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ISOLATION AND CHARACTERIZATION OF β-GLUCAN PRODUCING BACTERIA STRAINS FROM ANTARCTICA

SHARMMILA A/P PUBALAN

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A report submitted in fulfilment of the requirements for the degree of Bachelor of Applied Science (Bio-industrial Technology) with Honours

FACULTY OF BIOENGINEERING AND TECHNOLOGY

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DECLARATION

I declare that this thesis entitled "isolation and Characterization of β-Glucan producing Bacteria Strain from Antarctica" is the results of my own research except as cited in the references.

Signature : 

Student's Name : SHARMMILA A/P PUBALN

Date : _____

Verified by:

Signature : _____

Supervisor's Name : DR WEE SENG KEW

Stamp : _____

Date : _____

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Isolation and Characterization of β-Glucan producing Bacteria Strain from Antarctica

ABSTRACT

This thesis undertakes a study focused on bacterial isolates from Antarctica, which underwent a thorough examination aimed at isolating and characterizing strains capable of producing β-glucan. The β-glucan-producing bacteria from polar environments uncover novel strains with unique genetic and physiological characteristics, contributing to microbial diversity understanding, exploring potential biotechnological applications, gaining insights into adaptation to extreme conditions, understanding ecological interactions, and supporting conservation efforts. The methodology involved serial dilution, spread plate, isolation, Gram staining, biochemical tests, and gel electrophoresis. After initial plating, 20 distinct colonies were streaked on LB agar supplemented with Aniline Blue and sucrose, revealing the presence of β-glucan through blue staining. Its results that five blue-stained colonies (A1, A2, A3, A4, and A5). Gram staining and biochemical tests classified A1, A2, A4, and A5 as Gram-negative and A3 as Gram-positive. Catalase tests indicated A1, A2, and A3 as negative, while A4 and A5 were positive. Oxidase tests showed positive results for all isolates. Starch tests were negative for all strains. Gel electrophoresis after DNA extraction demonstrated successful DNA isolation, although the run was interrupted at 30 minutes, limiting fragment size determination. Despite the incomplete run, the visible presence of DNA fragments suggests successful extraction.

Keywords: β-glucan-producing strains, Gram staining, Biochemical Tests, DNA Extraction, Gel Electrophoresis.

Isolasi dan analisis ciri-ciri bakteia yang menghasilkan β-glucan dari Antarctica

ABSTRAK

Tesis ini menyajikan satu kajian mengenai isolat bakteria dari Antarctica yang telah dijalankan melalui penyelidikan menyeluruh dengan matlamat mengasingkan dan menganalisis ciri-ciri β-glucan yang dihasilkan. Bakteria penghasil β-glucan dari persekitaran kutub mengungkapkan strain baru dengan ciri-ciri genetik dan fisiologi yang unik, menyumbang kepada pemahaman kepelbagaian mikrob, meneroka potensi aplikasi bioteknologi, mendapatkan wawasan tentang penyesuaian kepada keadaan ekstrem, memahami interaksi ekologi, dan menyokong usaha pemuliharaan. Metodologi yang digunakan merangkumi pencairan berperingkat, plate rata, pengasingan, pewarnaan Gram, ujian biokimia, dan elektroforesis gel. Selepas penanaman awal, 20 koloni yang berbeza diratakan pada agar LB yang ditambah Aniline Blue dan sukrosa, mendedahkan kehadiran β-glucan melalui pewarnaan biru. Hasilnya menunjukkan lima koloni berwarna biru (A1, A2, A3, A4, dan A5). Pewarnaan Gram dan ujian biokimia mengelas A1, A2, A4, dan A5 sebagai Gram-negatif dan A3 sebagai Gram-positif. Ujian katalase menunjukkan A1, A2, dan A3 sebagai negatif, sementara A4 dan A5 adalah positif. Ujian oksidase menunjukkan keputusan positif untuk semua isolat. Ujian kanji menunjukkan keputusan negatif untuk semua strain. Elektroforesis gel selepas pengekstrakan DNA menunjukkan isolasi DNA yang berjaya, walaupun pusingan dihentikan pada minit ke-30, membataskan penentuan saiz fragmen. Walaupun pusingan yang tidak lengkap, kehadiran visual fragmen DNA mencadangkan kejayaan ekstraksi.

Kata Kunci: Strain Penghasil β-glucan, Pewarnaan Gram, Ujian Biokimia, Pengekstrakan DNA, Elektroforesis Gel.

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LIST OF ABBREVIATIONS

CO^2	Carbon Dioxide
LB	Luria Bertani
NaCl	Sodium Chloride
µL	Microliter
g/l	Gram per liter
w/v	Weight per volume

LIST OF SYMBOLS

β	B
%	Percentage
°C	Degree Celsius
V	Volt

CHAPTER 1

INTRODUCTION

1.1 Background of Study

β-glucan is type of polysaccharide which contains the D-glucose monomers, that is connected through β-(1,3) β-(1,4) and β-(1,6) glycosidic linkages (Maheshwari et al., 2017). β-glucans can be derived from various natural sources. They are found in the cell walls of bacteria (e.g., certain strains of *Bacillus* and *Agrobacterium*), fungi (e.g., species of *Saccharomyces*, *Candida*, and *Aspergillus*), yeast (e.g., species of *Saccharomyces* and *Candida*), algae (e.g., species of *Chlorella* and *Spirulina*), and plants (e.g., oats, barley, and mushrooms) (Mulyani, 2023)

B-glucan is produced through a biosynthetic process in certain microorganisms, including bacteria and fungi. The synthesis of β-glucan involves the activity of specific enzymes, such as glucosyltransferases and branching enzymes. These enzymes catalyse the formation of glycosidic bonds between glucose molecules, resulting in the linear or branched structure of β-glucan. The precise mechanism and regulation of β-glucan biosynthesis can vary among different organisms. The produced β-glucan can be either an intracellular component or an extracellular polysaccharide, depending on the organism. β-glucan serves various functions in microorganisms, including structural support, cell adhesion, and as an energy source.

Research suggests that β-glucans may have several health benefits. They have been studied for their potential in supporting antioxidant, antimicrobial, antitumor, and cholesterol-lowering activities (Yang & Huang, 2021). β-glucans have also been investigated for their application in functional foods, dietary supplements, pharmaceuticals, and biomedical materials (Du et al., 2019). The β-glucans has been extracted to be used in industrial scale with various strategies.

The samples that used to conduct this research has been collected from Antarctica. Currently, the polar environment has gained the scientist attention where the microbes from polar environment. Those microbes are called as psychrophilic microbes where they inhabit in the cold regions of the Earth including polar zones, high mountains, glaciers, and deep oceans. Psychrophiles are also well accumulated in pockets of sea ice with high pH, salinity, inorganic nutrients, dissolved gases and light Bacteria producing β-glucans in specific environments, such as polar environments, may have unique adaptations to survive under extreme conditions.(Nicolaus et al., 2010)

1.2 Problem Statement

This study has several problem statements that draw out the attention to isolate and study the characterization of the β-glucan. Polar environments, such as the Arctic and Antarctic regions, are known for their extreme conditions and unique ecosystems. By isolating and characterizing the bacteria that produce β-glucan as an extracellular product from these environments, we can uncover novel strains with unique genetic and physiological characteristics. This contributes to our understanding of microbial diversity and expands our knowledge of β-glucan producing bacteria beyond conventional habitats. This study also offers potential biotechnological applications, insights into adaptation to extreme conditions, understanding of ecological interactions, and support conservation efforts.

1.3 Objectives

1. To isolate the β-glucan producing bacteria strains from Antarctica.
2. To characterize the β-glucan producing bacteria strains from Antarctica.

1.4 Scope of Study

Isolation and characterization of β-glucan producing bacteria strains from polar environments involves the process of identifying and studying bacteria that have the ability to produce β-glucans in these extreme cold regions. The purpose of this research is to explore the biotechnological potential, understand the adaptation mechanisms, and investigate the ecological significance of these bacteria and their β-glucan products. Usage of Aniline Blu Dye has been studied and found out it can identify the presence of β-glucan. The isolation process begins with sample collection from polar environments, such as soil, marine sediments, or water. These samples are then processed in the laboratory to obtain the microbial population, which may include β-glucan producing bacteria strains. Various isolation techniques, such as serial dilution and plating on specific media, are employed to isolate individual bacterial strains. Meanwhile, biochemical test such as catalase test, oxidase test, and starch test, along with gram staining, DNA Extraction, Gel Electrophoresis, 16s rRNA sequencing with BLAST methods, are used to characterize those isolated bacterial strains.

1.5 Significances of Study

Firstly, β-glucans are polysaccharides with unique properties that have significant potential in various industries, including food, pharmaceuticals, cosmetics, and biotechnology. The demand of the polysaccharides is high. These polysaccharides have been found to possess immunomodulatory, antimicrobial, antioxidant, and anticancer properties, among others. By

identifying and studying bacteria strains that produce β-glucans in polar environments, researchers can explore new and potentially valuable sources of these bioactive compounds. Also, can provide β-glucans to fulfill the demand.

