicated structure than homogalacturonan (Niu & Dou et al., 2023). It has an alternating backbone of galacturonic acid (GalA) and rhamnose (Rha) units. The backbone of RG-I is made up of repeated units of GalA and Rha connected by -(1,4) glycosidic linkage (Kaczmarska & Pieczywek et al., 2022). Side chains, also known as arabinan chains, are joined to the backbone's rhamnose units. These arabinan chains are made up of arabinose (Ara) sugar units that can be combined with other sugars like galactose (Gal) and/or xylose (Xyl) (Widsten & West et al., 2024).

2.2.3 Rhamnogalacturonan II (RG-II)

Rhamnogalacturonan II (RG-II) is a complicated pectin structure that helps to plant cell structural integrity and performs critical roles in plant growth and development. It is essentially made up of galacturonic acid repeating units with various side chains connected (Tian & Qin et al., 2023).Sugar residues such as rhamnose, arabinose, apiose, and xylose are found in these side chains. This is impressive given that RG-II is made up of at least 12 different glycosyl residues connected by over 20 different glycosidic connections (O'Neill & Ishii et al., 2004). Because of its unique and complex structure, RG-II can form a cross-linked network within the plant cell wall, providing strength and stability to the wall structure (Wu & Qi et al., 2024).

2.3 Classification of pectinase

The classification of pectinases is based on their action on the pectin molecule. Pectinolytic enzymes are known as pectinases and are classified into three groups based on the following criteria: pectin, pectic acid, or oligo-galactutonate whether pectinases work by transelimation or hydrolysis; and cleavage is random/endwise (Shet & Desai et al., 2018). This classification system identified three main types of pectinases: pectinesterase, depolymerases, and protopectinases. The categorization further considered the mode of action on the pectin molecule, including whether the enzyme worked through transelimination or hydrolysis and whether cleavage was random or endwise (Yu, 2021). An enzyme catalyzing the de-esterification of pectin methoxyl groups fell into the pectinesterase category, while an enzyme breaking down complex polysaccharides by hydrolyzing glycosidic linkages belonged to the depolymerases category (Li & Peng et al., 2024). Subsequent sub-classification within depolymerases depended on whether the enzyme acted through hydrolysis or elimination and exhibited endo- or exo-activity. This systematic process enabled the effective correlation with the pectinase enzyme and the determination of its classification based on its specific action and mechanism (Zeuner & Thomsen et al., 2020).

2.3.1 Pectinesterase

pectinesterase (PE) catalyzes the de-esterification of pectin methoxyl groups, leading to pectic acid and methanol. Pectin molecules can be esterified, which means that some of the galacturonic acid units can be esterified with methyl groups (Garg & Singh et al., 2016). This esterification process can occur spontaneously or be stimulated during the ripening period of fruits (Phan & Bo et al., 2007). The function of pectinesterase removing the methyl ester groups from pectin. This is accomplished by hydrolyzing the ester bonds, resulting in the production of carboxyl groups. This de-esterification process becomes pectin into pectate, a partially methyl-esterified form. The degree of esterification changes the physicochemical properties of pectin, affects the texture, viscosity, and gel-forming capabilities of plant-based products (Maldonado and Strasser de Saad, 1998).

2.3.2 Depolymerases

Depolymerases are enzymes crucial for breaking down complex polysaccharides by hydrolyzing the (1,4)-glycosidic linkages in D-galacturonic acid, yielding smaller oligosaccharides or monosaccharides (Murad and Azzaz, 2011). These enzymes are classified based on their mechanism of action and include hydrolytic depolymerases, which break polymers through hydrolysis, and eliminative depolymerases, which cleave polymer chains via elimination without water addition (Berezina & Rykov et al. 2024). Their ability to cleave pectinaceous substrates into smaller, more manageable components has practical implications for industries reliant on enzymatic processes. Thus, investigating the presence and activity of depolymerases in soil bacteria near agricultural sites holds relevance for both academic research and industrial applications. These enzymes are produced by various organisms and play essential roles in numerous industrial processes, including food and beverage production and waste treatment (de Souza and Kawaguti 2021).

2.3.3 Protopectinase

Proteopectinases (PPases) are enzymes that breakdown insoluble protopectin and solubilize it as highly polymerized pectin. The primary function of protopectinase is to hydrolyze the protopectin molecule, leading to simpler pectins (Jeilu, 2017). The glycosidic linkages between the sugar units in the protopectin molecule are cleaved during this hydrolysis process. They are further classified as A-type PPases and B-type PPases depending on their reaction mechanisms. A-type PPases bind to protopectin's inner site, which is in the polygalacturonic acid region, whereas B-type PPases bind to the exterior site, which is on the polysaccharide chains that may connect the polygalacturonic acid chain and cell wall components (Hours and Sakai, 1994).

2.4 Source of pectinase

Pectinase enzymes are naturally found in fruits and vegetables and can be obtained directly from them. Fruits and vegetables with various amounts of pectinase include apples, oranges, and grapes, as well as carrots and tomatoes (Sandhya and Kurup, 2013). The pectinase enzymes present in these fruits and vegetables can be extracted along with the juice or pulp when they are crushed or juiced. This natural source of pectinase is used in the food processing industry, particularly in the production of fruit juices, jams, and jellies. The extracted juice or pulp can be treated with pectinase, which is obtained from fruit or vegetables, to break down pectin molecules, resulting in enhanced juice extraction, clarity, and desired texture in the final products (Schols & Visser et al., 2009).

Pectinase can be derived from a variety of microbes such as bacteria, fungus, and yeast. Bacterial sources that are commonly used such as *Bacillus subtilis, Bacillus licheniformis,* and *Pseudomonas s*pecies (Sharma & Rathore et al., 2013). Pectinase can be produced naturally or through genetic modification. Bacterial pectinases are strong and stable, making them suited for industrial applications. Pectinase is also widespread in fungi such as *Aspergillus niger, Penicillium spp.*, and *Trichoderma reesei*. Fungal pectinases have a wide substrate specificity and may effectively degrade several forms of pectin. They can be made by either solid-state or submerged fermentation (KC & Upadhyaya et al., 2020). Pectinase enzymes are produced by certain yeast species such as *Saccharomyces cerevisiae* and *Kluyveromyces marxianus*. These yeast-derived pectinases are used to improve extraction and clarity processes in winemaking and fruit juice processing (Alimardani-Theuil & Gainvors-Claisse et al., 2011).

Microorganisms are grown in large-scale fermentation tanks with a growth medium comprising carbon and nitrogen sources, vitamins, and minerals to create pectinase. For optimal enzyme synthesis, the fermentation process is carefully managed (Oumer, 2017). Following fermentation, the enzyme is extracted from the culture broth using filtering or centrifugation, and then purified to remove contaminants and concentrate the pectinase. Pectinase preparations generated from bacteria, fungus, or yeast are commercially accessible in liquid or powdered form (Srivastava and Malviya, 2011). These enzyme products are designed with specific properties such

as pH and temperature ranges to suit a variety of industrial applications such as fruit juice processing, textile processing, and fruit extract clarity (Wang & Xu et al., 2023).

2.5 Biochemical characterization and morphological identification of pectinolytic bacteria.

Biochemical characterisation and morphological identification of pectinolytic bacteria involve assessing their metabolic and physical properties, respectively, to identify and comprehend their potential to degrade pectin (Satapathy & Rout et al., 2020). Morphological identification studies the physical properties of bacterial colonies and cells. It involves evaluating colonies produced on agar plates for size, shape, colour, and texture (Rehman & Siddique et al., 2015). Gram staining is widely used to detect whether a bacteria's cell wall structure is gram-positive or gram-negative. Microscopic inspection of stained cells aids in the identification of their cellular morphology, which might be rod-shaped (bacillus), spherical (cocci), or spiral-shaped. Catalase tests, oxidase tests, and other relevant tests are used to examine the metabolic capacities of pectinolytic bacteria during biochemical characterization. These tests provide information regarding their enzymatic activity and substrate utilization (Öztürk, 2024).

2.6 Fermentation techniques for the production of pectinase

Pectinases can be produced either by submerged or solid-state fermentation. Submerged Fermentation (SmF) involves growing microorganisms in a liquid medium, while Solid-State Fermentation (SSF) involves growing microorganisms on solid substrates (López-Gómez and Venus 2021). SmF provides a uniform nutrient distribution and efficient oxygen transfer, while SSF is suitable for enzyme production, utilizing waste materials, and preserving microbial diversity (Solís-Pereira & Favela-Torres et al., 1993).

Microorganisms are cultured in a liquid medium in Submerged Fermentation (SmF) for pectinase production from soil, providing optimal conditions for enzyme production. A selected pectinase-producing microbe is inoculated into a nutrient-rich liquid medium and incubated under regulated circumstances (Kumar & Sharma et al. 2011). Microorganisms metabolise available

nutrients during fermentation, leading to the creation of pectinase enzymes. Temperature, pH, and nutrient quantities are carefully monitored to maximize enzyme synthesis. The liquid medium is separated from the microbial biomass during fermentation, yielding a crude enzyme extract containing pectinase enzymes. SmF has advantages such as increased enzyme yields, scalability, and better control over fermentation conditions, which make it a popular approach for large-scale pectinase production in industry (Hernández‐Beltrán & Acosta‐Saldívar et al., 2023).

Inoculation of the microbe, fermentation under controlled conditions, monitoring of enzyme output, and extraction of the crude enzyme extract are the important phases in SmF for pectinase production (Shrestha & Rahman et al., 2021). The method enables efficient enzyme production in a liquid environment while also ensuring homogeneous nutrient distribution and effective oxygen transfer (Patil and Dayanand 2006). SmF is suitable for large-scale pectinase synthesis, ensuring high enzyme yields and allowing for additional purification and characterization processes if necessary (Vaishnavi & Divyashri et al., 2023).

2.7 Analytical techniques for measuring pectinase activity by Spectrophotometric Assays method.

Spectrophotometric assays measure pectinase activity by monitoring changes in absorbance at specific wavelengths. The most commonly used spectrophotometric assay for pectinase activity is the dinitrosalicylic acid (DNS) method (Mohandas & Raveendran et al., 2018). In the DNS method, pectinase breaks down pectin into smaller sugar molecules, including reducing sugars such as glucose. The reducing sugars react with DNS reagent (a mixture of dinitrosalicylic acid and sodium hydroxide) to form a colored product that absorbs light at a specific wavelength (KC & Upadhyaya et al., 2020). The increase in absorbance is directly proportional to the amount of reducing sugars released by pectinase activity.

The spectrophotometer is used to measure the absorbance of the reaction mixture before and after the enzymatic reaction, and the difference is used to calculate the pectinase activity (Khan and Barate, 2016). A standard curve is typically generated using known concentrations of a reference sugar (e.g., glucose) to determine the amount of reducing sugars in the sample (de OK Franco & Suarez et al., 2021).

Pectinase activity is determined by comparing the viscosity of the sample with that of a control sample without the enzyme. The higher the pectinase activity, the greater the reduction in viscosity observed. This method provides a direct measure of the ability of pectinase to degrade pectin and is particularly useful for assessing the enzyme's effectiveness in various industrial applications (Said & Fonseca et al., 1991).

2.8 Application of pectinase

2.8.1 Animal feed

Intense research into the use of various enzymes in animal and poultry feeds started in the early 1980s (Shrestha & Rahman et al., 2021). The pectinase enzyme plays a crucial role in the animal feed industry by significantly enhancing the nutritional value and digestibility of plant-based feed ingredients. Pectinases are enzymes that are utilized in the digestion of animal feeds. Many plant-derived feed ingredients, including grains, legumes, and oil seeds, contain pectin, a complex polymer that animals can only partially digest. Pectinase, when added to animal feed, breaks down the pectin molecules into smaller, more digestible components, thereby increasing the availability of nutrients such as carbohydrates, proteins, and minerals. This improved digestibility leads to enhanced feed efficiency, better growth performance, and increased nutrient utilisation by animals (Shrestha & Rahman et al., 2021).

