Indonesia International Institute for Life Sciences

# ENRICHMENT PROGRAM REPORT

# PRODUCTION AND CHARACTERIZATION OF BIOCELLULOSE AND KERATIN AS DIABETIC WOUND DRESSING CANDIDATE COMBINED WITH TAMANU OIL

STUDY PROGRAM
**Biotechnology**

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INDONESIA INTERNATIONAL INSTITUTE FOR LIFE SCIENCES (i3L)
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*< Title Page for School of Life Sciences>*

**ENRICHMENT PROGRAM REPORT**

# Production and Characterization of Biocellulose and Keratin as a Diabetic Wound Dressing Candidate Combined with Tamanu Oil

By

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Submitted to

i3L – Indonesia International Institute for Life Sciences

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Biotechnology

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Jakarta, Indonesia

2022

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# CERTIFICATE OF APPROVAL

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Student : Ignasius Edwin Ciputra

Cohort 2019

Title of final thesis project : Internship di Indonesia International Institute for life sciences (i3l).

Internship at Indonesia International Institute for life sciences (i3l)

We hereby declare that this final thesis project is from the student's own work. The final project/thesis has been read and presented to i3L's Examination Committee. The final project/thesis has been found to be satisfactory and accepted as part of the requirements needed to obtain an i3L bachelor's degree.

Names and signature of examination committee members present:

Jakarta, 13 January 2023

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# ABSTRACT

Skin wound healing is a biological course of events where a series of molecular and cellular events works together to restore the damaged tissue in four phases. Several factors could interfere with the completion of those phases, causing delayed and abnormal wounds which may lead to ulcers. Commonly, the formation of ulcers is associated with type II diabetes mellitus (T2DM) disorder which affects the closure of the wound. Current treatments for diabetic ulcers are available, and one of them is wound dressing. Biocellulose hydrogels derived from gram-negative bacteria such as *Komagateibacter intermedius* are usually used as they offer protection from foreign particles, are able to maintain a moist wound healing condition, and are biocompatible. Recent studies revealed that biocellulose is often combined with other materials such as keratin and *Calophyllum inophyllum* (Tamanu) oil to enhance the wound healing process. The purpose of this project is to produce and characterize the keratin extract and biocellulose as well as analyze the antibacterial effect of tamanu oil and human hair keratin. The biocellulose and keratin were extracted from the *K. intermedius* using MRS broth and human hair keratin using shindai method respectively, followed by the characterization of KBC hydrogels. An antimicrobial assay was performed through the Kirby Bauer method to determine the antibacterial activity of tamanu oil and keratin extract. A zone of inhibition area is observed in both pure green and black tamanu oil against *S. aureus* but not in *P. aeruginosa*. KBC exhibits a spectrum that is similar to the combination of the BC and keratin solution peaks in the FT-IR spectra, demonstrating the presence of keratin. The BC showed a high % swelling and low % erosion index while a high concentration of keratin was calculated using BCA assay, indicating the success of BC and keratin extraction.

***Keywords:*** *Biocellulose, tamanu oil, ulcer, wound dressing, Diabetes Mellitus, and keratin.*

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# LIST OF ABBREVIATIONS

T2DM : Type 2 Diabetes Mellitus

BSC : Biological Safety Cabinet

MH: Muller -Hilton

SDS-PAGE : Sodium dodecyl-sulfate polyacrylamide gel electrophoresis

IF : Intermediate filaments

MRS : deMam-Rogosa-Sharpe

PBS : Phosphate buffered saline

NA : Nutrient Agar

BCA : Bichinconic acid

BSA : Bovine serum albumin

KBC : Keratin-biocellulose

FT-IR : Fourier Transformed Infrared Spectroscopy

ANOVA : Analysis of Variance

# CHAPTER I: INTRODUCTION

## 1.1 Institution Description

Indonesia International Institute for Life Sciences (i3L) is a leading science institution that offers advanced education in life sciences. I3L was first founded in 2014 in Jakarta, Indonesia. For undergraduate studies, i3L currently offers two departments: the i3L School of Life Sciences and the School of Business (iSB), while the Master in Bio Management degree is the only graduate program available. The i3L School of Life Sciences offers Pharmacy, Bioinformatics, Food Science and Nutrition, Biomedicine, Food Technology, and Biomedicine. The iSB offers International Business Management, Business & Entrepreneurship in Life Sciences, Creative Digital Marketing, and International Applied Accounting. Through its vision to be a leading and globally-connected interdisciplinary institution that impacts society through science and innovation, i3L pursues to shape a well-rounded generation by providing an interdisciplinary, integrative, and unique learning environment with its high-quality faculty, and state-of-the-art facilities. In addition, strong international collaborations with academics, industries, and governments also allow i3l's students to have a chance to create networking and gain experiences.