Secondly, polar environments, such as the Arctic and Antarctic regions, are extreme and harsh environments characterized by low temperatures, high salinity, and limited nutrient availability. Bacteria that can thrive in these conditions possess specialized adaptations and metabolic capabilities. Studying bacteria strains from polar environments can provide insights into their unique biochemical and physiological characteristics, as well as their ability to produce valuable compounds like β-glucans. This knowledge can contribute to our understanding of microbial diversity, adaptation, and bioprospecting in extreme environments. Further studies can be done one this to identify usage of the β-glucan from the polar bacterial microbes.

CHAPTER 2

LITERATURE REVIEW

2.1 β – Glucan

β-glucan is type of polysaccharide which contains the D-glucose monomers, that is connected by β-(1,3) β-(1,4) and β-(1,6) glycosidic linkages (Maheshwari et al., 2017). The functionality of β-glucan basically depends upon types of linkages, degree of branching, and structural arrangement that, in turn, manipulate its biological activity. It is mentioned that the introduction of suitable ionic groups with appropriate degrees of substitution enhanced the water solubility of the polysaccharides along with the change in the conformation of the polymer chain in a solution that, in turn, was responsible for their improved biological activities. (Du et al., 2019)

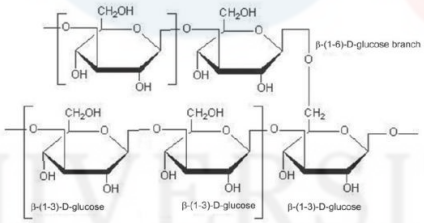


Figure 2.1: β-glucans glycosidic Bond

2.2 Biosynthesis of β-Glucan

β-glucan synthesis occurs in the cell wall through the action of the GLS protein complex. The key subunit involved in this process is FKS 1/2, which catalyzes the elongation of growing β-glucan chains by adding UDP-glucose monomers. The FKS 1/2 subunit contains an endoplasmic hydrophilic loop, which is considered to be responsible for the catalytic activity of the complex. (Papaspyridi et al., 2018)

The activity of FKS 1/2 is regulated by the protein RHO, which functions as a GTP-dephosphorylase. RHO controls the action of FKS 1/2 through the regulation of GDP (guanosine diphosphate). The presence of GDP inhibits the activity of FKS 1/2, while the conversion of GDP to GTP (guanosine triphosphate) activates FKS 1/2 and allows for β-glucan synthesis. This regulatory mechanism ensures that β-glucan synthesis occurs only when the necessary conditions are met. (Papaspyridi et al., 2018)

Additionally, the protein ROM plays a role in β-glucan synthesis by serving as a cell wall-associated GDP-GTP exchange protein. ROM is responsible for the regeneration of GTP from GDP, allowing for a continuous supply of GTP to activate FKS 1/2. This ensures the sustained synthesis and elongation of β-glucan chains in the cell wall. (Papaspyridi et al., 2018)

The schematic representation of β-glucan synthesis in the fungal cell wall highlights the involvement of the GLS protein complex, with FKS 1/2 as the catalytic subunit. The regulatory proteins RHO and ROM play crucial roles in controlling the activity of FKS 1/2 and maintaining the supply of GTP for β-glucan synthesis. This process is essential for the formation and integrity of the fungal cell wall, providing structural support and protection for the fungal cell.(Papaspyridi et al., 2018)

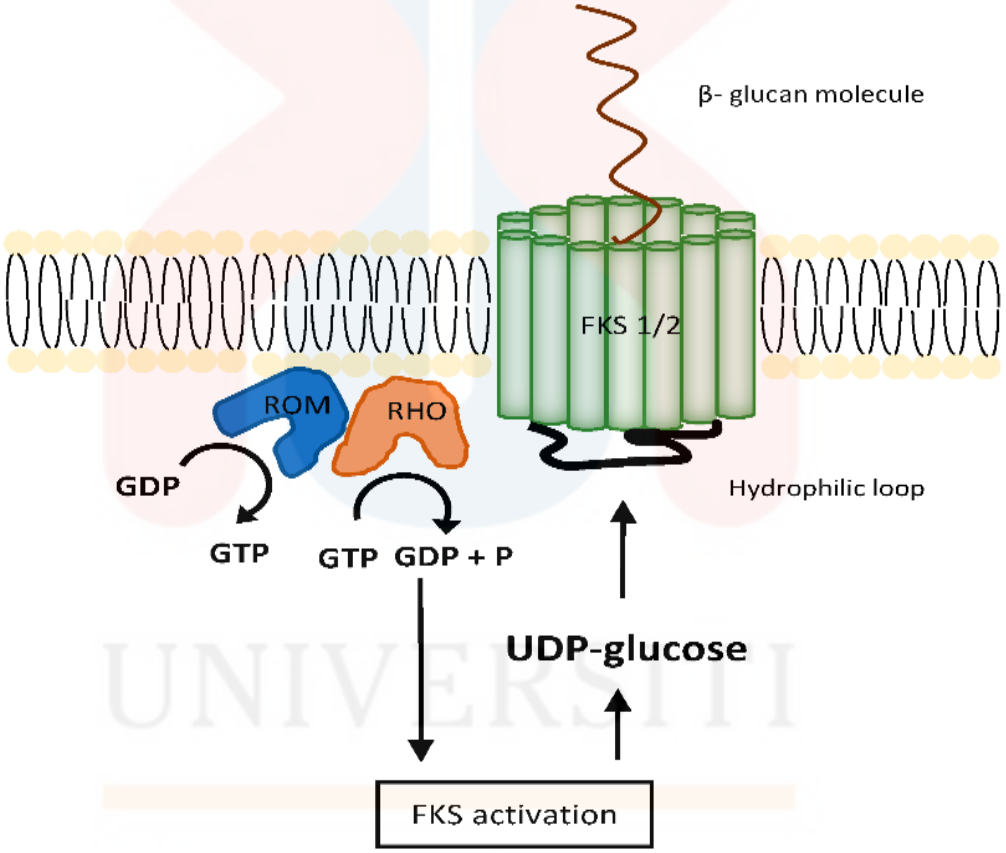


Figure 2.2: β-glucan synthesis in the fungal cell wall

2.3 Polar Environment

The polar environment is characterized by extreme temperature conditions, with average temperatures often reaching below freezing. However, during the short summer season, temperatures can rise to around 0°C or slightly above. The cold temperatures in the polar environment pose significant challenges to living organisms, as they need to adapt to survive in such extreme conditions (Post et al., 2019).

Carbon dioxide (CO^2) levels in the polar environment are typically lower compared to other regions. This is mainly due to limited vegetation and minimal human activity in these areas. The absence of large-scale plant life reduces the amount of CO^2 in the atmosphere. Additionally, the polar regions are characterized by vast ice sheets and frozen surfaces, which limit the release of CO^2 from the ground.

The pH level in the polar environment varies depending on the specific location and ecosystem. In general, the pH levels in polar soils and water bodies tend to be lower or more acidic. This is often attributed to factors such as the presence of organic acids, decomposition of organic matter, and weathering processes in the polar regions. However, it's important to note that pH levels can still vary within different microhabitats and ecosystems within the polar environment(Thomas, 2019).

In polar regions, the amount of precipitation is typically limited, and the primary form of moisture is in the form of snowfall. The cold temperatures prevalent in these areas restrict the evaporation process, leading to an overall arid environment. However, over time, the continuous accumulation of snow plays a crucial role in the formation of expansive ice sheets and glaciers that are characteristic of polar regions. This accumulation process contributes to the unique landscape and geological features found in these areas.

The polar environment is characterized by extremely low temperatures, relatively low carbon dioxide levels, and varying pH levels. These factors contribute to the unique and challenging conditions that organisms face in these regions, shaping their adaptations and survival strategies.