2.8.2 Wine production

Pectinase, an enzyme derived from *Aspergillus*, enzyme that first used in the wine industry (Espejo, 2021). Over the past forty years, efforts have been made to enhance yeast strains and microbial enzymes for wine production. Pectinase plays a crucial role by breaking down pectin molecules, resulting in clearer and more stable wines. Additionally, pectinase increases the terpene content of the wine. It facilitates the separation of juice from solid components by breaking down pectin and promoting the formation of larger particles, which can be easily removed through settling or filtration (Mojsov, 2013). This clarification process enhances the visual appearance and stability of the wine. Moreover, pectinase affects the sensory qualities of wine, improving its mouthfeel and overall texture by breaking down pectin (KC & Upadhyaya et al. 2020).

2.8.3 Fermentation of tea and coffee

Pectinase aids in breaking down the pectin found in tea leaves when used in tea fermentation, resulting in improved extraction of flavor compounds and enhanced release of polyphenols (Kashyap & Vohra et al., 2001). This enzyme contributes to the overall fermentation process, leading to better quality tea. Similarly, in coffee fermentation, pectinase enzymes are employed to break down pectin present in coffee cherries. This enzymatic treatment accelerates the removal of the pectin-rich mucilage layer, facilitating faster and more efficient fermentation of coffee beans (Oumer, 2017). The enzymatic treatment reduces the fermentation time from approximately 80 hours to 20 hours. By utilizing pectinase enzymes, the extraction of desirable flavor compounds can be optimized, ultimately enhancing the taste and quality of the final products (Hoondal & Tiwari et al., 2002).

CHAPTER 3

MATERIALS AND METHODS

3.1 Apparatus

Conical flask, test tubes, pH meter, autoclave, petri dishes, glass spreader, sterile pipette, bunsen burner, microscope, rotary shaker incubator, centrifuge, visible spectrometer, magnetic stirrer, volumetric flask, dark bottle with a tight seal, centrifuge tubes, gel casting tray, well-forming comb, microwave oven, micropipette, electrophoresis chamber, water bath, UV transilluminator, collection tubes, microcentrifuge tubes.

3.2 Materials

Nutrient agar, distilled water, sodium nitrate, potassium chloride, magnesium sulfate, dipotassium phosphate, pectin, yeast extract, ammonium chloride, sodium hydrogen phosphate dodecahydrate, potassium dihydrogen phosphate, magnesium sulfate heptahydrate, iodine potassium iodide solution, 3,5-dinitrosalicylic acid (DNS) reagent, galacturonic acid, citric acid, sodium hydroxide, crystal violet, gram's iodine, acetone, safranine, Luria-Bertani (LB) broth, Glucose-Tris-EDTA (GTE) buffer, 1X TAE buffer, 6X loading dye, agarose, DNA Marker, Binding buffer (NTI),Washing buffer (NT3),Elution buffer (NE), Sodium chloride.

3.3 Sample collection

The soil sample collected from cempedak farm near to Taman Pinggiran UMK (5.7493° North, 101.8657° East) taken from underneath soil containing rotten cempedak. Temperature and pH of the site have been checked. To maintain sterility, proper precautions are taken, including wearing gloves and using sterile tools for handling. After collection, the soil sample appropriately label with relevant information, such as the date, time, and any additional details necessary for identification. Finally, the samples are transported to the laboratory under appropriate storage conditions, ensuring their integrity for further analysis and experimentation.

3.4 Isolation of potential pectinolytic producing bacteria.

A set of 6 sterilized test tubes was prepared. The first test tube contained 10 ml of distilled water, while the remaining test tubes each contained 9 ml of distilled water. Initially, 1 gram of soil was combined with 50 ml of distilled water, forming a concentrated solution. Subsequently, 1 ml from this mixture was transferred to the first test tube, where it was diluted with 9 ml of additional distilled water and thoroughly mixed. Following this, 1 ml of the suspension was removed using a sterile pipette and transferred to the second test tube containing 9 ml of distilled water. This dilution step was repeated, with 1 ml of the previous suspension transferred to each subsequent test tube. The tubes were sequentially labeled from 10^{-1} until 10^{-6}.

In the primary screening of pectinolytic bacteria, pre-prepared nutrient agar plates labeled as 10^{-1} to 10^{-6} were utilized. Sample tubes corresponding to these plates were vortexed to ensure proper suspension, and 0.1 ml of each suspension was spread onto the respective nutrient plates, resulting in a tenfold dilution. The plates were then incubated at 37 °C for 24 hours. Colonies were chosen based on morphological characteristics, including size, form, color, margin, consistency, and elevation, until pure isolates were obtained.

3.5 Screening of pectinolytic bacteria

After isolating a single colony, pure colonies were transferred from activated plates to pectin agar media using the single streaking method. The pectin agar plates were incubated at 30°C for 48 hours. Subsequently, Gram's iodine solution was flooded onto the Petri dishes to identify the clear zone around colonies indicative of pectinase production. The clear zone diameter reflected the bacteria's relative pectinase production capacity. To ensure isolated purity, colony morphology and microscopy were employed. The clear zone ratio is measured using the equation 3.1:

$$clear\ zone\ ratio = \frac{diameter\ of\ clear\ zone\ (mm)}{diameter\ of\ colony\ (mm)}$$

3.6 Seed culture

For the establishment of the seed culture, a single colony from each sample was added into 5 ml of pectin broth in individual test tubes. These test tubes underwent overnight incubation at 30°C with constant agitation at 150 rpm. Subsequently, the cultivated samples from the test tubes were transferred to pectin broth. The pH was adjusted to the desired range, typically between 4.0 and 6.0, in conical flasks of 50 ml individually and incubated for an additional 3 to 5 hours until reaching an optical density (OD) of 0.5. This meticulous procedure ensured the development of a robust seed culture, setting the stage for optimal pectinase production in the subsequent fermentation process.

3.7 Fermentation of pectinolytic bacteria

The production of pectinase using submerged fermentation was carried out following the method described in a study by Kumar and Sharma in 2012. Conical flasks were used, with each flask containing 100 ml of pectin broth. The pH values were maintained between 4.0 and 6.0 to support optimal enzyme production. To initiate the fermentation process, 5ml was added to each flask. The flasks were then placed in a rotary shaker incubator and maintained at a temperature of 30°C for 36 hours. The shaker incubator ensured continuous mixing and aeration of the cultures at a speed of 120 rotations per minute (rpm).

3.8 Extraction of crude enzyme

After fermentation, the cultures underwent centrifugation at 6,000 rpm for 10 minutes, effectively separating the solid cell mass from the liquid fraction. The resulting supernatants, rich in crude enzymes, were collected and stored at -20 °C for further evaluation. These crude enzymes served as the basis for assessing the efficiency of bacterial isolates in producing pectinase activities. This preliminary extraction step provided a concentrated source of enzymes from the fermentation process, facilitating subsequent analyses and applications in various industries.

3.9 Enzyme assay to determine pectinolytic activity

In the enzyme assay aimed at determining pectinolytic activity, a systematic procedure was adhered to for accurately assessing the efficacy of the enzyme in degrading pectin substrates. Initially, a reaction mixture was meticulously prepared by combining 1 ml of the crude enzyme solution with 1 ml of a 1% pectin substrate in a 0.1 M sodium acetate buffer with a pH of 7, ensuring an optimal environment for enzymatic activity. Control was established by substituting the enzyme solution with distilled water which provides a baseline for comparison. After the reaction mixture had been incubated at 30°C for 10 minutes, the enzymatic reaction was halted by adding 2 ml of the 3,5-dinitrosalicylic acid (DNS) reagent to each tube. Boiling the tubes induced a noticeable color change, indicating the completion of the reaction. The absorbance of the resulting solutions, both from the reaction tubes and the control tubes, was precisely measured at 540 nm using a visible spectrometer.

To determine the concentration of reducing sugars, a standard curve was constructed using galacturonic acid solutions of known concentrations, following the same meticulous procedure. Each unit of enzymatic activity (U) was defined as the release of 1 µmol of reducing sugars per minute. By adhering to standardized protocols and utilizing established units of measurement, this method facilitated the accurate assessment of pectinolytic activity, providing valuable insights into the effectiveness of the enzyme in degrading pectin substrates. The generated standard curve served as a reference for determining the concentration of reducing sugars in the assay samples based on their absorbance readings. This approach ensured precise quantification of pectinolytic activity, offering valuable insights into the enzyme's efficacy in degrading pectin substrates. Therefore, the pectinase activity was determined using equation 3.2:

$$\text{Pectinase activity (U/mL)} = \frac{reducing\ sugar\ concentration\ x\ 1000\ x\ dilution\ factor}{molecular\ weight\ of\ galacturonic\ acid\ x\ incubation\ time(minutes)}$$

3.10 Identification and characterization of isolated colony

3.10.1 Colonial observation

The colonial properties of pectinolytic bacterial colonies were observed. This method involved describing the characteristics of individual colonies grown on agar, including their size, shape, elevation, surface appearance, and opacity. The colonies were examined in a petri dish, and their colonial properties were carefully documented for further analysis and identification of pectinolytic bacteria.

3.10.2 Phenotypic characterization

(a) Gram staining

Firstly, a colony of pectinolytic bacteria was taken with a sterile loop from agar plates and smeared on the slide. Then, the smears were stained with crystal violet for 1 minute. The slide was rinsed with distilled water for no more than 5 seconds to remove any unattached crystal violet. Gram's iodine was added for 1 minute. The slide was rinsed with distilled water again, and smears were treated with acetone for 30 seconds, followed by rinsing. The alcohol decolorized the sample if it was Gram-negative, eliminating the crystal violet. Gram-positive cells might have been stained if the alcohol was kept on the sample for too long. Finally, the smear was counter-stained for 60 seconds with safranine and then rinsed with water. The smear was air-dried and examined with a light microscope under oil immersion (100X).

3.11 Molecular identification for isolate colony

3.11.1 Genomic DNA extraction

The selected pectinolytic colony was added to 5 ml of Luria-Bertani (LB) broth in individual test tubes. These test tubes underwent 24 hours of incubation at 30 degrees Celsius with constant agitation at 150 rpm. Subsequently, the cultivated samples from the test tubes were transferred to 50 ml of LB broth in individual conical flasks and incubated for an additional 3 to 5 hours until they reached an optical density (OD) of 0.7 to 0.9. After the incubation period, a 2 ml pure bacteria culture was obtained and centrifuged for 5 minutes at 8,000 rpm to collect the pellet for DNA extraction. The PrimeWay Genomic DNA Extraction Kit was utilized for DNA extraction following the kit's protocol, including preparation of chemicals, and following the steps accordingly.

For Gram-positive bacteria, pellet was resuspended in a mixture of 50 mM EDTA and Bacteria Pre-Lysis Buffer, followed by incubation at 37°C and subsequent centrifugation. The pellet was then resuspended in GL1 Buffer and treated with Proteinase K Solution at 60°C. Subsequently, GL2 Buffer and RNase A Solution were added, and after incubation at room temperature, absolute ethanol was introduced to precipitate DNA. The lysate was transferred to a PrimeWay Genomic II Column for purification, followed by washing steps with Wash Buffer G1 and G2 to remove impurities. Finally, the DNA was eluted with preheated Elution Buffer.

For Gram-negative bacteria, the pellet was resuspended in GL1 Buffer and treated with Proteinase K Solution at 60°C. After adding GL2 Buffer and RNase A Solution, the mixture was incubated at room temperature. Absolute ethanol was added to precipitate DNA, and the lysate was transferred to a PrimeWay Genomic II Column for purification. The column was washed with Wash Buffer G1 and G2 to remove contaminants, followed by centrifugation to dry the membrane. Elution Buffer was then added to the column to release the genomic DNA, which was collected after centrifugation. These procedures ensured the isolation of high-quality genomic DNA from both Gram-positive and Gram-negative bacterial sources.

3.11.2 Measurement of DNA concentration and purity

In the determination of DNA concentration and purity, a NanoDrop 2000 spectrophotometer was employed following a meticulous protocol. The spectrophotometer was set up and calibrated to ensure accurate measurements. Constituting 1 µl distilled water, was utilized as a blank to establish a baseline for subsequent readings. After removing the blank, 1 µl of the DNA sample was loaded onto the NanoDrop platform, and the instrument measured the absorbance of light at 260 nm to quantify DNA concentration. The resulting value was expressed in nanograms per microliter (ng/µl), offering crucial insights into the amount of DNA present in the sample. Additionally, the NanoDrop software determined the 260/280 ratio, a key metric indicating DNA purity. A ratio close to 1.8 signified pure double-stranded DNA, while deviations suggested contamination by proteins or other impurities. This comprehensive measurement approach enabled researchers to assess both the quantity and quality of DNA in a sample, ensuring suitability for subsequent molecular biology applications such as PCR or sequencing.