## 1.2 Student's department

This internship project is done under the pharmacy study program at i3L. As one of the life sciences study programs, Pharmacy is an interdisciplinary education that focuses on drug studies, not only its benefit for the human body but also on how its manufactured and analyzed its economics and sustainability aspects.

## 1.3 Products of host institution

Beside scientists, one of the outcomes of the life sciences department is this opportunity to be a part of this project as a research intern in which the student is responsible for producing and characterizing keratin, biocellulose, and tamanu oil for diabetic wound healing.

## 1.4 Organizational Structure

i3L is structurally led by the rector of i3l and assisted by the first and second vice-rectors. The i3l consisted of two school programs: i3l school of life sciences and school of business. The life

sciences are led by the Dean of the school of life sciences and it has seven departments in which each of them are led by its own head of department. The school of business has two departments each also has its own head of each department.

# CHAPTER II: INTERNSHIP ACTIVITIES

The research internship at i3L began from 29th June until 1st November 2022. Throughout the internship program, the intern had a similar working schedule as the regular i3L employee. It is commonly seven-eight hours per day, weekdays, starting from 08.00 to 17.00.

During the internship period, the intern had various tasks to help in the in vivo research of keratin-tamanu-biocellulose for diabetic wound healing in mice and rabbits. These routine activities are the combination of the following activities:

a. Biocellulose production and extraction: the activity consists of subculturing bacteria, producing biocellulose, harvesting the biocellulose from the previous batch, neutralizing the harvested biocellulose, and performing the sterility testing.
b. Biocellulose characterization: the activity includes measuring the swelling and erosion capability of the biocellulose and performing Fourier Transform-Infrared Spectroscopy (FT-IR).
c. Keratin Extraction: the activity consists of extracting the keratin from human hair every two weeks
d. Keratin Evaluation: the activity includes measuring the concentration of all the batches of keratin extract, conducting SDS-PAGE and Fourier Transform-Infrared Spectroscopy (FT-IR), and also the antimicrobial activity of keratin.
e. Tamanu oil evaluation: the activity includes assessing the antimicrobial activity of black and green tamanu oil.

A biweekly meeting was conducted to present the intern's progress to the field supervisor, thus the intern could deliver the challenge he faced as well as gain comments and advice from the supervisor to improve his work quality.

In general, the knowledge and theories gained by the intern from over the three years of study in i3L has helped in conducting the project works during the internship. Although most of the lecture and laboratory were conducted online, it was still significantly beneficial to build a great foundation in understanding the principle of analysis and procedure used in these projects. The knowledge gained during the molecular biology as well as bioprocessing course has equipped the intern with a lot of basic knowledge that were helpful in conducting the harvestion and characterization during the internship. With all being said, the knowledge learned by the intern during their study at i3l has impacted a lot in completing the internship.

Throughout the internship period, the intern was faced with challenges in conducting the project works at first as the intern only had barely experience in operating laboratory types of

equipment. The workload is also sometimes a little bit overwhelming for the intern as, after a month of the internship period, two of the interns in the team left the project and thus the intern has to do the project mostly all by himself. Some contaminations in the biocellulose production and extraction were quite challenging as they caused the death of the bacteria used in this experiment which led to some delay in the production of the products. Another difficulty encountered is the result obtained is not as desired, therefore the intern has to re-conducted the work multiple times which is quite stressful.

# CHAPTER III: PROJECT DESCRIPTION

## 3.1 Project of Internship

### 3.1.1 Project background

The skin is defined as the human body's topmost layer and has a critical role in several mechanisms such as thermal regulation, protection from pathogens and chemicals, and also excretion (Tottoli et al., 2020). Physical trauma such as burns, pressure, cuts, or surgical incisions that cause skin damage therefore could be life-threatening. The human body has its unique mechanism of repairing an injured epidermal layer of the skin (Gonzalez et al., 2016). Wound healing is described as the natural biological process where a sequence of cellular, humoral and molecular events contribute together to restore the damaged tissue in four structural phases (Ozgok & Rengan, 2022). However, several factors can interfere with one or more of these phases, leading to delayed and abnormal wound healing. Such wounds may lead to ulcers that are frequently associated with type II diabetes Mellitus (T2DM) which is a chronic condition marked by insufficient insulin secretion by pancreatic beta-cells and improper tissue responsiveness to blood insulin. (Olokoba et al., 2012). The defects in those mechanisms in diabetic patients may lead to high levels of glucose blood level termed hyperglycemia. Hyperglycemia can cause vascular dysfunction and angiopathy, which leads to neuropathy (Baldassarro et al., 2022). In fact, about 80% of chronics wounds are linked with diabetes Mellitus and about 15% of T2DM patients develop Diabetic Foot Ulcers (DFU) in which 14-24% of those patients undergo a lower-limb amputation, with an increase in mortality rate to 50-59% after five years following the amputation (Okonkwo & DiPietro, 2017). As it is next to impossible to greatly reduce diabetes prevalence, a better innovation to treat diabetic foot ulcers should be developed.