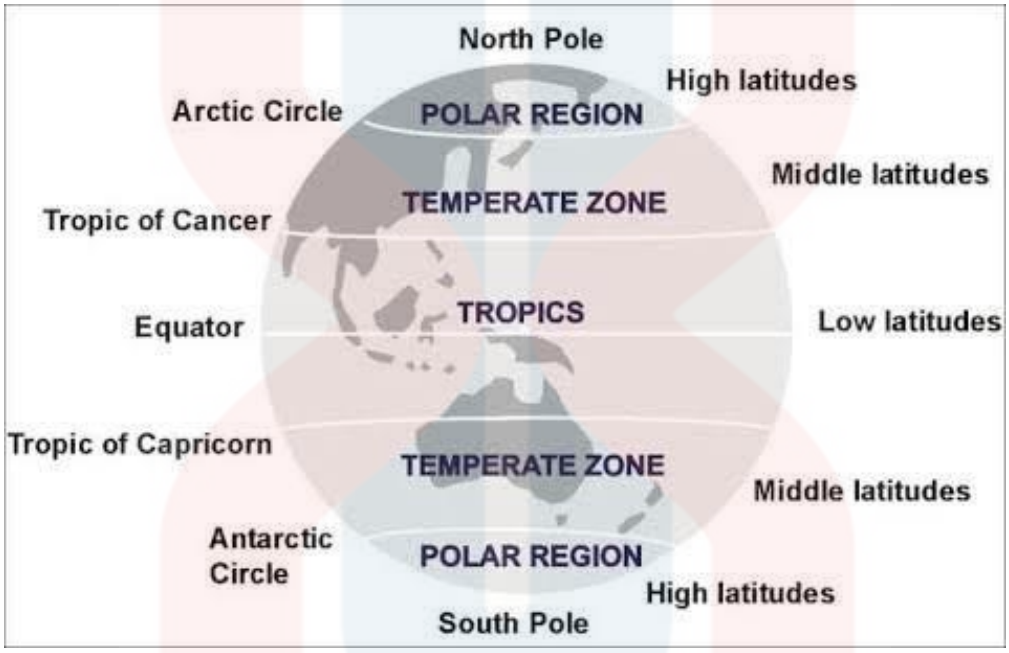


Figure 2.3: Polar Region on Earth

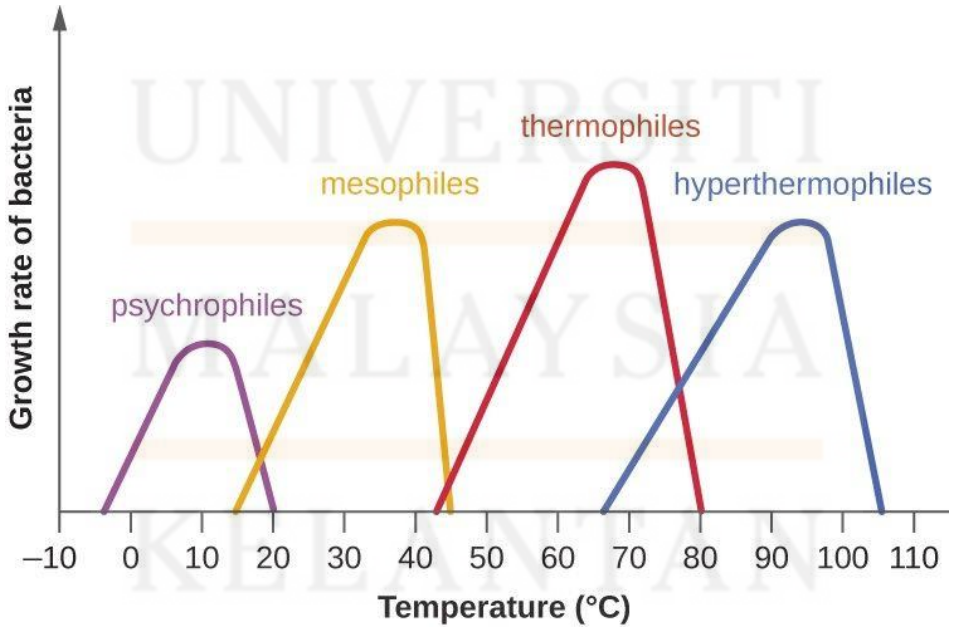


Figure 2.4: Name of the Microbes according to temperature

2.4 Luria Bertani (LB) Agar

Luria Bertani (LB) agar is a commonly used growth medium in microbiology for the cultivation and maintenance of bacteria. The LB agar medium consists of several components, including tryptone, yeast extract, and sodium chloride. Tryptone and yeast extract serve as sources of amino acids, peptides, vitamins, and minerals, providing the necessary nutrients for bacterial growth (MacWilliams & Liao, 2006). Agar, a polysaccharide derived from seaweed, is added to solidify the medium, allowing the bacteria to grow as visible colonies.

2.5 Aniline Blue

Aniline Blue is a cationic dye that can be used to stain and identify β-glucans, which are polysaccharides composed of glucose units linked by β-glycosidic bonds. Aniline Blue specifically binds to the β-glucan structure, allowing for its visualization. They do provide blue stains on the bacteria colonies and show the β-glucan producing bacteria strain(Wood & Fulcher, 1984).

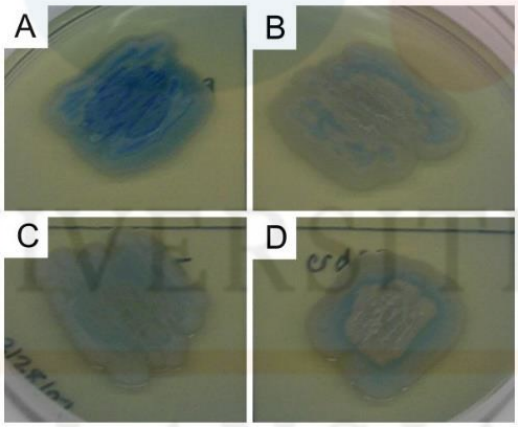


Figure 2.5: Production of blue strain to show the presence of β-glucan

2.6 Biochemical Test

2.6.1 Catalase Test

The nitrate reduction test is performed to determine if bacteria can reduce nitrate (NO3-) to nitrite (NO2-) or further to other nitrogenous compounds. The test involves adding a reagent containing sulfanilic acid and alpha-naphthylamine to the bacterial culture after incubation with nitrate. If nitrate has been reduced to nitrite, a colour change occurs, turning the medium red. Additional tests can be performed to distinguish between different nitrogenous end products.

2.6.2 Oxidase Test

The oxidase test is a biochemical test used to determine the presence of the enzyme cytochrome c oxidase in bacteria. The test involves the addition of an oxidase reagent, such as tetramethyl-p-phenylenediamine dihydrochloride, to a bacterial culture. If the bacteria possess cytochrome c oxidase, the enzyme will catalyze the oxidation of the oxidase reagent, resulting in a color change, typically to dark purple or blue. This color change indicates a positive oxidase reaction, suggesting the presence of cytochrome c oxidase in the bacteria. The oxidase test is a simple and rapid method that aids in bacterial identification and classification, particularly in differentiating between oxidase-positive and oxidase-negative bacteria.

2.6.3 Starch Test

The starch test is a simple microbiological technique used to determine whether bacteria produce the enzyme amylase, which breaks down starch. In this test, a sample of bacteria is streaked onto a solid agar medium containing starch. After incubation, the agar is flooded with iodine solution. If the bacteria produce amylase, they will hydrolyse the starch in the agar, creating a clear zone around the bacterial growth where the iodine-starch complex is disrupted. This results in a visible colour change from dark blue or black to clear in the zone where starch has been broken down. On the other hand, if the bacteria do not produce amylase, there will be no change, and the agar surrounding the bacterial growth will remain dark. The starch test is commonly used in

microbiology to differentiate bacterial species based on their ability to utilize starch as a carbon source.

2.7 DNA extraction

The genetic material, DNA, serves as a fundamental repository of information encoding the specific traits and functionalities of the bacteria. Isolating DNA allows researchers to delve into the genetic composition of the strains, providing insights into the presence and organization of genes. Through techniques such as PCR and DNA sequencing, one can unravel the specific genetic markers and pathways linked to β-glucan production. This genetic information not only aids in confirming the identity of the strains but also facilitates a comprehensive understanding of the underlying mechanisms involved. Additionally, DNA extraction sets the stage for various downstream analyses, including enzymatic profiling, taxonomic identification, and structural characterization.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

In the laboratory, a range of equipment and materials was employed for various experimental procedures. Storage and incubation were facilitated by a chiller set at 4ºC, a cold room operating between 4ºC and 15ºC, a -20ºC freezer, a 30ºC incubator, and a 37ºC incubator. The culture media included LB agar and starch agar for bacterial growth, with supplementation involving Aniline Blue and sucrose. LB broth served as a liquid medium for bacterial cultivation. Staining procedures utilized crystal violet, iodine, acetone, and safranin. Additionally, hydrogen peroxide was employed for catalase tests, and tetra-methyl-p-phenylenediamine dihydrochloride aided in oxidase tests. DNA extraction was conducted using the PrimeWay Genomic Kit, with supporting materials such as EDTA, absolute ethanol, and deionized water. For gel electrophoresis, TAE buffer and agarose gel were utilized, and visualization was achieved using FloroSafe. Also the hindll marker to study the size of the DNA fragments.