3.11.3 Gel electrophoresis

a) Preparation of Agarose Gel

The agarose gel (1%) was prepared for use by inserting the well-forming comb into the slots on the casting tray. Approximately 0.3 g of agarose was weighed out and dissolved in 39.5 ml of 1X TAE buffer. The suspension was heated in a microwave oven until no agarose particles were visible. Once the solution had cooled to around 50˚C, 1µl of flurosafe was added to the solution. The agarose was poured into the tray, and a forming comb was inserted into the casting tray and allowed to harden for approximately 20 minutes. After carefully removing the comb, the gel was placed in the electrophoresis chamber and covered with sufficient 1X TAE running buffer.

b) Electrophoresis of DNA samples

For the electrophoresis of DNA samples, 1µl of loading dye and 1µl of lambda DNA/Hindlll were added to 4µl of deionized water to create a DNA marker, which was prepared using a parafilm strip. Carefully, it was loaded into the first lane of the gel. Following this, 1µl of loading dye was added to 1µl of DNA sample and loaded into the subsequent wells of the gel using a micropipette. This process was repeated for all samples. Once the gel tank was closed and the power source was switched on, the gel was run at 80V for 45 minutes. The progress of the gel was monitored by observing the DNA marker dye, and the run was stopped when the bromophenol blue had migrated approximately ¾ the length of the gel. Afterward, the power supply was turned off, the leads were disconnected, and the gel was removed from the casting tray. Finally, the gel could be directly analyzed by viewing it on a UV transilluminator.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Physical characterization of soil sample

The soil collected from underneath of soil containing the rotten cempedak at Taman Pinggiran Cempedak farm exhibited specific pH and temperature conditions. The pH of the soil sample was measured and recorded as 5.7, while the temperature was observed to be 28°C. The soil sample was maintained under room temperature atmospheric conditions after its collection. Table 4.1 the pH and temperature of the soil before sample collection.

Table 4.1 pH and temperature of the soil sample

Soil Sample	**pH**	**Temperature (°C)**
Soil containing rotten cempedak	5.7	28

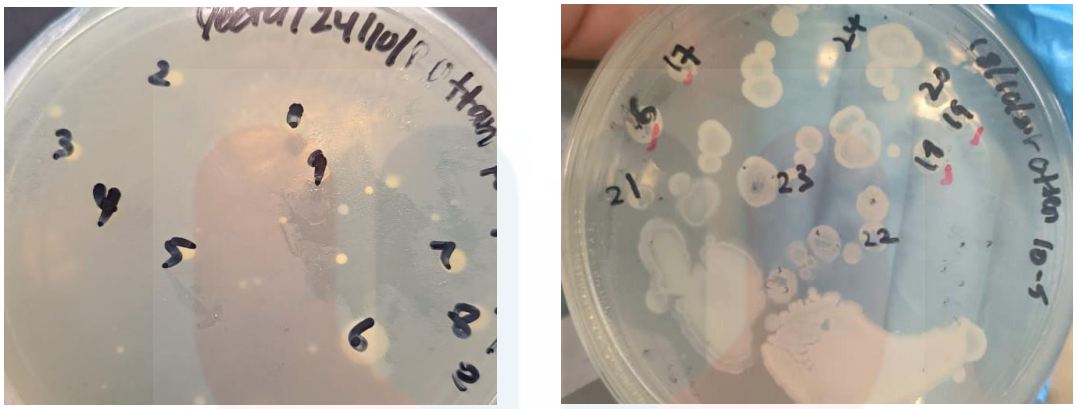
The selection of the location and sample from underneath the rotten cempedak at Taman Pinggiran Cempedak farm was justified by the availability of resources and proximity, as well as the abundance of nutrients in the soil, particularly those influenced by the presence of the rotten cempedak. The ease of accessibility to the Taman Pinggiran Cempedak farm facilitated efficient sample collection and experimentation. Accessing the site readily reduced logistical challenges and ensured timely data collection. Additionally, the proximity of the farm made it convenient for monitoring and follow-up studies, enhancing the feasibility of long-term research endeavors.

Furthermore, the choice of soil containing rotten cempedak was driven by the significant nutrient content found in such soil. Rotten fruits like cempedak contained organic compounds,

including pectin, which served as a substrate for microbial activity. (Barth, Hankinson et al. 2009). Pectinase enzymes produced during the decomposition process broke down pectin, releasing nutrients and influencing soil composition. (Kjøller and Struwe 2002). By studying soil samples from underneath, the rotten cempedak, researchers investigated the nutrient dynamics and microbial processes associated with organic decomposition. Therefore, the selection of this location and sample type aligned with the objective of leveraging available resources while exploring the rich nutrient content and microbial activity associated with rotten cempedak decomposition.

4.2 Isolation of pectinolytic producing bacteria from soil sample on nutrient agar

The isolation procedure commenced with the spread plating of a serially diluted soil sample onto nutrient agar plates, as nutrient agar was selected for its capacity to provide essential nutrients necessary for microbial growth. This medium facilitated the cultivation and isolation of a broad spectrum of microorganisms from the soil sample. Serial dilutions were crucial in preventing overcrowding and facilitating the isolation of individual bacterial cells or colonies, laying the foundation for subsequent screening. The strategic aim was to achieve discrete bacterial colonies on nutrient agar. Following this, single colonies were meticulously chosen from the nutrient agar plates, specifically targeting dilutions of 10^{-4}, 10^{-5}, and 10^{-6}. These isolated colonies were identified as potential candidates for further evaluation of their pectinase-producing ability. Figure 4.2 illustrates the isolated colonies grown on nutrient agar plates.



(a) (b)

(c)

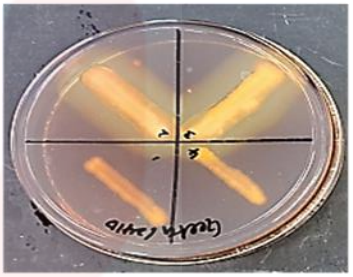
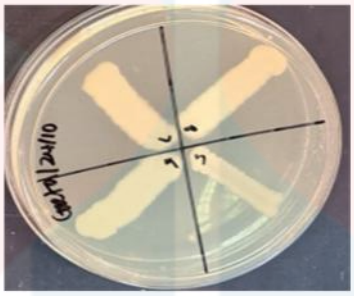
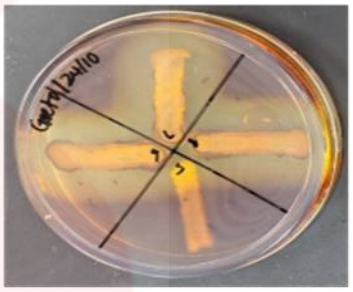
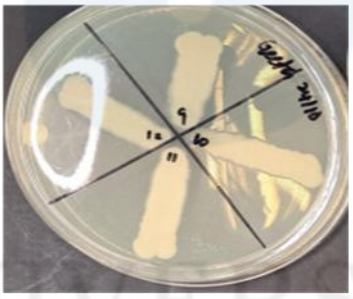
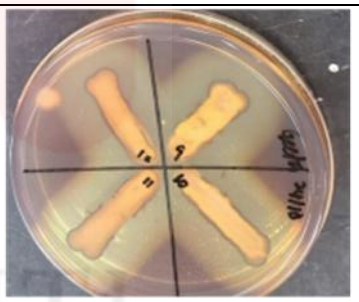
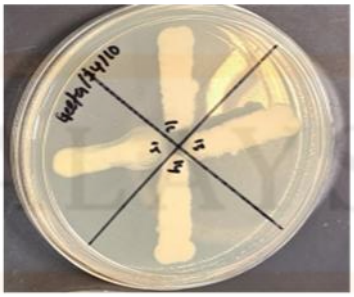
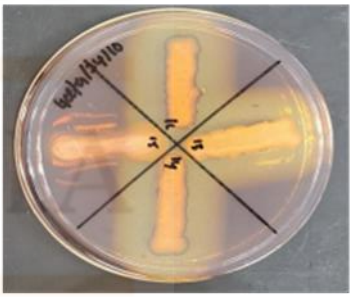
Figure 4.1: Grown colonies on nutrient agar plates that spread from different dilution is (a) 10^{-4}, (b) 10^{-5} and (c) 10^{-6}.

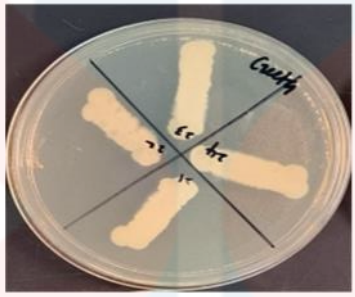
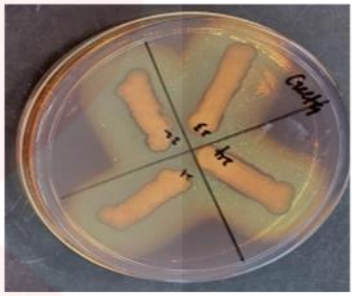
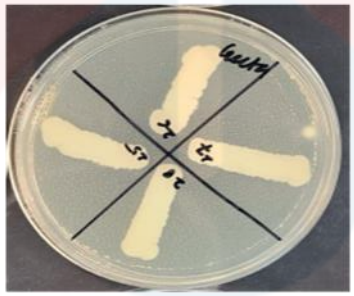
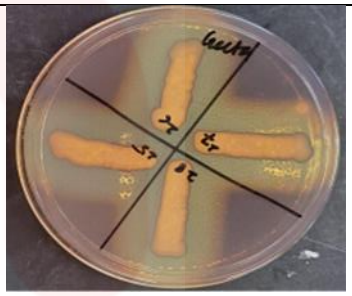
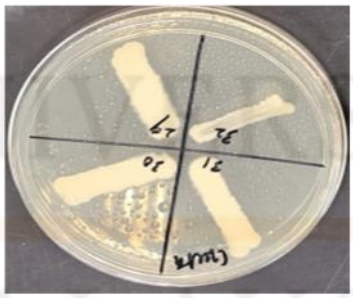
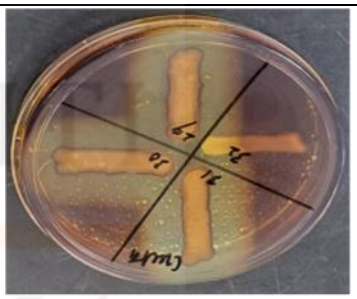
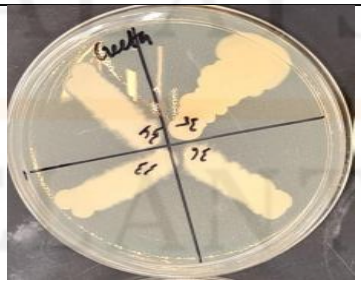
4.3 Screening and selecting pectinolytic bacteria using iodine test.

After distinct colonies were identified from the nutrient agar plates, they were streaked onto pectin-supplemented agar plates. Pectin plates were chosen as the selective medium because pectin served as the sole carbon source, promoting the growth of bacteria capable of utilizing pectin and potentially producing pectinase enzymes. The streak plate method was employed to streak individual colonies onto the pectin plates, ensuring the separation of bacterial cells to obtain isolated colonies. The pectin agar plates were then incubated at 30 °C for 48 hours. Following the incubation period, potential pectinase-producing colonies were observed.

To confirm the presence of pectinase activity, the pectin plates were flooded with iodine solution after incubation. Iodine interacted with the undigested pectin in the agar, resulting in the formation of a brown halo or clear zone around colonies that had utilized or broken down the pectin. This flood test aided in identifying colonies exhibiting pectinase activity, as indicated by the absence or reduction of the brown halo zone around the colonies that produced pectinase. The table below presents the screening plates growth and the halo zone after Gram's iodine solution flooded.