Current treatment of diabetic foot ulcers comprises wound care with a number of dressings, wound off-loading, wound debridement, antibiotic treatment, and blood glucose control (Lim, Ng, & Thomas, 2017). Although topical treatment is often classified as secondary care over surgical and systemic care, wound dressing plays a crucial role in the management of diabetic foot ulcers as it is able to maintain moist wound healing conditions and protect the wound from foreign particles. A wide range of topical regimens is available for various diabetic wound ulcers including hydrogels, alginates, foams, hydrocolloids, etc (Kavitha, K. V, 2014). Among all the dressings, bio-cellulose derived from gram-negative bacteria such as *Komagateibacter intermedius* is preferable due to its properties such as being non-toxic, biocompatible, having high water content, and water retention ability (Baldassarro et al., 2022) . The use of biocellulose hydrogels is also considered to be more

painless compared with the conventional wound care as it does not cause any damage upon the removal of the dressing (Konop et al., 2021).

Biocellulose itself is a natural biopolymer produced by gram-negative bacteria by utilizing the presence of nitrogen and carbon sources in the medium of culture. In the hydrogel form, biocellulose will have a high water uptake of up to 99%, good water retention, and high chemical purity (Petersen, & Gatenholm, 2011; Jankau et al., 2022). Unfortunately, biocellulose lacks a biologically active compound to promote tissue modeling as well as an antimicrobial effect. Therefore, some researchers in previous studies often combined the biocellulose with keratin extract to enhance the wound-healing process. Recent studies conducted by Sadeghi et al., (2020) show that keratin biocellulose dramatically increases wound closure, as keratin improves fibroblast attachment, cell adhesion, and proliferation. Besides that, the addition of natural antimicrobial compounds is often used to prevent infections, and tamanu oil is one of them.

The oil of the tamanu tree (*Calophyllum inophyllum*) also called Nyamplung in Indonesia, is well-known for its traditional uses, especially to treat skin problems. The oil extracted from the tamanu seed has been utilized to treat various skin conditions and injuries including infections, eczema, dermatosis, scars, and burns (Léguillier et al., 2015). Literature data showed that *Calophyllum inophyllum* contains calophyllolide, a bioactive component that exhibits anti-inflammatory and antibacterial properties that may speed up the healing of wounds. (Nguyen et al., 2017). Calophyllolide combined with xanthone compound in the tamanu oil was found to be effective against a strain of *Staphylococcus aureus* that is mostly found in skin infections. A previous study revealed that tamanu oil is capable of enhancing the growth of keratinocytes and fibroblasts which are essential for the process of tissue regeneration (Raharivelomanana et al., 2018).

All of these reports suggest that keratin-bio cellulose combined with tamanu oil can be used as an advanced wound dressing that could help diabetic foot ulcers progress. Therefore, this project aims to assist the scope of the main projects by producing and characterizing keratin and biocellulose as well as analyzing the antimicrobial activity of keratin extract, black, and green tamanu oil.

### 3.1.2 The scope of the project

The Pharmacy, Biotechnology, and Biomedicine departments of i3L collaborated on this project with a grant from DIKTI. The Biotechnology department take a part in producing and characterizing the keratin and biocellulose as well as assessing the antimicrobial activity of tamanu Oil, whereas the Pharmacy and Biomedicine department assesses the ability of keratin tamanu BC hydrogel as a wound dressing in vivo testing using diabetic mice and rabbit.

#### 3.1.3 Objectives.

The purpose of this project is to produce and evaluate the keratin and biocellulose and also analyze the antibacterial effect of tamanu oil and human hair keratin extract.

#### 3.1.4 Problem Formulation.

As previously stated, the ability of biocellulose combined with keratin and an antimicrobial agent such as tamanu oil has become the potential wound dressing for diabetic wound healing. Therefore, in this experiment, the production, and characterization of keratin and biocellulose and also antimicrobial activity evaluation of tamanu oil and keratin have been conducted as preparation for further in-vivo testing.