3.2 Methods

The objective of this project is to isolate and characterize bacteria strains capable of producing β-glucan from Antarctic samples. The methodology involves a series of systematic steps to achieve this goal. Initially, a serial dilution technique is employed to dilute the bacterial content from the samples, followed by the spread plate method for even distribution and isolation of individual bacterial colonies. The isolates are then screened for β-glucan production, and the selected strains undergo further characterization through Gram staining and a series of biochemical tests, including the oxidase and catalase tests. The capability of the strains to hydrolyze starch is assessed through the starch test. Culturing techniques are employed to maintain and propagate the isolated strains. Finally, DNA extraction is performed to enable molecular analysis and identification of the β-glucan-producing bacteria. This comprehensive methodology aims to not only isolate these specialized bacteria from the unique Antarctic environment but also to characterize their key biochemical and genetic traits for potential applications in various fields.

3.2.1 Soil Sampling

Soil sample was collected from Antarctica. The collected sample were kept in freezer -20˚C.

3.2.2 Serial Dilution

1g of soil sample was mixed with 9ml saline water. Then serially diluted as shown in Figure 3.1. 1ml of stock sample suspension was transferred to 9ml of saline water and the similar step was repeated for the rest. 0.1ml of saline water with dilution factors of 10^{-1}, 10^{-2},10^{-3},10^{-4}, 10^{-5} & 10^{-6} were spread evenly on LB agar plates (10g/l Peptone, 10g/l NaCl, 5g/l Yeast Extract, 16g/l Agar Powder) and incubated at 9°C until single colonies emerged on the plates.

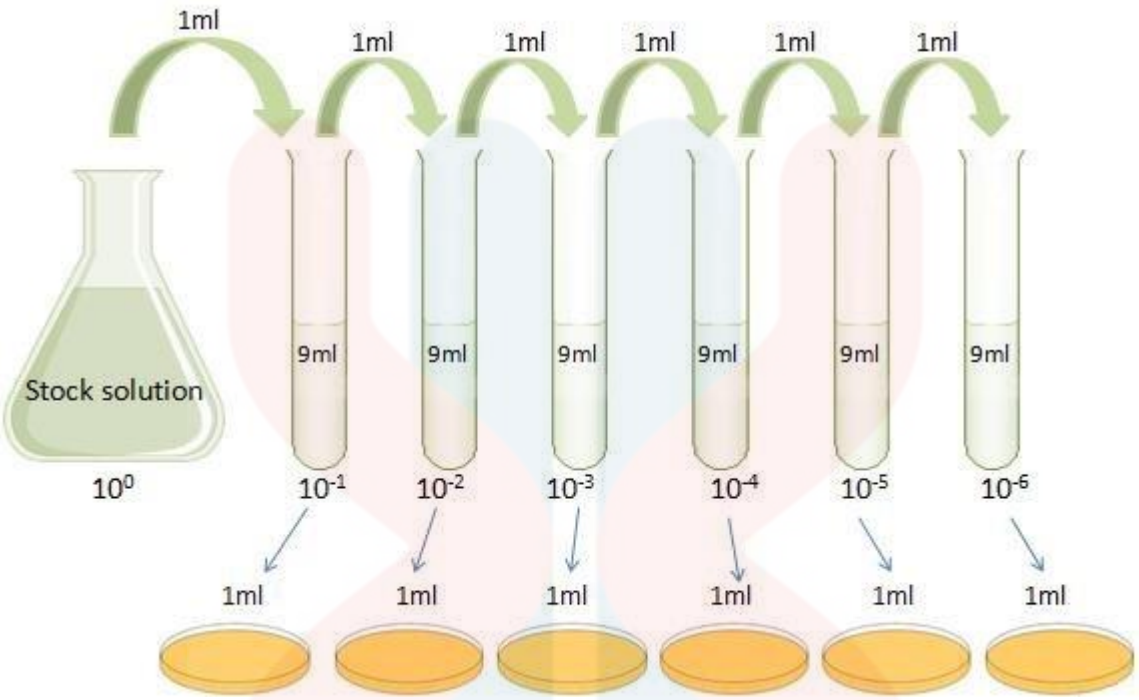


Figure 3.1: Serial Dilution and Spread Plate

3.2.3 Screening/Isolation

All colonies were streaked on separated LB Agar which was supplemented with 0.005% (w/v) Aniline Blue and 5% sucrose. Excessive EPS production strain, *Agrobacterium sp* ATCC 31749 was used as positive control while *Shewanella oneidensis* MR-1 strain was used as negative control. The bacteria colonies were streaked on different plates as shown in Figure 3.2 and the blue stained colonies were screened as shown in figure 3.3.

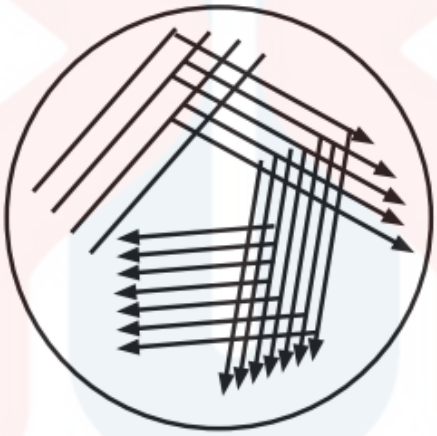


Figure 3.2: Streaked Plate

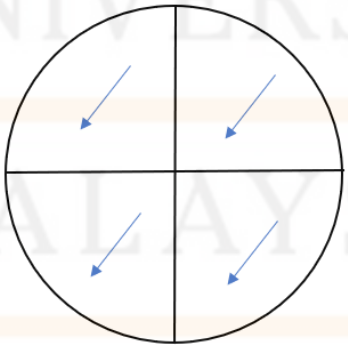


Figure 3.3: Screened Plate

3.2.4 Gram Staining

The gram-reaction of bacterial colonies was determined by using staining procedures. A thinly spread bacterial colonies were smeared on a clean slide, dried in air and heat to be fixed. Then, the slide was flooded of dried smear with crystal violet solution for 30 seconds and washed with distilled water for few seconds. The slide was flooded with iodine solution again for 30 seconds and washed with distilled water and dried. It was decolourized with acetone by applying drop by drop method until there are no more colour liquid flows from the smear and washed and dried. Finally, the slide was counter stained for about 10 seconds with safranin, washed and examined under microscope by using oil immersion objective.

3.2.5 Biochemical Test

a. Catalase Test

   Small loop of bacterial colonies from bacterial culture plates was transferred to clean microscopic slides by using inoculating loop. The production of catalase was determined by adding few drops of 3% H_2O2 appropriately to the cells on microscopic

b. Oxidase Test

   Small loop of bacterial colonies were rubbed on a filter paper that already immersed with oxidase reagent, aqueous Kovacs reagent (N, N, N, N-tetramethyl-p-Phenylenediamine dihydrochloride) solution. The colour changes determined the species of oxidase-positive and oxidative-negative results.

c. Starch Test

   A small loop of bacterial colonies is typically streaked onto a solid agar medium supplemented with starch as shown in Figure 3.4. After incubation, the medium is flooded with iodine solution. The iodine reacts with starch to form a dark blue or black colour complex. The presence or absence of this colour change is used to determine the ability of the bacteria to hydrolyse or break down starch.

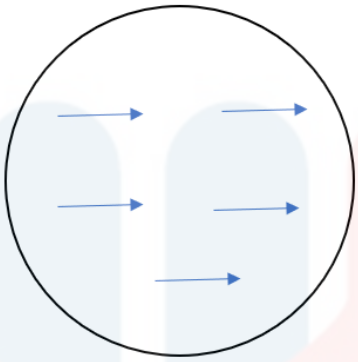


Figure 3.4: Starch Test Streaking

3.2.6 Culturing

15 mL of LB broth were autoclaved before use together with magnetic stir bar. Inoculated the broth with a single bacterial colony. The conical flask, containing the broth and bacterial culture, was then placed on the magnetic stirrer. This setup facilitated continuous and uniform agitation of the culture for a duration of three days in cold room. The controlled environmental conditions and stirring mechanism aimed to promote the growth and proliferation of the bacterial culture, allowing for an efficient cultivation process. This method is commonly employed in microbiological laboratories to ensure consistent and reproducible results in bacterial culture development.

3.2.7 DNA Extraction

The PrimeWay Genomic DNA Extraction Kit were used to extract the DNA. Based on the kit Protocol the chemicals were prepared and steps are followed.

3.2.8 Gel Electrophoresis

1% (w/v) agarose gel was prepared by mixing 0.3g of agarose powder in 39.5mL of 1X TAE buffer and microwaved for 1 minutes until the agarose was completely dissolved. The agarose

solution was swirled to cool down the temperature to about 50°C and 1µL flurosafe was added to the solution. The agarose was poured into a tray with well comb in place and cooled down to temperature for 20-30 minutes for solidification. The solidified gel is filled into gel box with 1X TAE buffer completely covered the gel.