Table 4.2: Screening of pectinolytic bacteria and halo zone after iodine solution flooded

No strain	Before	After
ST1,ST2,ST3,ST4		
ST5,ST6,ST7,ST8		
ST9,ST10,ST11,ST12		
ST13,ST14,ST15,ST16		

ST17,ST18,ST19,ST20		
ST21,ST22,ST23,ST24		
ST25,ST26,ST27,ST28		
ST29,ST30,ST31,ST32		
ST33,ST34,ST35,ST36		

The investigation aimed to assess the pectinase-producing capabilities of 36 bacterial strains from ST1 to ST36. Positive results for pectinase production were observed in 30 strains: ST2, ST3, ST6, ST7, ST8, ST9, ST10, ST11, ST12, ST13, ST14, ST16, ST17, ST18, ST19, ST20, ST21, ST22, ST23, ST24, ST25, ST26, ST27, ST28, ST29, ST30, ST31, ST33, ST34, and ST36. The presence of clear zones around the colonies on pectin agar plates indicated the successful hydrolysis of pectin by these strains, rendering them promising candidates for various pectin hydrolysis processes in industrial applications.

However, 6 strains, namely ST1, ST4, ST5, ST15, ST32, and ST35, did not exhibit positive pectinase-producing characteristics. The absence of clear zones around their colonies on pectin agar plates suggested a limited capacity to hydrolyze pectin or indicated that they were not pectinase-producing bacterial strains. This implies that these strains may lack or have reduced pectinase activity.

To further evaluate the pectinase-producing capabilities, specific colonies showing positive results were selected. These colonies were streaked on pectin agar plates to observe their ability to break down pectin. The absence or presence of clear zones around the colonies was noted, and their diameters were measured. The efficiency of these bacteria in generating pectinase enzyme was assessed by analyzing the clear zone ratio around their colonies on pectin agar plates. A larger clear zone indicated a superior ability to produce more pectinase, making these strains promising candidates for various pectin hydrolysis processes in industries.

Table 4.3: clear zone ratio of pectinolytic bacteria

No	Strain	Pectinolytic activity	Diameter of clear zone (mm)	Diameter of colony (mm)	Clear zone ratio
1	ST1	No	-	-	-
2	ST2	Yes	25	8	3.1
3	**ST3**	**Yes**	**39**	**2**	**19.5**
4	ST4	No	-	-	-
5	ST5	No	-	-	-
6	ST6	Yes	30	5	5
7	ST7	Yes	30	6	5
8	ST8	Yes	27	8	3.4
9	ST9	Yes	34	7	4.9
10	**ST10**	**Yes**	**38**	**2.2**	**17.2**
11	ST11	Yes	34	7	4.9
12	ST12	Yes	30	5	6
13	**ST13**	**Yes**	**35**	**2.3**	**15.2**
14	ST14	Yes	32	4	8
15	ST15	No	-	-	-
16	**ST16**	**Yes**	**39**	**2**	**19.5**
17	ST17	Yes	32	5	6.4
18	**ST18**	**Yes**	**35**	**2**	**17.5**
19	ST19	Yes	30	7	4.3
20	ST20	Yes	30	7	4.3
21	ST21	Yes	30	4	7.5
22	ST22	Yes	30	6	5
23	**ST23**	**Yes**	**35**	**2**	**17.5**
24	ST24	Yes	35	15	2.3
25	ST25	Yes	30	18	1.7
26	ST26	Yes	30	5	6

27	ST27	Yes	28	3	9.3
28	ST28	Yes	28	2	14
29	ST29	Yes	29	2	14.2
30	ST30	Yes	29	24	1.2
31	ST31	Yes	29	4	7.25
32	ST32	No	-	-	-
33	ST33	Yes	30	8	3.8
34	ST34	Yes	30	10	3
35	ST35	No	-	-	-
36	ST36	Yes	29	20	1.5

Among the 30 positive pectinolytic bacterial strains examined, six strains stood out with notably high clear zone ratios: ST3, ST13, ST16, ST18, ST23, and ST10. These strains demonstrated exceptional capabilities in hydrolyzing pectin, as evidenced by their significantly larger clear zones compared to other strains. The elevated clear zone ratios suggest a superior ability to produce pectinase enzymes or a more efficient enzymatic activity in breaking down pectin molecules. This heightened enzymatic efficiency could be attributed to various factors, including the genetic makeup of the strains, optimal growth conditions provided during the experiment, and their adaptation to efficiently utilize pectin as a substrate. Additionally, these strains may regulatory mechanisms that promote enhanced pectinolytic activity, contributing to their superior performance in pectin hydrolysis processes. The remarkable pectinase-producing capabilities of these strains highlight their potential for industrial applications requiring efficient degradation of pectin-rich substrates, such as in the food, textile, and biofuel industries.

4.4 Pectinase enzyme activity

Pectinase assays play a crucial role in assessing the efficacy of enzymes responsible for breaking down pectin, a complex plant cell wall polysaccharide. The method employed in this experiment involved a meticulous approach to measuring pectinase activity. A reaction mixture comprising 1 ml of crude enzyme solution and 1 ml of 1% pectin in 0.1 M sodium acetate buffer at pH 7 was incubated at 30°C for 10 minutes. The enzymatic reaction was quenched by adding 2 ml of 3,5-dinitrosalicylic acid (DNS) reagent, and subsequent boiling induced a color change indicating the release of reducing sugars. Visible spectrophotometry at 540 nm allowed for absorbance measurements, with the concentration of reducing sugars determined using a standard curve generated with galacturonic acid.

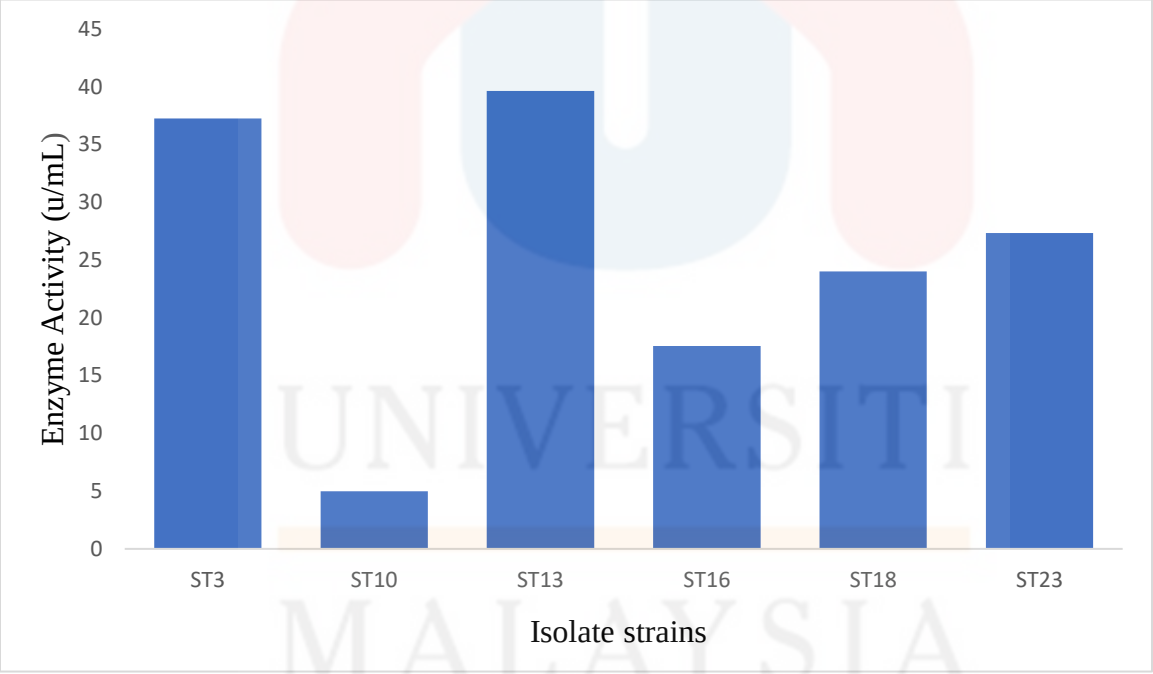


Figure 4.2: pectinase enzyme activity

The calculated enzyme activities demonstrated variability among samples (ST3, ST10, ST13, ST16, ST18, ST23). Such variability could have arisen from differences in the enzyme preparations, sample conditions, or inherent enzyme characteristics. Integral components in the calculation included a dilution factor of 1, the molecular weight of galacturonic acid, and an incubation time of 30 minutes, emphasizing the importance of standardized conditions in enzyme assays.

When comparing the enzymatic activities among the samples (ST3, ST10, ST13, ST16, ST18, ST23), it's notable that samples ST3 and ST13 demonstrated the highest pectinase activities, with values of 37.25 U/ml and 39.62 U/ml, respectively. The elevated activities in these samples could be attributed to a potentially higher concentration of active pectinase, optimal environmental conditions promoting effective enzyme-substrate interactions, and ample substrate availability leading to increased concentrations of reducing sugars. These findings presented promising prospects for industrial applications, suggesting the potential utility of these samples in processes where enhanced pectinase activity was desirable.

Conversely, sample ST10 displayed a notably lower pectinase activity of 4.99 U/ml. The lower activity in ST10 may have been indicative of a lower concentration of active pectinase, suboptimal environmental conditions, and the presence of inhibitors. Importantly, a critical factor contributing to the low enzyme activity in ST10 appeared to be limited substrate availability. If the concentration of pectin in ST10 had been diminished, the enzyme may not have fully realized its catalytic potential, resulting in reduced production of reducing sugars. Understanding these variations in enzyme activity and substrate availability in ST10 was crucial for refining processes that depended on optimal pectinase performance.

4.5 Characterization of isolated colonies of pectinolytic producing bacteria by morphology study

After the isolation and screening processes, individual colonies that exhibited pectinase-producing capabilities were selected for further analysis. The characterization of isolated colonies of pectinolytic-producing bacteria was a crucial step in understanding their distinct features and potential applications. This involved a detailed examination of colony morphology, where physical characteristics offered valuable insights into the diversity of isolated bacteria. Thorough characterization was conducted on the 30 strains identified as pectinolytic-producing bacteria to explore their potential for pectinase production. By characterizing these isolated colonies, researchers laid the foundation for elucidating the diversity within the pectinolytic-producing bacterial community and gaining insights that may be instrumental in industrial applications or further research endeavors.

4.5.1 Colony morphology

The focus was on observing colony morphology of the selected bacterial strain to gain insights into its physical characteristics. Bacterial colonies were observed on a nutrient agar medium, chosen for its non-selective and non-differentiating properties, allowing optimal bacterial growth. When streaked on fresh nutrient agar, attention was given to the colors, forms, margins, and sizes of the colonies. This visual scrutiny aimed to discern unique features of bacterial isolates, providing valuable information about their physical appearance. Nutrient agar, selected for its versatility in supporting diverse bacterial growth, facilitated comprehensive observation of the strains. A summary of the colony morphology characteristics of 30 strains observed for the bacterial colonies grown on the agar plates is presented in the following table.