### 3.2 Problem solution

#### 3.2.1 Production of biocellulose hydrogel

##### 3.2.1.1 *Komagataeibacter intermedius* preparation

The *K. intermedius* bacteria were collected from i3L's earlier project. In order to utilize the bacteria, first the bacteria were subcultured in MRS broth. Type III distilled water was used to dissolve the MRS powder until a concentration of 55g/L was achieved, and the solution was then autoclaved for 15 minutes at 121$^{o}$C to obtain the MRS broth. After the autoclave is completed, an MRS broth of 4900 µl was transferred to several test tubes and then a 100 µl of the bacteria was added inside the BSC. The culture was then sealed with aluminum foil and parafilm and incubated at 30$^{o}$C for 5-7 days. This incubation time was chosen since the bacteria only takes 5 days to generate a BC (Fernandez et al., 2019). However, in this experiment, the BC was subcultured every 10 days in the same period as the biocellulose was harvested to minimize the work. To ensure the quality of the culture, a gram staining was performed before the culture is being used in BC production. As a gram-negative bacteria, *K. intermedius* should have a pink appearance and a rod shape.

##### 3.2.1.1 Biocellulose production and extraction

In a 24-well plate, MRS broth media was used to produce the biocellulose. Initially, 1400 µl of MRS broth, 100µl of the *K. intermedius*, and resuspended inside the BSC. The 24-well plate was sealed and stored for 10 days at 30$^{o}$C.

After the BC is formed as desired, the BC was then harvested from each well using a stainless spatula and immersed into 150 ml of NaOH 2M solutions overnight at 30°C to remove any undesired bacteria cells, chemicals, and residual medium from the cellulose fibers. After that, the pH level of the biocellulose hydrogel was adjusted to 6-7 by submerging in the concentrated acetic acid solution. Before being immersed in 96% ethanol for 24 hours, the biocellulose was washed using distilled water to eliminate any remaining acid-base solution. After 24 hours, the sterility of the biocellulose was tested by placing the BC in the agar and incubating it at 30°C for 24 hours. The presence of bacteria growth was then observed, if the bacteria were present in the plate, the biocellulose will be incubated in the ethanol for the next 24 hours. For the sterile biocellulose then stored in phosphate buffer saline (PBS) solution at the refrigerator for long-term storage.

### 3.2.2. Characterization of isolated bio cellulose

#### 3.2.2.1 Swelling and Erosion Test

The swelling and erosion experiment was used for the evaluation of the biocellulose's ability to absorb and release water respectively. The biocellulose was divided into three subgroups, 1 minute, 10 minutes, and 100 minutes of immersion. Each group was assessed in triplicate for both swelling and erosion tests. Before being swelled in the PBS, the biocellulose was placed in a desiccator for 24 hours to remove the excess liquid, and then their initial weight was recorded. Afterward, the biocellulose of each subgroup was submerged in 5ml PBS according to their time point in the petri dish. The swelled weight of biocellulose was recorded after the testing is done and the % swelling index was computed using the formula below:

$$\%\, Swelling = \frac{Wt - Wo}{Wo}\, x\, 100\%$$

Wo = Dry BC weight

Wt = swelled BC weight

Since PBS has an identical property to human body fluids or isotonic to human solutions, it was chosen as the solvent. These solutions act as wound exudate which may cause the degradation of biocellulose (Chen et al., 2011).

Following the swelling test, the erosion test was conducted by layering all of the biocellulose subgroups on top of the filter paper and storing them for 24 hours in a 37°C oven. After the biocellulose is completely dried, the % erosion was calculated using the following formula:

$$\%\, Erosion = \frac{Wo - Wx}{Wo}\, x\, 100\%$$

Wo = Dry BC weight

Wx = Dry Bc weight (2nd drying process)