1µL of loading dye was added to 5µL of deionized water and was loaded into first lane of the gel carefully. 1µL of loading dye was added to 5µL of sample and loaded into the subsequent wells of the gel. The gel was then run at 80 V for 45 minutes. After that, electrodes was disconnected from power source and the gel was removed from the gel and observed under ultraviolet light.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Isolation of β-glucan

After the serial dilution three (i. ii, iii) plates were spread from each and every factor 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. After 1 week, there was 20 different colonies were observed and isolated as shown in table 4.1. The observation of 20 distinct colonies after the initial serial dilution and subsequent plating suggests a diverse microbial population in the original sample. The serial dilution technique allowed for the isolation and enumeration of viable colonies at various dilution factors, providing a representative sampling of the microbial community.

Those 20 colonies were streaked on separated LB agar which supplemented with 0.005% Aniline Blue and 5% Sucrose. Previous studies revealed that aniline blue stained specifically with β-(1,3)-glucan (Keller, M., Muller, P., Simon, R., & Puhler, A. (1988)). PI18 and MR1 was streaked on the LB Agar before the streaking to confirm the production of Blue Stains (Chee, M.T (2016)). The higher amount of β-(1,3)-glucan present, the deeper the blue colour intensity accumulated.

The blue staining observed in the colonies after three days on this specialized medium indicates the presence of β-glucan. The appearance of blue-stained colonies provides valuable information about the microbial community's composition and highlights the potential presence of bacteria with distinctive biochemical properties.

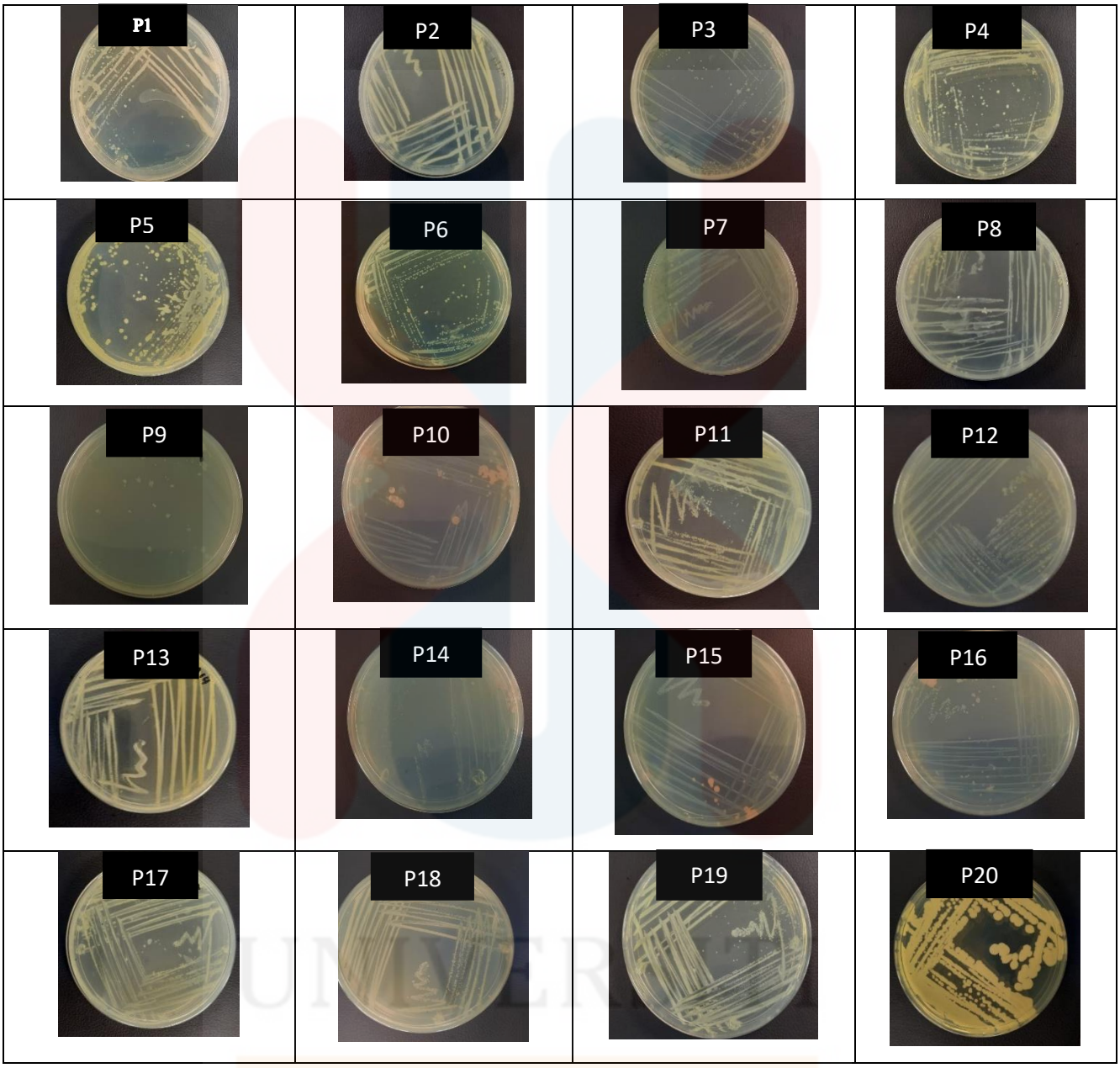


Table 4.1: Isolated ColoniesTable

4.2 Screened Colonies

Among the 20 single colonies there was 5 colonies were blue stained. Those stains were screened as shown in figure 4.1.

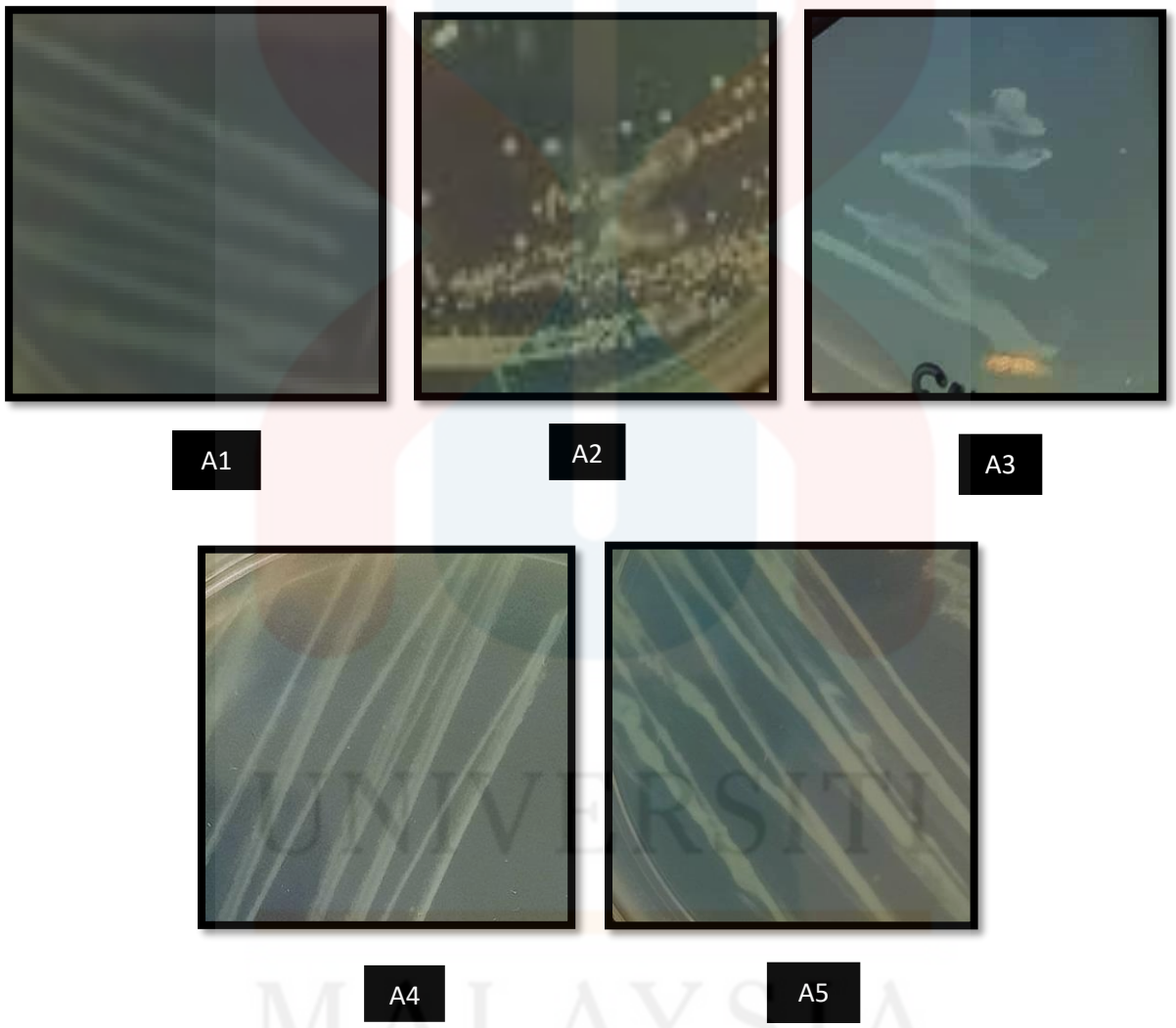
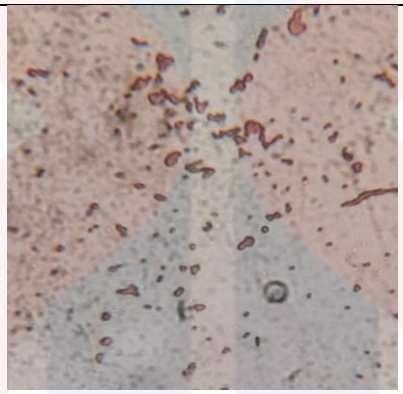
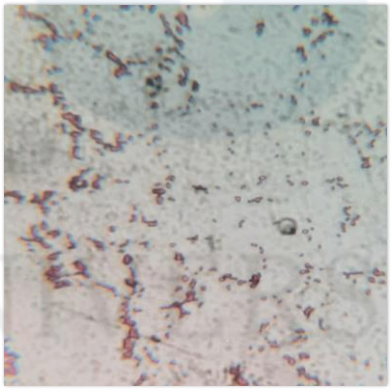
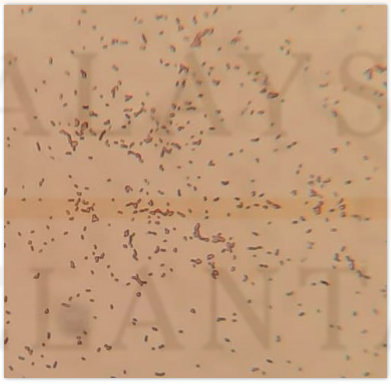