Table 4.4: Colony morphology of pectinolytic strain on nutrient agar

Isolate	Pectinolytic activity	Colony shape	Surface	Colour	Opacity	Elevation	Margin	Diameter of halo zone (cm)
ST2	Yes	Circular	mucoid	White creamy	Opaque	Raised	Entire	2.5
ST3	Yes	Circular	mucoid	White creamy	Opaque	Raised	Entire	3.9

ST6	Yes	Circular	Rough	White creamy	Transparent	Crateriform	Entire	3
ST18	Yes	Circular	Rough	White creamy	Transparent	Crateriform	Entire	3.5
ST19	Yes	Circular	Rough	White creamy	Transparent	Crateriform	Entire	3
ST10	Yes	Circular	smooth	White	Translucent	Raised	Entire	3.8
ST11	Yes	Circular	Smooth	White	Translucent	Raised	Entire	3.4
ST7	Yes	Circular	Smooth	White	Transparent	Crateriform	Entire	3
ST8	Yes	Circular	Smooth	White	Transparent	Crateriform	Entire	2.7
ST9	Yes	Circular	Smooth	White	Transparent	Crateriform	Entire	3.4
ST12	Yes	Circular	Smooth	White	Transparent	Crateriform	Entire	3
ST13	Yes	Circular	Smooth	White	Transparent	Crateriform	Entire	3.5
ST14	Yes	Circular	Smooth	White	Transparent	Crateriform	Entire	3.2
ST33	Yes	Circular	Smooth	White	Transparent	Crateriform	Entire	3
ST34	Yes	Circular	smooth	White	Transparent	Crateriform	Entire	3
ST36	Yes	Circular	Smooth	White	Transparent	Crateriform	Entire	2.9
ST16	Yes	Circular	Rough	White	Transparent	Crateriform	Entire	3.9
ST17	Yes	Circular	Rough	White	Transparent	Crateriform	Entire	3.2
ST20	Yes	Circular	Rough	White	Transparent	Crateriform	Entire	3
ST21	Yes	Circular	Rough	White	Transparent	Crateriform	Entire	3
ST24	Yes	Circular	Rough	White	Transparent	Crateriform	Entire	3.5
ST26	Yes	Circular	Rough	White	Transparent	Crateriform	Entire	3
ST27	Yes	Circular	Rough	White	Transparent	Crateriform	Entire	2.8
ST28	Yes	Circular	Rough	White	Transparent	Crateriform	Entire	2.8
ST31	Yes	Circular	Rough	White	Transparent	Crateriform	Entire	2.9
ST22	Yes	Circular	Smooth	White creamy	Translucent	Flat	Entire	3
ST23	Yes	Circular	smooth	White creamy	Translucent	Flat	Entire	3.5
ST25	Yes	Circular	Smooth	White creamy	Translucent	Flat	Entire	3
ST29	Yes	Circular	smooth	White creamy	Translucent	Flat	Entire	2.9
ST30	Yes	Circular	smooth	White creamy	Translucent	Flat	Entire	2.9

The observed distinctions in colony morphology on nutrient agar are crucial indicators of potential variations and similarities among the bacterial strains. For instance, strains ST2 and ST3 not only shared pectinolytic activity but also exhibited identical features such as circular colonies, mucoid surface, white creamy color, opaque opacity, raised elevation, and entire margin. These consistent characteristics strongly suggest a high likelihood that ST2 and ST3 are indeed the same bacterial species.

Similarly, strains ST6, ST18, and ST19, which displayed rough colonies with a transparent surface, white creamy color, crateriform opacity, raised elevation, and entire margin, exhibited common features indicative of a potential shared identity. The presence of these consistent traits raises suspicions of these strains being closely related or even the same bacterial species. Strains ST7, ST8, ST9, ST12, ST13, and ST14, with their smooth circular colonies, white transparent color, and crateriform opacity, demonstrated similarities suggesting a potential commonality. This shared set of features along with the pectinolytic activity implies a closer relationship among these strains (Norris & Falagán et al., 2020).

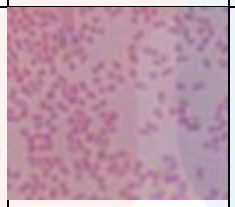
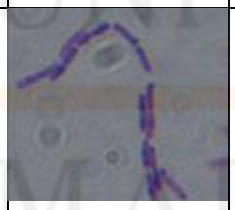
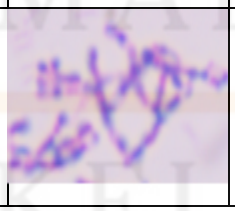
Strains ST10 and ST11, with circular smooth colonies, translucent surface, raised elevation, and entire margin, showcased comparable characteristics, reinforcing the notion of a potential similarity or shared lineage. Furthermore, the identified pattern among strains ST16, ST17, ST20, ST21, ST24, ST25, ST27, ST28, and ST31, all displaying circular rough colonies with transparent features and crateriform opacity, strongly suggests a common lineage or closely related strains. In contrast, strains ST22, ST23, ST25, ST29, and ST30, with smooth circular colonies, translucent surface, flat elevation, and entire margin, exhibited distinct traits from the groups, indicating a potential divergence into a different bacterial strain or lineage.

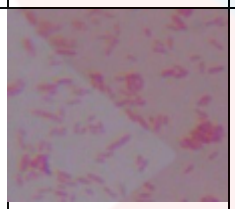
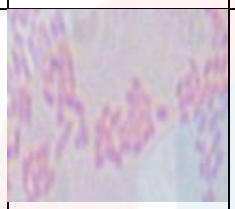
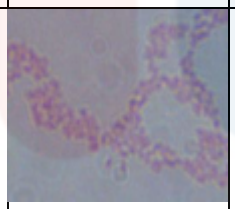
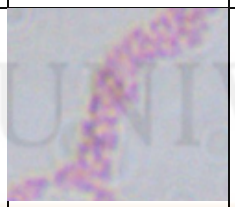
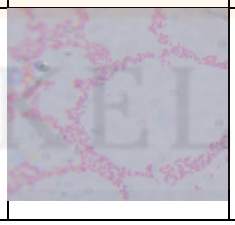
Therefore, the justification for potential relationships among these bacterial strains is grounded in the consistent observations of their colony morphology features, providing a basis for further exploration into their genetic and ecological connections.

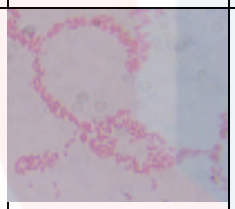
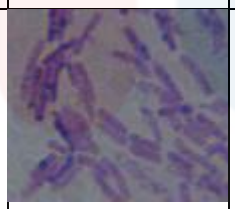
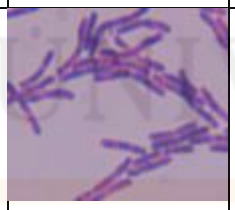
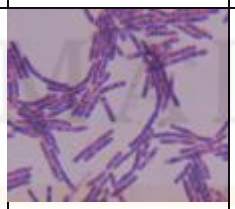
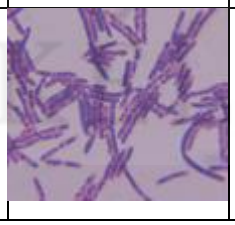
4.5.2 Gram staining

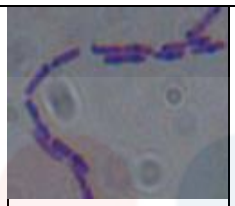
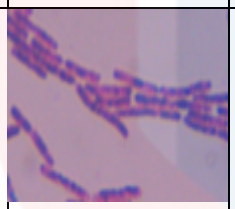
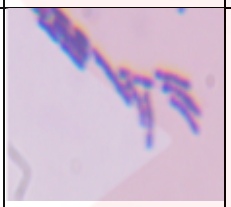
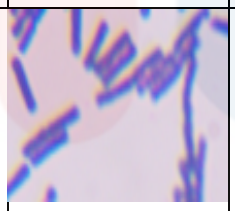
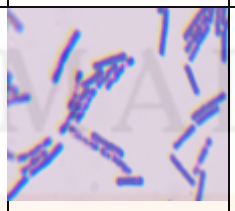
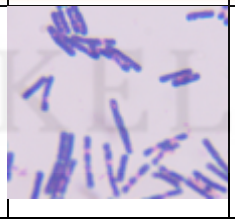
The gram staining of the isolated bacterial strains involves visual observations, Gram's reaction, color, and cellular morphology. The Gram staining procedure involves classifying bacteria into two main groups based on the characteristics of their cell walls: Gram-positive, which retains the crystal violet stain, and Gram-negative, which take up the safranin counterstain. For visual observation, used a microscope with a 100x objective lens magnification, combined with oil immersion. The results, as summarized in Table 4.5, provide insights into the diversity of the bacterial community and potential relationships among strains.

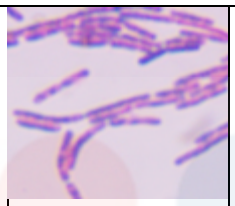
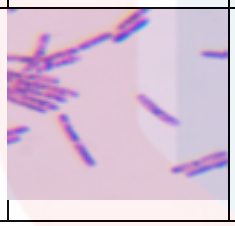
Table 4.5: result based on gram staining of pectinolytic strain

Isolate	Visual observation	Gram's reaction	Colour	Cellular morphology
ST2		Negative	Pink	Coccus
ST3		Negative	Pink	Coccus
ST6		Positive	Purple	Rod
ST18		Positive	Purple	Rod

ST19		Positive	Purple	Rod
ST10		Negative	Pink	Coccus
ST11		Negative	Pink	Coccus
ST7		Negative	Pink	Rod
ST8		Negative	Pink	Rod
ST9		Negative	Pink	Rod
ST12		Negative	Pink	Rod
ST13		Negative	Pink	Rod

ST14		Negative	Pink	Rod
ST33		Negative	Pink	Rod
ST34		Negative	Pink	Rod
ST36		Negative	Pink	Rod
ST16		Positive	Purple	Rod
ST17		Positive	Purple	Rod
ST20		Positive	Purple	Rod
ST21		Positive	Purple	Rod

ST24		Positive	Purple	Rod
ST26		Positive	Purple	Rod
ST27		Positive	Purple	Rod
ST28		Positive	Purple	Rod
ST31		Positive	Purple	Rod
ST22		Positive	Purple	Rod
ST23		Positive	Purple	Rod
ST25		Positive	Purple	Rod

ST29		Positive	Purple	Rod
ST30		Positive	Purple	Rod

4.6 Characterization of isolated colonies of pectinolytic producing bacteria by molecular identification

4.6.1 Gel electrophoresis using Agarose.

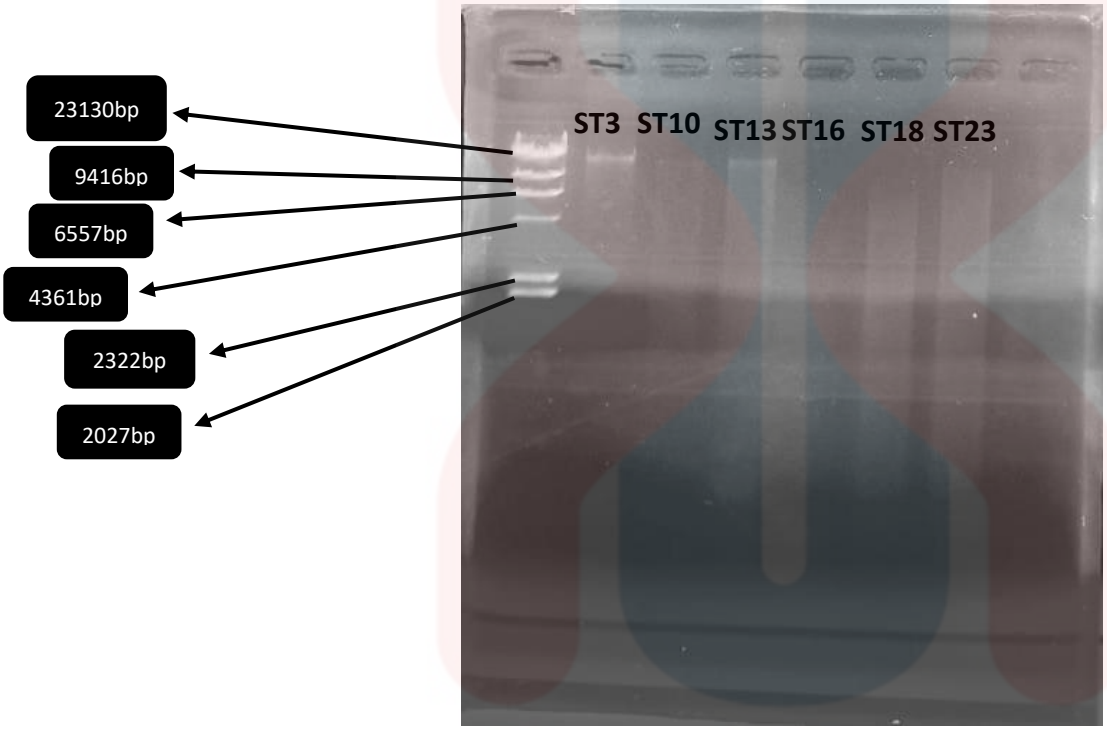


Figure 4.3: Gel electrophoresis using 1% agarose after observed on a UV transilluminator at 365 nm. Lane 1: ladder of DNA marker, Lane 2:ST3 , Lane 3:ST13 , Lane 4:ST13 , Lane 5:ST16 , Lane 6: ST18 , Lane 7: ST23

After extraction of the DNA of the chosen six isolates which are ST3, ST10, ST13, ST16, ST18 and ST23. Size of the DNA of chosen isolates were measured by running agarose gel electrophoresis and staining with flourosafe DNA stain. Agarose gel electrophoresis stands as a foundational technique in molecular biology, facilitating the separation and characterization of nucleic acids based on their size. The experimental design involved the application of an 80-volt electric field over a 45-minute duration, utilizing agarose as the gel matrix for its porous structure.

Agarose gel electrophoresis is a widely used technique for the measurement of DNA size. This method relies on the principles of electrophoresis, a process in which charged molecules, such as DNA fragments, migrate through a gel matrix under the influence of an electric field. Agarose, a polysaccharide extracted from seaweed, is the gel material commonly employed in this technique due to its ability to form a porous matrix with uniform pore sizes. The first step involves preparing an agarose gel by dissolving agarose powder in a buffer solution and then solidifying the mixture into a gel mold. The gel is then submerged in a buffer-filled electrophoresis chamber.

To measure the size of DNA molecules, a sample containing DNA fragments of interest is loaded into wells at one end of the gel. When an electric current is applied, the DNA fragments move through the gel matrix at a rate proportional to their size; smaller fragments migrate more quickly than larger ones. After a suitable electrophoresis period, the gel is stained with a flurosafe which binds to DNA and makes the fragments visible under ultraviolet light. A DNA ladder, consisting of known-size fragments, is often included for reference. This research plays a pivotal role in advancing our comprehension of the molecular composition specific to the selected isolates, potentially influencing fields such as genetics, microbiology, and epidemiology.

In the gel electrophoresis, the DNA marker, as described consists of various fragments with different sizes. The highest molecular weight fragment is 23,130bp, and the smaller fragments range from 9,416bp to 2,027bp. In the gel, the smaller fragments migrate faster and appear closer to the bottom, while the larger fragments migrate more slowly and appear higher up the gel.

Results of ST3 and ST13, which align with the first line in the marker (23,130bp), indicate that these samples contain DNA fragments of a size similar to the highest molecular weight fragment in the marker. This provides confidence in the integrity of the larger DNA fragments in these samples. The challenges observed in ST10 and ST16, where no bands or lines were visible, may suggest that the DNA in these samples is not present in sufficient quantity for detection, or there may be issues with the DNA extraction process. For ST18 and ST23, where bands were present without clear lines, it suggests the presence of DNA fragments, but the lack of well-defined lines could indicate incomplete digestion or potential DNA degradation.

4.6.2 Purity and concentration of DNA extraction through NanoDrop Spectrophotometry

Table 4.6: Purity and concentration of DNA extraction

Sample	Concentration of DNA sample (ng/µl)	Purity ratio (260/280)
ST3	24.674	1.93
ST10	1.922	-1.51
ST13	23.358	2.29
ST16	21.169	1.25
ST18	16.882	1.74
ST23	19.269	2.06

Following DNA extraction, NanoDrop spectrophotometry was employed to assess the purity and concentration of the DNA samples. Notably, the purity ratios 260/280 and concentrations varied across the isolates. The NanoDrop 200 spectrophotometer was utilized to assess the purity and concentration of DNA samples extracted from six bacterial isolates. For instance, ST3 has both the highest concentration of DNA and the highest purity ratio, indicating a high amount of pure DNA in the sample. ST10 has the lowest concentration of DNA and the lowest purity ratio, suggesting low DNA content and potential contamination or impurities in the sample. ST13 also has a high concentration of DNA and a relatively high purity ratio, indicating a good quality DNA sample. ST16, ST23, and ST18 have moderate concentrations of DNA and purity ratios within an acceptable range, indicating reasonable DNA content and purity. The negative purity ratio for ST10 is concerning and suggests significant contamination or issues with the sample, which may require further investigation or purification steps. ST13 appears to have the highest concentration of DNA and the highest purity ratio, while ST10 has the lowest concentration of DNA and a negative purity ratio, indicating potential issues with the sample.

CHAPTER 5

CONCLUSION & RECOMMENDATIONS

5.1 Conclusion

This study has explored the complex realm of bacteria that produce pectinase, revealing their possible uses in different industries. After carefully screening, 30 bacterial strains were found to have positive pectinase-producing traits, indicating their potential for industries that rely on breaking down pectin. The visible clear zones around colonies on pectin agar plates confirmed the strains' ability to break down pectin, making them suitable for biotechnological applications. Additionally, tests showed significant differences in pectinase activity among the strains, highlighting the importance of understanding enzyme production and the need for efficiency in industrial settings.

Moreover, examining the physical characteristics of isolated colonies provided insights into the appearance and possible connections between bacterial strains. This understanding not only enriches our knowledge of microbial diversity but also sets the stage for future research targeting specific traits for industrial purposes. However, challenges like variability in enzyme activity and DNA quality emphasize the ongoing need for optimization and standardization efforts. Improving extraction and purification techniques and exploring innovative methods like metagenomics could uncover new bacterial strains with improved pectinase-producing abilities, driving further advancements in industrial biotechnology.

5.2 Recommendation

It is recommended to optimize the screening process for pectinase-producing bacterial strains to identify high-performing candidates more efficiently. This could involve the development of high-throughput screening methods that leverage automation and robotics to handle large sample sizes rapidly. Additionally, implementing advanced molecular techniques such as metagenomics and next-generation sequencing can enhance the discovery of novel pectinolytic enzymes and provide insights into the genetic mechanisms underlying pectin degradation. Furthermore, there is a critical need to standardize enzyme activity assays and fermentation protocols to ensure reproducibility and scalability in industrial settings. Collaborative efforts between academia, industry, and regulatory bodies are essential to establish guidelines and best practices for pectinase production and application, facilitating the adoption of these enzymes in various industrial sectors, including food processing, textile manufacturing, and biofuel production.

REFERENCES

A Collmer, a. and N. T. Keen (1986).The Role of Pectic Enzymes in Plant Pathogenesis. Annual Review of Phytopathology **24**(1): 383-409.

Abdollahzadeh, R., et al. (2020). Screening of pectinase-producing bacteria from farmlands and optimization of enzyme production from selected strain by RSM. Folia microbiological **65**: 705-719.

Alimardani-Theuil, P., et al. (2011). Yeasts: An attractive source of pectinases from gene expression to potential applications: A review. Process Biochemistry **46**(8): 1525-1537.

Artur, Z. a., et al. (2021). The primary, secondary, and structures of higher levels of pectin polysaccharides. Comprehensive Reviews in Food Science and Food Safety **20**(1): 1101-1117.

Barth, M., et al. (2009). Microbiological spoilage of fruits and vegetables. Compendium of the microbiological spoilage of foods and beverages: 135-183.

Berezina, O. V., et al. (2024). Xanthan: enzymatic degradation and novel perspectives of applications. Applied Microbiology and Biotechnology **108**(1): 227.

Bhatt, K., et al. (2022).New insights into engineered plant-microbe interactions for pesticide removal. Chemosphere **309**: 136635.

Bonnin, E., et al. (2014). Pectin-modifying enzymes and pectin-derived materials: applications and impacts. Applied Microbiology and Biotechnology **98**: 519-532.

Collmer, A., et al. (2012). Pectase lyase regulation and bacterial soft-rot pathogenesis. Phytopathogenic prokaryotes **1**: 395-422.

de OK Franco, M., et al. (2021). A novel digital image method for determination of reducing sugars in aged and non-aged cachacas employing a smartphone. Food Chemistry **338**: 127800.

de Souza, T. S. and H. Y. Kawaguti (2021). Cellulases, hemicellulases, and pectinases: Applications in the food and beverage industry. Food and Bioprocess Technology **14**(8): 1446-1477.

Espejo, F. (2021). Role of commercial enzymes in wine production: a critical review of recent research.
Journal Food Science Technology **58**(1): 9-21.

Garg, G., et al. (2016). Microbial pectinases: an ecofriendly tool of nature for industries. Biotechnology **6**(1): 47.

Hernández-Beltrán, J. U., et al. (2023). Bioreactor Scale Strategy for Pectinase Production. Microbial Bioreactors for Industrial Molecules: 103-130.

Hoondal, G., et al. (2002). Microbial alkaline pectinases and their industrial applications: a review. Applied Microbiology and Biotechnology **59**: 409-418.

Hours, R. A. and T. Sakai (1994). Protopectinase production in solid state culture of *Aspergillus awamori*. Biotechnology Letters **16**(7): 721-726.

Jeilu, O. (2017). Pectinase: Substrate, Production and their Biotechnological Applications. International Journal of Environment, Agriculture and Biotechnology (IJEAB) **2**.

Kaczmarska, A., et al. (2022). Structure and functionality of Rhamnogalacturonan I in the cell wall and in solution: A review. Carbohydrate Polymers **278**: 118909.

Kashyap, D., et al. (2001). Applications of pectinases in the commercial sector: a review. Bioresource Technology **77**(3): 215-227.

KC, S., et al. (2020). Production, characterization, and industrial application of pectinase enzyme isolated from fungal strains. Fermentation **6**(2): 59.

Khan, I. and D. Barate (2016). Effect of various parameters on activity of pectinase enzyme. International Journal of Advanced Research **4**(1): 853-862.

Kim, C. C., et al. (2019). Genomic insights from *Monoglobus pectinilyticus*: a pectin-degrading specialist bacterium in the human colon. The ISME Journal **13**(6): 1437-1456.

Kjøller, A. H. and S. Struwe (2002). Fungal communities, succession, enzymes, and decomposition. Enzymes in the environment: Activity, ecology and applications **1**: 267-284.

Kumar, S., et al. (2011). Effect of substrate and fermentation conditions on pectinase and cellulase production by *Aspergillus niger* NCIM 548 in submerged (SmF) and solid state fermentation (SSF). Food Science and Biotechnology **20**: 1289-1298.

Li, J., et al. (2024). An overview of microbial enzymatic approaches for pectin degradation. International Journal of Biological Macromolecules **254**: 127804.

López-Gómez, J. P. and J. Venus (2021). Potential role of sequential solid-state and submerged-liquid fermentations in a circular bioeconomy. Fermentation **7**(2): 76.

Luzio, G. A. and R. G. Cameron (2008). Demethylation of a model homogalacturonan with the salt-independent pectin methylesterase from citrus: Part II. Structure–function analysis. Carbohydrate Polymers **71**(2): 300-309.

Mahto, R. B., et al. (2023). Biochemical properties and application of a novel pectinase from a mutant strain of *Bacillus subtilis*. Biomass Conversion and Biorefinery **13**(12): 10463-10474.

Maldonado, M. C. and A. M. Strasser de Saad (1998). "Production of pectinesterase and polygalacturonase by *Aspergillus niger* in submerged and solid state systems. Journal Industry Microbiol ology Biotechnology **20**(1): 34-38.

Millan-Linares, M. C., et al. (2021). Pectins and olive pectins: From biotechnology to human health. Biology **10**(9): 860.

Mohandas, A., et al. (2018). Production of pectinase from *Bacillus sonorensis MPTD1*. Food Technology and Biotechnology **56**(1): 110.

Mojsov, K. (2013). "Use of enzymes in wine making: a review. International Journal of Marketing and Technology **3**: 112-127.

Moore, J. P., et al. (2008). A role for pectin-associated arabinans in maintaining the flexibility of the plant cell wall during water deficit stress. **3**(2): 102-104.

Murad, H. and H. Azzaz (2011). Microbial pectinases and ruminant nutrition. Research Journal of Microbiology **6**(3): 246-269.

Mutter, M., et al. (1994). Rhamnogalacturonan alpha-L-rhamnopyranohydrolase. A novel enzyme specific for the terminal nonreducing rhamnosyl unit in rhamnogalacturonan regions of pectin. Plant Physiology **106**(1): 241-250.

Niu, H., et al. (2023). A critical review of RG-I pectin: sources, extraction methods, structure, and applications. Critical Reviews in Food Science and Nutrition: 1-21.