Figure 4.1 Screened Blue Stained Colonies

4.3 Gram Staining

All the 5 stains were characterized via gram staining and biochemical test. A1, A2, A4 and A5 were identified as gram negative as they appeared in red colour. Meanwhile A3 were identified as gram positive where they appeared in purple colour (Table 4.2).

Isolates	Microscope view	Observation
A1		Gram Negative
A2		Gram Negative
A3		Gram Positive

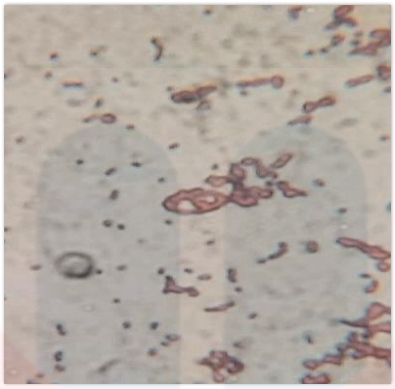
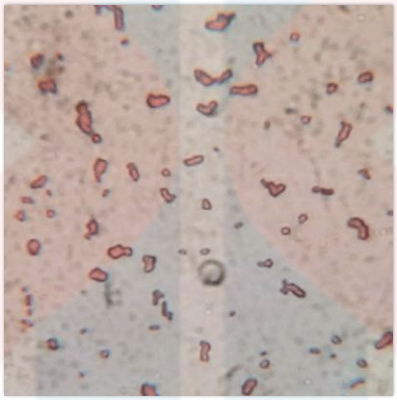
A4		Gram Negative
A5		Gram Negative

Table 4.2: Gram Staining

4.4 Biochemical Test

a. Catalase Test

The A1, A2, and A3 exhibited a negative catalase reaction, indicating the absence of catalase activity. Conversely, A4 and A5 demonstrated a positive catalase reaction, producing bubbles upon the addition of hydrogen peroxide. The presence or absence of catalase can be linked to the bacteria's metabolic and respiratory characteristics. Bacteria with catalase activity can efficiently eliminate toxic hydrogen peroxide, whereas those without catalase may employ alternative mechanisms for peroxide detoxification. These catalase test results provide valuable information about the biochemical profile of the bacterial isolates and contribute to their identification and classification in microbiological studies.

Isolate	Image	Result
A1		Negative
A2		Negative

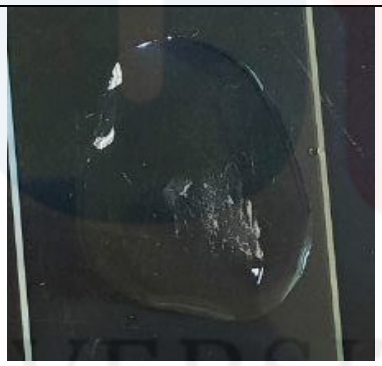
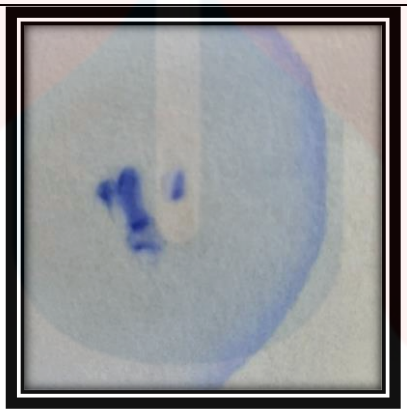
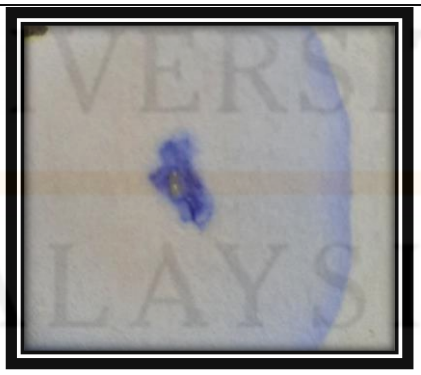
A3		Negative
A4		Positive
A5		Positive

Table 4.3: Catalase Test Result

b. Oxidase Test

All isolates A1 to A5 exhibited positive oxidase reactions. A positive oxidase test for bacterial isolates A1 to A5 indicates the presence of the enzyme oxidase in these strains. The oxidase test is designed to detect the presence of cytochrome c oxidase, an enzyme involved in the electron transport chain. When an oxidase reagent is applied to a bacterial colony, a positive reaction is observed if the bacteria contain cytochrome c oxidase, leading to a colour change.

Isolates	**Image**	**Result**
A1		Positive
A2		Positive

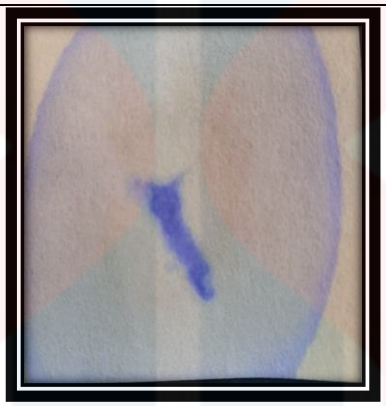
A3		Positive
A4		Positive
A5		Positive

Table 4.4: Oxidase Test Result

c. Starch Test

The starch test detect the presence of amylase, an enzyme that breaks down starch into simpler sugars. In the context of characterizing β-glucan-producing strains, the starch test serves as a negative control. Since β-glucans and starch are both polysaccharides, the absence of starch hydrolysis (negative result as in figure 4,2) indicates that the strains are not producing significant levels of amylase. This is important because it suggests that any β-glucan production observed is not due to contamination by organisms capable of breaking down starch into simpler sugars, which could potentially interfere with the interpretation of β-glucan production.

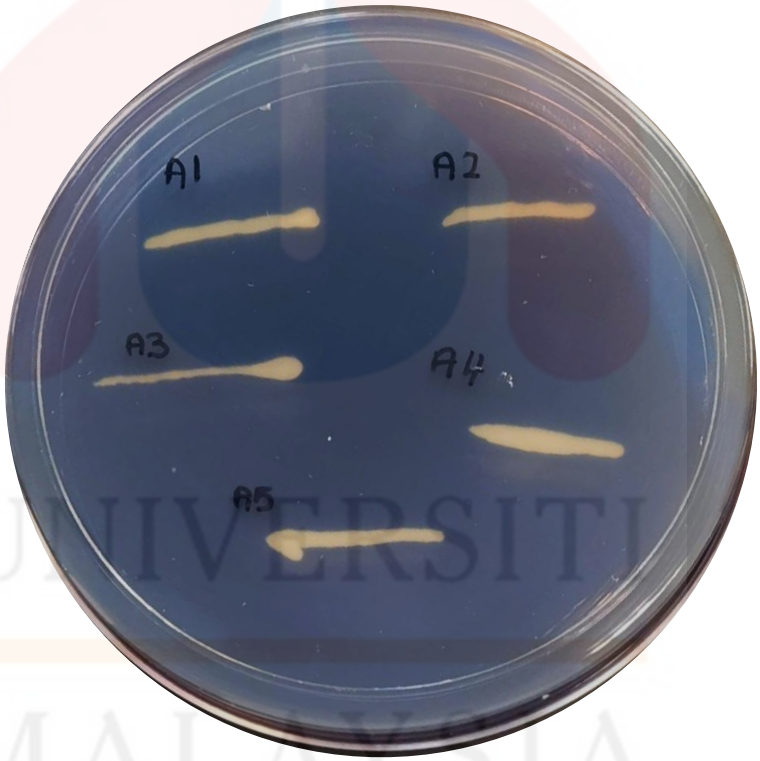


Figure 4.2: Starch Test Result

Isolates	Result
A1	Negative
A2	Negative
A3	Negative
A4	Negative
A5	Negative

Table 4.5: Starch Test Result

The table 4.6 collectively provide insight into the biochemical characteristics and classification of the bacterial isolates. The Gram staining helps differentiate bacteria based on their cell wall structure, while the catalase and oxidase tests provide information about metabolic pathways and enzyme production. The starch test indicates the ability of the isolates to hydrolyse starch, and proven it is not contaminated. Table 4.6 also provides information that all 5 colonies are different in some way where they do have different characterizations.