Noor, N., et al. (2021). Food biopolymers: Structural, functional and nutraceutical properties: 127-171.

Norris, P. R., Falagan, (2020). *Acidithiobacillus ferrianus*: an ancestral extremely acidophilic and facultatively anaerobic chemolithoautotrophy. 24(2), 329–337.

O'Neill, M. A., et al. (2004). Rhamnogalacturonan II: structure and function of a borate cross-linked cell wall pectic polysaccharide. **55**: 109-139.

Oumer, O. J. (2017). Pectinase: substrate, production and their biotechnological applications. International Journal of Environment, Agriculture and Biotechnology **2**(3): 1007-1014.

Oumer, O. J. and D. Abate (2018). Comparative Studies of Pectinase Production by *Bacillus subtilis* strain Btk 27 in Submerged and Solid-State Fermentations. Biomedical Resources **2018**: 1514795.

Öztürk, M. (2024). Isolation, identification, and characterization of *Pectobacterium actinidiae* isolated from infected sugar beet leaves. Journal of Phytopathology **172**(1): e13275.

Patidar, M. K., et al. (2018). Pectinolytic enzymes-solid state fermentation, assay methods and applications in fruit juice industries: a review. Biotechnology **8**(4): 199.

Patil, S. R. and A. Dayanand (2006). Production of pectinase from deseeded sunflower head by *Aspergillus niger* in submerged and solid-state conditions. Bioresource Technology **97**(16): 2054-2058.

Phan, T. D., et al. (2007). Silencing of the major salt-dependent isoform of pectinesterase in tomato alters fruit softening. Plant Physiology **144**(4): 1960-1967.

Pieczywek, P. M., et al. (2021). Aggregation and weak gel formation by pectic polysaccharide homogalacturonan. Carbohydrate Polymers **256**: 117566.

Prado, S. B. d., et al. (2016). Physiological degradation of pectin in papaya cell walls: release of long chains galacturonans derived from insoluble fractions during postharvest fruit ripening. Frontiers in plant science **7**: 1120.

Reddy, P. L. and A. Sreeramulu (2012). Isolation, identification and screening of pectinolytic fungi from different soil samples of Chittoor district. International Journal of Life Sciences Biotechnology and Pharmaceutical Research **1**(3): 1-10.

Rehman, H. U., et al. (2015). Morphological and molecular based identification of pectinase producing *Bacillus licheniformis* from rotten vegetable. Journal of Genetic Engineering and Biotechnology **13**(2): 139-144.

Robinson, P. K. (2015). Enzymes: principles and biotechnological applications. Essays Biochemistry **59**: 1-41.

Ropartz, D. and M.-C. Ralet (2020). Pectin structure. Pectin: Technological and Physiological Properties: 17-36.

Ropartz, D. and M.-C. Ralet (2020). Pectin Structure. Pectin: Technological and Physiological Properties. V. Kontogiorgos. Cham, Springer International Publishing**:** 17-36.

Ruiz, H. A., et al. (2017). Pectinolytic enzymes. Current developments in biotechnology and bioengineering, Elsevier**:** 47-71.

Said, S., et al. (1991). Pectinase production by *Penicillium frequentans*. World Journal of Microbiology and Biotechnology **7**: 607-608.

Samanta, S. (2019). Microbial pectinases: a review on molecular and biotechnological perspectives. The Journal of Microbiology, Biotechnology and Food Sciences **9**(2): 248.

Sandhya, R. and G. Kurup (2013). Screening and isolation of pectinase from fruit and vegetable wastes and the use of orange waste as a substrate for pectinase production. Int. **2**(9): 34-39.

Satapathy, S., et al. (2020). Biochemical prospects of various microbial pectinase and pectin: an approachable concept in pharmaceutical bioprocessing. Frontiers in Nutrition **7**: 117.

Schols, H. A., et al. (2009). Pectins and pectinases, Wageningen Academic Publishers.

Shahapurkar, S. O. (2023). Production And Optimization Of Pectinases Produced By Some Fungi From Fruit Peels Of Nanded District.

Sharma, N., et al. (2013). Microbial pectinase: sources, characterization and applications. Reviews in Environmental Science and BioTechnology **12**(1): 45-60.

Sharma, P., et al. (2021). Pectin. Biomass, Biofuels, Biochemicals, Elsevier**:** 101-128.

Shet, A., et al. (2018). PECTINOLYTIC ENZYMES: CLASSIFICATION, PRODUCTION, PURIFICATION AND APPLICATION. **4**: 337-348.

Shi, L., et al. (2022). Exploring the effects of pectate and pectate lyase on the fruit softening and transcription profiling of Solanum lycopersicum. Food Control **133**: 108636.

Shrestha, S., et al. (2021). New insights in pectinase production development and industrial applications. Applied Microbiology and Biotechnology **105**: 9069-9087.

Solís-Pereira, S., et al. (1993). Effects of different carbon sources on the synthesis of pectinase by *Aspergillus niger* in submerged and solid state fermentations. Applied Microbiology and Biotechnology **39**: 36-41.

Srivastava, P. and R. Malviya (2011). Sources of pectin, extraction and its applications in pharmaceutical industry for an overview.

Tapre, A. and R. Jain (2014). Pectinases: Enzymes for fruit processing industry. International Food Research Journal **21**(2): 447.

Thakur, B. R., et al. (1997). Chemistry and uses of pectin for A review. Critical Reviews in Food Science & Nutrition **37**(1): 47-73.

Tian, G., et al. (2023). Effect of Side-Chain Functional Groups in the Immunogenicity of Bacterial Surface Glycans. Molecules **28**(20): 7112.

Vaishnavi, V., et al. (2023). Microbial Production Strategies for the Production of Industrially Important Pectinases: Bioreactor Consideration and its Applications in Various Sectors. Journal of Mines, Metals & Fuels **71**(3).

Van Buren, J. (1991). Function of pectin in plant tissue structure and firmness. The Chemistry and Technology of Pectin: 1-22.

Wang, F., et al. (2023). Application of Immobilized Enzymes in Juice Clarification. Foods **12**(23): 4258.

Widsten, P., et al. (2024). Isolation of Arabinose and Galactose from Industrial Sidestreams in High Yield and Purity. BioResources **19**(1).

Winkelmann, G. (1992). Microbial degradation of natural products.

Wu, C.-l., et al. (2024). Study on low methoxyl pectin (LMP) with varied molecular structures cross-linked with calcium inducing differences in the gel properties. Food Hydrocolloids **146**: 109271.

Yu, G. (2021). Interactions of Chitin and Lignin Thin Films with Other Molecules, Virginia Tech.

Zeuner, B., et al. (2020). Comparative characterization of *Aspergillus pectin lyases* by discriminative substrate degradation profiling. Frontiers in Bioengineering and Biotechnology **8**: 873.

APPENDIX A

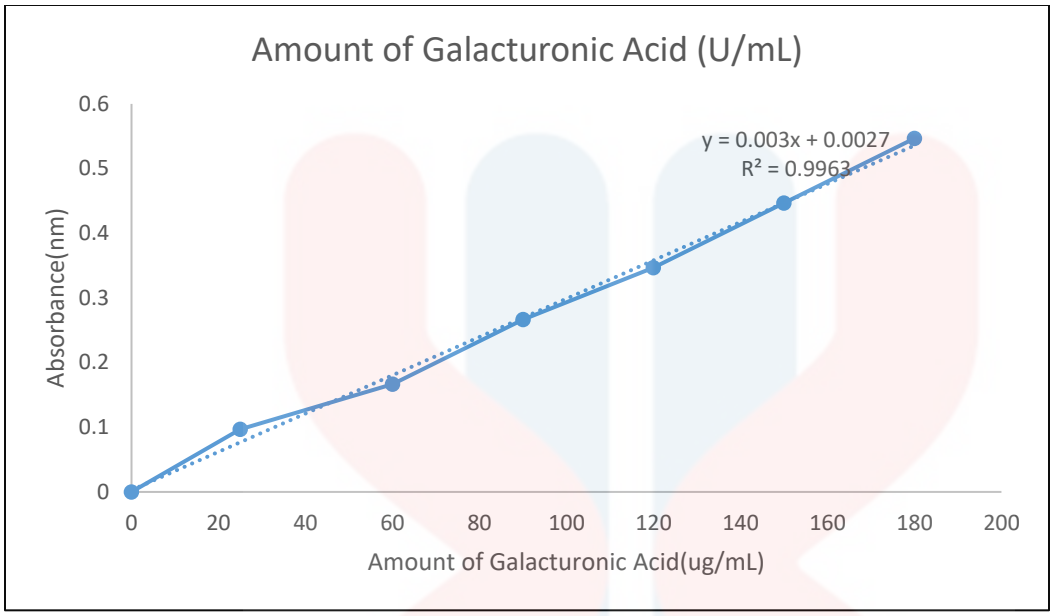
CALCULATION

(a) Clear zone ratio

$$clear\ zone\ ratio = \frac{diameter\ of\ clear\ zone\ (mm)}{dxdiameter\ of\ colony\ (mm)}$$

(b) Standard curve of galacturonic acid

Amount of galacturonic acid (μ/ml)	Absorbance (nm)
0	0
25	0.0966
60	0.1666
90	0.2666
120	0.3466
150	0.4466
180	0.5466



(c) absorbance values of strain

Strain	Absorbance (nm)
ST 3	0.654
ST 10	0.090
ST 13	0.695
ST 16	0.310
ST 18	0.423
ST 23	0.480

c) calculation of reducing sugar concentration of strain

formula y= mx+c

Equation of galacturnic standard curve

y= 0.003x+0.0027

R^2= 0.9963

i) ST 3

0.654= 0.003x+0.0027

x= 217.1

ii) ST 10

0.090= 0.003x+0.0027

x= 29.1

iii) ST 13

0.695= 0.003x+0.0027

x= 230.77

iv) ST 16

0.310= 0.003x+0.0027

x= 102.43

v) ST 18

0.423= 0.003x+0.0027

x= 104.1

vi) ST 23

0.480= 0.003x+0.0027

x= 159.1

(E) enzyme activity

Pectinase activity (U/mL) = $\frac{reducing\ sugar\ concentration\ x\ 1000\ x\ dilution\ factor}{molecular\ weight\ of\ galacturonic\ acid\ x\ incubation\ time(minutes)}$

Dilution factor = 1

Molecular sugar of galacturonic acid = 194.139g/mol

Incubation time = 10minutes

ST 3 Pectinase activity (U/mL) = $\frac{217.1\ x\ 1000\ x\ 1}{194.139x\ 10}$

= 37.28 U/mL

ST 10 Pectinase activity (U/mL) = $\frac{29.1\ x\ 1000\ x\ 1}{194.139x\ 10}$

= 4.99 U/mL

ST 13 Pectinase activity (U/mL) = $\frac{230.77\ x\ 1000\ x\ 1}{194.139x\ 10}$

= 39.62 U/mL

ST 16 Pectinase activity (U/mL) = $\frac{102.43\ x\ 1000\ x\ 1}{194.139x\ 10}$

= 17.57 U/mL

ST 18 Pectinase activity (U/mL) = $\frac{104.1\ x\ 1000\ x\ 1}{194.139x\ 10}$

= 24 U/mL

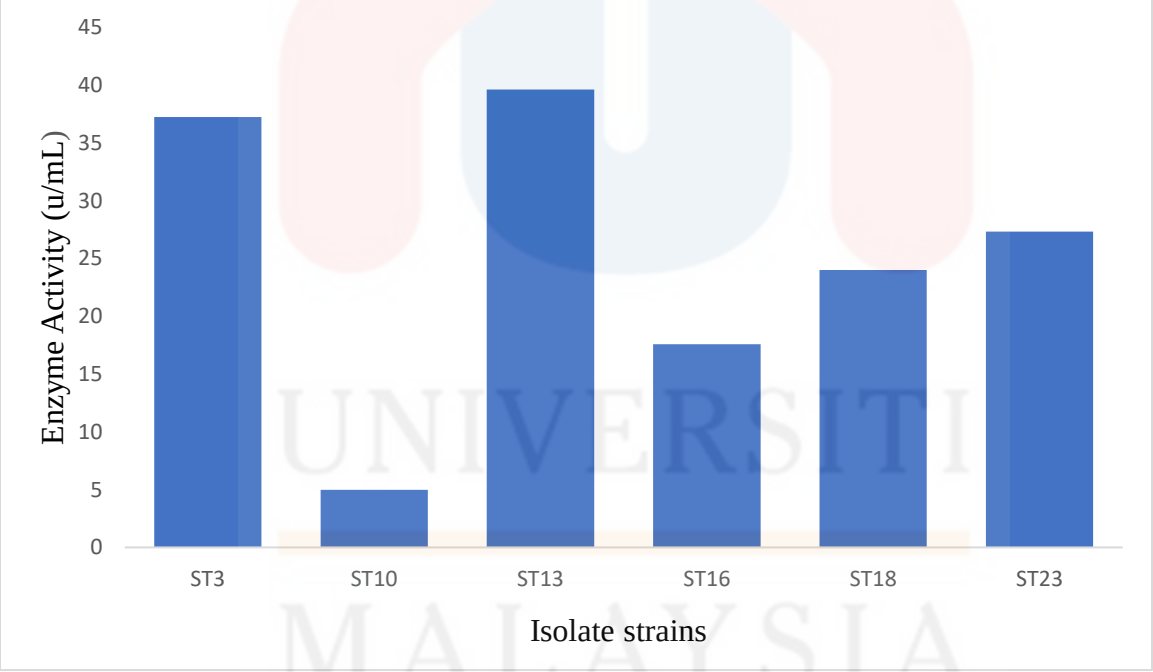
ST 23 Pectinase activity (U/mL) = $\frac{159.1\ x\ 1000\ x\ 1}{194.139x\ 10}$

= 27.32 U/mL

F) table shows enzyme activity

Isolated strain	Enzyme Activity (ug/mL)
ST3	37.25
ST10	4.99
ST13	39.62
ST16	17.57
ST18	24
ST23	27.32

G) Graph shows enzyme activity of 6 strain



APPENDIX B

Nutrient Agar

Add 28g of nutrient agar to a flask containing 1000 ml of distilled water. Mix the contents of the flask thoroughly to ensure proper dissolution. Heat the flask gently while stirring to dissolve it completely. Once the ingredients are dissolved, adjust the pH of the mixture to around 7 using a pH meter or pH indicator strips. After adjusting the pH, remove the flask from the heat source. Sterilize the nutrient agar by autoclaving at 121°C for 15 minutes. After autoclaving, allow the agar to cool down to approximately 45–50 °C before pouring into petri dishes. Carefully pour the sterilized nutrient agar into sterile petri dishes, filling them about halfway. Let the agar solidify by leaving the petri dishes undisturbed at room temperature.

Pectin Agar Medium

Sodium nitrate (2 g), 0.5 g of potassium chloride, 10g of pectin,0.5 g of magnesium sulfate, and 1.0 g of dipotassium phosphate will be added to a flask containing 1000 ml of distilled water. The contents of the flask will be thoroughly mixed to ensure proper dissolution. The flask will be gently heated while stirring to dissolve the ingredients completely. Once the ingredients are dissolved, 20 g of agar will be added to the flask. The heating and stirring continued until the agar is completely dissolved. The pH of the mixture will be adjusted to around 7 using a pH meter or pH indicator strips. After adjusting the pH, the flask will be removed from the heat source. The nutrient agar will be sterilized by autoclaving at 121°C for 15 minutes. After autoclaving, the agar will be allowed to cool down to approximately 45–50 °C. Before pouring it into petri dishes. The sterilized nutrient agar will be carefully poured into sterile petri dishes, filling them about halfway. The agar will be left undisturbed at room temperature to solidify.

Preparation of LB broth

The required amounts of 10g tryptone, 5g yeast extract, and 10g sodium chloride will be weighed and measured. These ingredients will be added to a glass or heat-resistant container. A small amount of distilled water will be added to the container, and the mixture will be stirred thoroughly to dissolve the ingredients. Gradually, more distilled water will be added while continuously stirring until all the ingredients are completely dissolved. The solution will be stirred until it appears homogeneous, ensuring there are no visible particles or clumps. The solution will then be transferred to a suitable container, such as a glass bottle or Erlenmeyer flask. If necessary, the pH of the LB broth will be adjusted to approximately 7.0 using a pH meter or pH indicator paper, and small amounts of acid (e.g., hydrochloric acid) or base (e.g., sodium hydroxide) will be added as needed. Distilled water will be added to the container to reach the desired final volume, typically 1 liter, while leaving enough headspace to prevent spillage during autoclaving. The container will be securely closed with a cap or stopper. To sterilize the LB broth, it will be autoclaved at 121 degrees Celsius for 15-20 minutes. Autoclaving ensures the removal of any potential contaminants. After autoclaving, the LB broth will be allowed to cool down to room temperature before it is used for bacterial culture or storage.

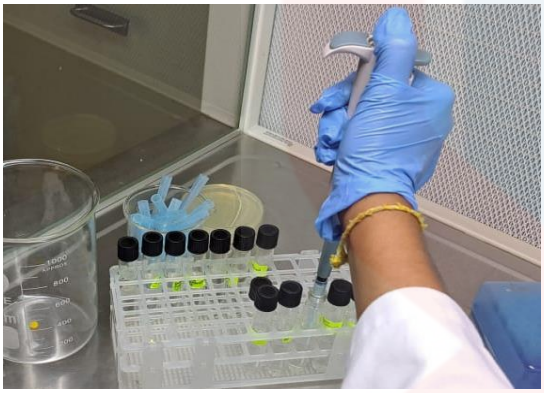
Pectin broth

The pectin broth of 100ml composed of 0.5 g of pectin, 0.1 g of yeast extract, 0.715 g of Ammonium chloride, 0.45 g of sodium hydrogen phosphate dodecahydrate, 0.63 g of potassium dihydrogen phosphate, 0.075 g of potassium chloride, and 0.025 g of magnesium sulfate heptahydrate. The pH values will be maintained between 4.0 and 6.0 to support optimal enzyme production.

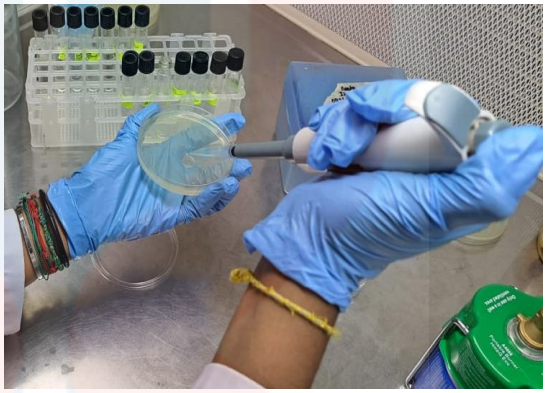
Preparation of DNS Reagent

To prepare the DNS reagent, start by diluting 10 g of 3,5-dinitrosalicylic acid in 200 ml of distilled water. Thoroughly mix the solution and gradually add more distilled water until the volume reaches 600 ml. Then, slowly add 30 g of sodium potassium tartrate to the solution while ensuring the temperature stays below 40°C. Heat the mixture to aid the dissolution of the ingredients. Finally, add distilled water to reach a total volume of 1000 ml. Store the prepared DNS reagent in a dark, tightly sealed bottle and keep it refrigerated.

APPENDIX C

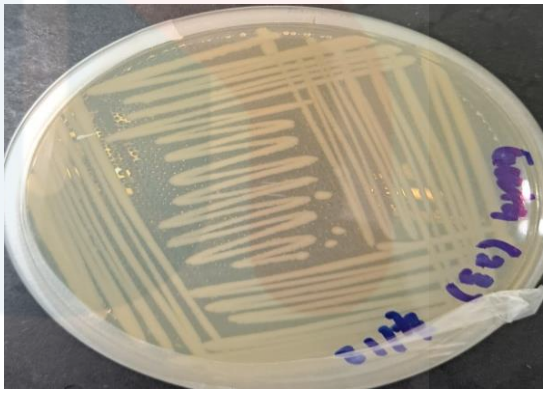


Serial dilution

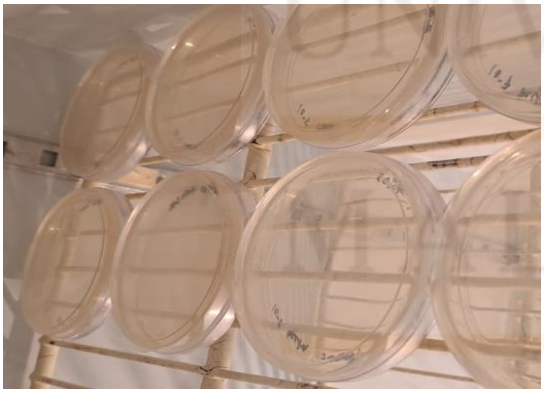


Pour plate

Spread plate

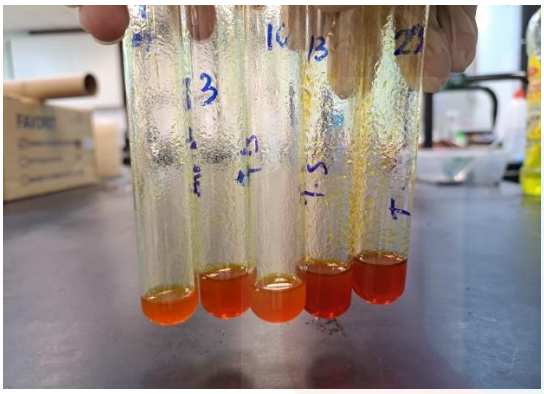


Streak plate

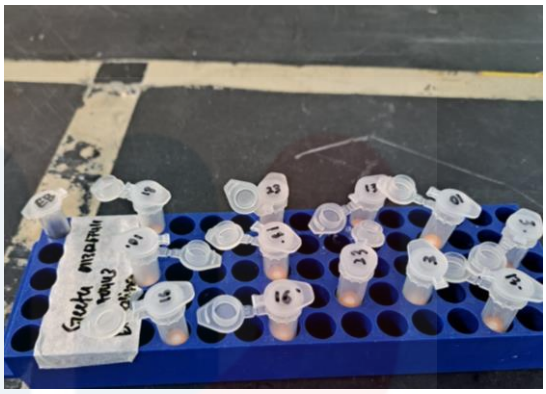


Incubate streak plate

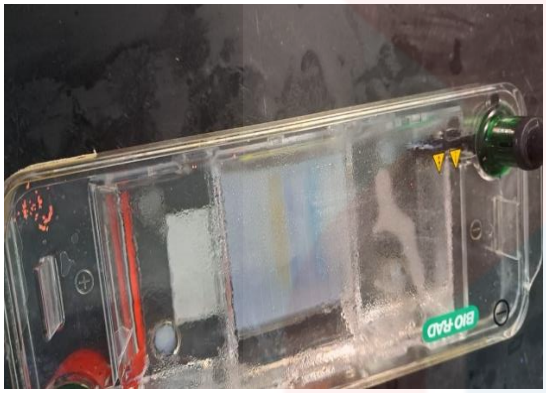
Fermentation



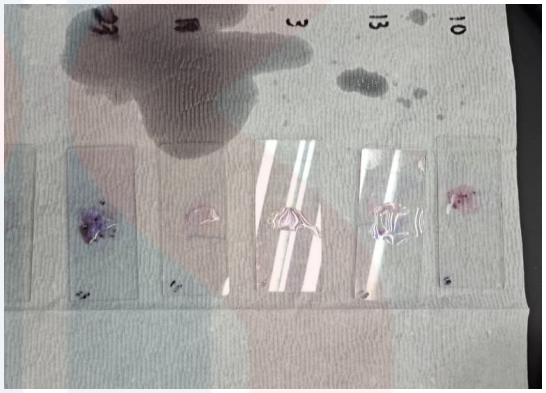
Pectinolytic assay



Sample of DNA extraction



Running gel electrophoresis



Gram Staining

Pour agar

pectin agar preparation

Nutrient agar preparation

pectin broth preparation

Lambda HindIII

DNA Loading Dye