Isolates	Gram Staining	Catalase Test	Oxidase Test	Starch Test
A1	-	-	+	-
A2	-	-	+	-
A3	+	-	+	-
A4	-	+	+	-
A5	-	+	+	-

Table 4.6: Gram Staining, and Biochemical Test Result for A1 to A5

4.5 Gel Electrophoresis

The bacterial isolates from Antarctica were cultured for DNA extraction. The DNA extraction was carried out following the protocol PrimeWay Genomic Kit. However, due to time constraints, the gel electrophoresis process was interrupted after a 30-minute run instead of the planned 45 minutes. Consequently, the DNA fragments appeared darker on the gel, indicative of their presence, but the incomplete run prevented accurate determination of fragment sizes. The interrupted electrophoresis hinders the precise characterization of DNA fragments, as their migration patterns were not fully realized within the gel matrix. Despite this limitation, the visible presence of DNA fragments implies a successful extraction process. While the incomplete run precludes the determination of specific fragment sizes, the qualitative presence of DNA can still offer insights into the genomic content of the isolated bacteria.

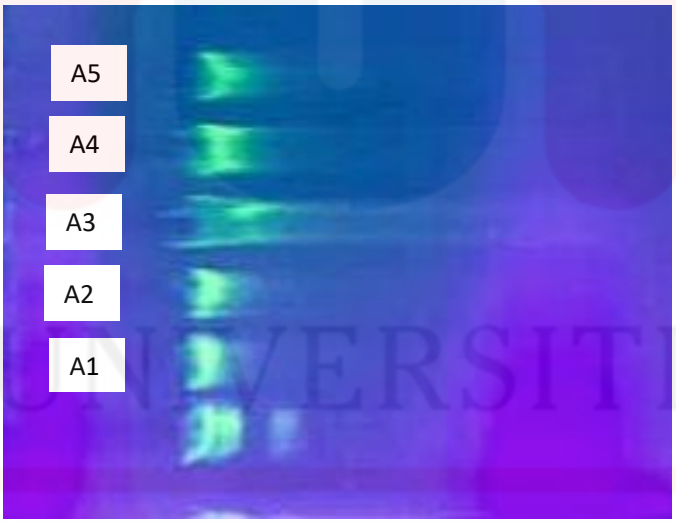


Figure 4.3: Gel Electrophoresis Rest after DNA Extraction

4.6 16s RNA Sequencing

The BLAST results exhibit diverse similarity percentages, ranging from 70.30% to 96.89%, indicating various levels of genetic relatedness between the isolated strains and known bacterial species. Notably, strains A1, A2, and A3 share a high similarity percentage of 89.71%, primarily with Klebsiella pneumoniae, Klebsiella oxytoca, and Escherichia coli strains, suggesting a close genetic relationship within these genera. On the other hand, strains A4 display a significantly higher similarity percentage of 96.75% to 96.89% with species from the genera Lelliottia, Enterobacter, and Citrobacter, indicating a distinct genetic relatedness. Additionally, strain A5 exhibits a similarity percentage of 70.30% to 70.44% with Citrobacter freundii strain CF09 and various Raoultella sp. enrichment culture clones, indicating a separate genetic lineage. These findings highlight the presence of diverse bacterial species within the analyzed samples, emphasizing the importance of further molecular analysis to elucidate their taxonomic classification and genetic diversity.

The evidence from the 16S rRNA sequence results aligns with previous studies, reinforcing the notion that the identified strains are indeed capable of producing β-glucan. For instance, Klebsiella aerogenes strain ZJB-17003, which exhibited a high similarity percentage of 92.07%, has been documented by (Jawad et al., 2023) as a β-glucan producer. Similarly, the strains showing similarity to Citrobacter freundii strain CF09 and Raoultella sp. enrichment culture clones (70.30% to 70.44%) have been reported by (Mielecki et al., 2024) to possess β-glucan production capabilities. This alignment between our findings and previous research adds credibility to the notion that these strains harbor the necessary genetic machinery for β-glucan synthesis. Moreover, it underscores the potential biotechnological significance of these strains, as β-glucans are valued for their various industrial and health-related applications. Further experimental validation, such as β-glucan quantification assays and gene expression studies, would provide conclusive evidence regarding the β-glucan production abilities of these strains, thus advancing our understanding of their biotechnological potential.

A1

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Lelliottia amnigena strain JZY1-8 16S ribosomal RNA gene, partial sequence	Lelliottia am...	1376	1376	49%	0.0	94.91%	1184	MT071359.1
Enterobacter sp. strain MBWS10.(5) 16S ribosomal RNA gene, partial sequence	Enterobacte...	1376	1376	49%	0.0	94.91%	1245	OP990448.1
Erwinia sp. CBU054A 16S ribosomal RNA gene, partial sequence	Erwinia sp. ...	1376	1376	49%	0.0	94.91%	1399	KM021304.1
Enterobacter sp. RA-15 16S ribosomal RNA gene, partial sequence	Enterobacte...	1376	1376	49%	0.0	94.91%	1420	KJ152098.1
Bacterium strain AGE_YJ_G87 16S ribosomal RNA gene, partial sequence	bacterium	1376	1376	49%	0.0	94.91%	1457	MW037659.1
Enterobacter sp. strain M321 16S ribosomal RNA gene, partial sequence	Enterobacte...	1371	1371	49%	0.0	94.80%	1403	MH669187.1
Lelliottia aquatilis strain JZY1-2 16S ribosomal RNA gene, partial sequence	Lelliottia aq...	1371	1371	49%	0.0	94.80%	1434	MT071355.1
Enterobacter sp. strain IAUK3010 16S ribosomal RNA gene, partial sequence	Enterobacte...	1371	1371	49%	0.0	94.80%	1412	MK968094.1
Enterobacter sp. strain IAUK3009 16S ribosomal RNA gene, partial sequence	Enterobacte...	1371	1371	49%	0.0	94.80%	1427	MK968093.1
Enterobacter sp. strain IAUK3003 16S ribosomal RNA gene, partial sequence	Enterobacte...	1371	1371	49%	0.0	94.80%	1421	MK968091.1

A2

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Klebsiella aerogenes strain ZJB-17003 16S ribosomal RNA gene, partial sequence	Klebsiella aerog...	782	782	92%	0.0	92.07%	1394	MN198096.1
Citrobacter gillenii strain R2-55 16S ribosomal RNA gene, partial sequence	Citrobacter gillenii	782	782	92%	0.0	92.07%	974	MF111266.1
Bacterium strain HB64 16S ribosomal RNA gene, partial sequence	bacterium	782	782	92%	0.0	92.07%	1384	ON387051.1
Enterobacter sp. B200 16S ribosomal RNA gene, partial sequence	Enterobacter sp....	778	778	93%	0.0	91.52%	1424	JX680982.1
Enterobacter sp. strain M321 16S ribosomal RNA gene, partial sequence	Enterobacter sp.	776	776	92%	0.0	91.91%	1403	MH669187.1
Lelliottia amnigena strain JZY1-8 16S ribosomal RNA gene, partial sequence	Lelliottia amnigena	776	776	92%	0.0	91.91%	1184	MT071359.1
Lelliottia aquatilis strain JZY1-2 16S ribosomal RNA gene, partial sequence	Lelliottia aquatilis	776	776	92%	0.0	91.91%	1434	MT071355.1
Enterobacter sp. strain IAUK3010 16S ribosomal RNA gene, partial sequence	Enterobacter sp.	776	776	92%	0.0	91.91%	1412	MK968094.1
Enterobacter sp. strain IAUK3009 16S ribosomal RNA gene, partial sequence	Enterobacter sp.	776	776	92%	0.0	91.91%	1427	MK968093.1
Enterobacter sp. strain IAUK3003 16S ribosomal RNA gene, partial sequence	Enterobacter sp.	776	776	92%	0.0	91.91%	1421	MK968091.1

A3

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Klebsiella pneumoniae wild strain P20 blaLAT gene for CMY-2 family cephalosporin-hydrolyzing class C beta-l...	Klebsiella pneu...	520	520	90%	2e-142	89.71%	1346	NG_049265.1
Klebsiella pneumoniae blaCMY gene for class C beta-lactamase CMY-31, complete CDS	Klebsiella pneu...	520	520	90%	2e-142	89.71%	1146	NG_048826.1
Klebsiella oxytoca NMI4204_10 unnamed blaCMY gene for class C beta-lactamase CMY-177, complete CDS	Klebsiella oxytoca	520	520	90%	2e-142	89.71%	1146	NG_078046.1
Klebsiella oxytoca strain NMI4204_10 class C beta-lactamase CMY-177 (blaCMY) gene, blaCMY-177 allele, c...	Klebsiella oxytoca	520	520	90%	2e-142	89.71%	1146	OK217282.1
Salmonella enterica subsp. enterica serovar Newport strain AM17274 plasmid pA172, complete sequence	Salmonella ente...	520	520	90%	2e-142	89.71%	8197	EU331425.1
Klebsiella pneumoniae class C beta-lactamase CMY-31 (blaCMY-31) gene, complete cds	Klebsiella pneu...	520	520	90%	2e-142	89.71%	1146	EF622224.1
Escherichia coli plasmid pMEL2 DNA for bla LAT-4 gene	Escherichia coli	520	520	90%	2e-142	89.71%	1159	Y15412.1
K.pneumoniae LAT-1 Beta-lactamase plasmid gene	Klebsiella pneu...	520	520	90%	2e-142	89.71%	1395	X78117.1
Proteus mirabilis strain HN2p chromosome HN2p, complete sequence	Proteus mirabilis	514	514	90%	9e-141	89.46%	4172466	CP046048.1
Escherichia coli F070 DNA, complete genome	Escherichia coli	514	514	90%	9e-141	89.46%	4873850	AP023237.1

A4

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Citrobacter gillenii strain S2-243 16S ribosomal RNA gene, partial sequence	Citrobacter gillenii	1181	1181	97%	0.0	96.89%	982	MF111854.1
Lelliottia amnigena strain JZY1-8 16S ribosomal RNA gene, partial sequence	Lelliottia amnigena	1173	1173	97%	0.0	96.75%	1184	MT071359.1
Enterobacter sp. strain IAUK3010 16S ribosomal RNA gene, partial sequence	Enterobacter sp.	1173	1173	96%	0.0	96.88%	1412	MK968094.1
Enterobacter sp. strain IAUK3009 16S ribosomal RNA gene, partial sequence	Enterobacter sp.	1173	1173	97%	0.0	96.75%	1427	MK968093.1
Lelliottia sp. WB101 chromosome, complete genome	Lelliottia sp. WB101	1173	9273	97%	0.0	96.75%	4607442	CP028520.1
Lelliottia aquatilis strain 9827-07 16S ribosomal RNA gene, partial sequence	Lelliottia aquatilis	1173	1173	97%	0.0	96.75%	1536	MG916974.1
Lelliottia aquatilis strain 6331-17 16S ribosomal RNA gene, partial sequence	Lelliottia aquatilis	1173	1173	97%	0.0	96.75%	1536	MG916969.1
Lelliottia jeotgali strain PFL01 chromosome, complete genome	Lelliottia jeotgali	1173	9378	97%	0.0	96.75%	4603334	CP018628.1
Citrobacter gillenii strain S2-270 16S ribosomal RNA gene, partial sequence	Citrobacter gillenii	1173	1173	97%	0.0	96.75%	959	MF111880.1
Citrobacter gillenii strain S2-200 16S ribosomal RNA gene, partial sequence	Citrobacter gillenii	1173	1173	97%	0.0	96.75%	942	MF111813.1

A5

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Citrobacter freundii strain CF09 16S ribosomal RNA gene, partial sequence	Citrobacter freundii	68.0	68.0	38%	2e-06	70.30%	1346	PP264343.1
Uncultured bacterium clone H78 16S ribosomal RNA gene, partial sequence	uncultured bacterium	66.2	66.2	36%	7e-06	70.44%	731	KU241280.1
Raoultella sp. enrichment culture clone TB44_10 16S ribosomal RNA gene, partial sequence	Raoultella sp. enric...	66.2	66.2	36%	7e-06	70.44%	1500	JX403617.1
Raoultella sp. enrichment culture clone TB44_9 16S ribosomal RNA gene, partial sequence	Raoultella sp. enric...	66.2	66.2	36%	7e-06	70.44%	1500	JX403616.1
Raoultella sp. enrichment culture clone TB44_8 16S ribosomal RNA gene, partial sequence	Raoultella sp. enric...	66.2	66.2	36%	7e-06	70.44%	1501	JX403615.1
Raoultella sp. enrichment culture clone TB44_7 16S ribosomal RNA gene, partial sequence	Raoultella sp. enric...	66.2	66.2	36%	7e-06	70.44%	1502	JX403614.1
Raoultella sp. enrichment culture clone TB44_6 16S ribosomal RNA gene, partial sequence	Raoultella sp. enric...	66.2	66.2	36%	7e-06	70.44%	1501	JX403613.1
Raoultella sp. enrichment culture clone TB44_5 16S ribosomal RNA gene, partial sequence	Raoultella sp. enric...	66.2	66.2	36%	7e-06	70.44%	1503	JX403612.1
Raoultella sp. enrichment culture clone TB44_4 16S ribosomal RNA gene, partial sequence	Raoultella sp. enric...	66.2	66.2	36%	7e-06	70.44%	1503	JX403611.1
Raoultella sp. enrichment culture clone TB44_3 16S ribosomal RNA gene, partial sequence	Raoultella sp. enric...	66.2	66.2	36%	7e-06	70.44%	1502	JX403610.1

Table 4.7 16s rRNA Result

A1: Klebsiella aerogenes strain ZJB-17003 (Klebsiella aerogenes) - 92.07%

A2: Various Klebsiella, Klebsiella oxytoca, and Escherichia coli strains - 89.71%

A3: Various Klebsiella, Klebsiella oxytoca, and Escherichia coli strains - 89.71%

A4: Various Lelliottia, Enterobacter, and Citrobacter strains - 96.75% to 96.89%

A5: Citrobacter freundii strain CF09 and Raoultella sp. enrichment culture clones - 70.30% to 70.44%

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Five β-glucan producing bacteria stains were isolated successfully as they were streaked on LB agar supplemented with Aniline Blue and Sucrose. The presence of β-glucan has been confirmed through the observation of blue-stained colonies. It indicates β-(1,3)-glucan production. The Antarctica bacteria were grown well in the chiller at 4˚C. The isolate was characterized. They were further characterized through Gram staining and biochemical tests. Despite encountering a time constraint during gel electrophoresis after DNA extraction, the process yielded visible DNA fragments. The incomplete run hindered precise fragment size determination, but the qualitative presence of DNA suggests a successful extraction.

5.2 Recommendations

In order to advance our knowledge of Antarctic microbial diversity and harness the biotechnological potential of β-glucans, future studies should prioritize several key areas of research. Firstly, comprehensive genetic characterization through techniques like whole-genome sequencing or targeted gene sequencing can provide invaluable insights into the genetic makeup of isolated Antarctic bacteria. By elucidating the genetic pathways involved in β-glucan production, researchers can identify potential genetic markers essential for strain identification and manipulation, thus paving the way for tailored biotechnological applications and enhanced understanding of microbial diversity in extreme environments. Additionally, optimizing cultivation conditions to maximize β-glucan production represents a crucial avenue for exploration. By systematically exploring different growth parameters such as temperature, pH, and nutrient availability, as well as investigating the effects of various carbon sources and supplements, researchers can fine-tune cultivation strategies to achieve optimal β-glucan yield and quality.

Furthermore, future studies should delve into the biotechnological applications of β-glucans produced by isolated Antarctic bacteria. Evaluating their diverse bioactive properties, including antioxidant, antimicrobial, immunomodulatory, and other functionalities, can unlock their potential for applications in food, pharmaceutical, cosmetic, and agricultural industries. Moreover, investigating the ecological significance of β-glucan-producing bacteria in Antarctic ecosystems is essential for understanding their roles in nutrient cycling, ecosystem resilience, and interactions with other organisms. Utilizing β-glucan production as a biomarker for environmental monitoring in polar regions can provide valuable insights into the impacts of climate change, pollution, and other stressors on microbial communities and associated ecosystems. By addressing these recommendations, future research endeavors can significantly contribute to our understanding of Antarctic microbial diversity and the multifaceted applications of β-glucans in various fields, propelling advancements in biotechnology and pharmaceuticals.

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