### 3.2.3 Extraction and Characterization of human hair keratin

#### 3.2.3.1 Keratin extraction

The Shindai method adapted from Wong et al., (2016) with a few modifications was performed to isolate keratin from hair. The human hair sample was provided from i3L's earlier project. The human hair was priorly washed with soap and tap water to remove any residual waste and then air dried in the fume hood for a night. Afterward, A 100grams hair sample was immersed in 96% ethanol for 24 hours, followed by chloroform-methanol (1:1) solution for delipidization for 24 hours inside the fume hood. Before being submerged using the Shindai solution for 24 hours, the hair was air dried inside the fume hood and then trimmed into small pieces (5mm). The Shindai solution was made by mixing 25mM Tris-hydrochloric acid, 5M urea, 2.6M thioureas, and 2-mercaptoethanol (5%). The solution was then collected using cheesecloth followed by filtration using Whatman®No.1 Filter Paper twice with the assistance of a vacuum pump to filtrate and remove any remaining hair. The extracted solution was then undergone a dialysis method to isolate the keratin. In short, the samples were transferred into 10kDa dialysis tubes and submerged in type III distilled water with a constant stirring under the following settings: 450rpm for 30 minutes repeated 4 times, 450rpm for 1 hour repeated twice, 450rpm for 2 hours and 360rpm overnight. The extracted keratin solution was collected and stored in a container covered with aluminum foil to prevent light exposure and stored at 4°C.

#### 3.2.3.2 SDS-PAGE

Qualitative assessment of keratin in the solution was performed by using SDS-PAGE which is a method to separate proteins based on their molecular sizes and charges. Firstly, the gel cassette was assembled and tested using type I distilled water to ensure there was no leakage before being filled with the 10% resolving gel which was generated by mixing type I distilled water (1980µL), 1.5M Tris-HCl pH 8.8 (1250µL), 10% SDS (50µL), 30% acrylamide(1660µL), 10% APS (5µL). After the cassette was filled 80%, the isopropanol was added to remove any air bubbles in the gel and then let the gel polymerized for 15 minutes. Subsequently, after removing the isopropanol, the 4% stacking gel was added to the cassette which was a mix of type I water (1100µL), 0.5M Tris-HCL pH 6.8 (500µL), 10% SDS (20 µL), 30% acrylamide (340µL), 10% APS (20µL), and TEMED (8µL). The stacking gel was filled until it was full and the comb was then placed carefully on top of the gel. Before filling the comb with the samples, the sample was prepared by mixing the 10µL of keratin extract with Laemmli buffer (9.5µL) and β-

mercaptoethanol (0.5µL) followed by incubation at 95$^{o}$C for 15 minutes. Afterward, the gel was put in the SDS cascade, and placed inside the SDS tank which was then filled with a 1x SDS running buffer. The ladder and sample were loaded for 10µL and 15µL into wells respectively and then ran at 120V and 440A for 90 minutes. After the run was completed, The gel was stained using a staining agent containing coomassie blue for 4 hours, followed by immersed with a destaining agent for 4 hours.

#### 3.2.3.3 Bicinchoninic Acid (BCA) Assay

BCA assay was performed to assess the concentration of keratin solution. A 0, 125, 250, 500, 1000, and 2000 µg/mL concentration of bovine serum albumin (BSA) was utilized as a protein standard. The keratin samples were diluted with a dilution factor of 10, 20, 30, and 40 by mixing them with type 1 water. The BCA working reagent was then created by mixing BCA Reagent A and B with the ratio of 50 Reagent A: 1 Reagent B. Into the 96 well-plate, the 25µL of samples and standards were transferred into each well in triplicate and added with 200µL of working reagent. Afterward, the plate was protected with aluminum foil to prevent light exposure and incubated at 37$^{o}$C for 30 minutes. Following a measurement of the sample's absorbance at 562nm, the concentration of keratin was determined using a standard curve.

#### 3.2.3.4 Fourier Transform-Infrared Spectroscopy (FT-IR) of keratin

Characterization of keratin extract was carried out using PerkinElmer Spectrum$^{TM}$ Fourier Transform-Infrared Spectroscopy (FT-IR). The Spectrum Two$^{TM}$ FT-IR was prepared according to the manual instruction for tablet form samples. The keratin tablets were priorly made by mixing the Potassium Bromide (KBr) powder with keratin extract with a ratio of 1: 100. The mixture was then grounded using a mortar and then dried inside the desiccator overnight. Afterward, the powder was pressed into a tablet form using a hydraulic press machine. The tablet was then analyzed using a KBr pellet holder and the data previewed by PerkinElmer Spectrum IR software Ver 10.6.2 was recorded.

### 3.2.4 Keratin Biocellulose (KBC) hydrogels production and characterization

#### 3.2.4.1 Production of Keratin Biocellulose (KBC) hydrogels.

The production of KBC hydrogels was performed by immersing the biocellulose in 10mg/ml of keratin extract for at least 24 hours prior to being used. The 10mg/ml of keratin concentration was achieved by diluting the keratin with type III distilled water.

#### 3.2.4.2 Fourier Transform-Infrared Spectroscopy (FT-IR) of biocellulose and KBC

Characterization of biocellulose hydrogels was performed through PerkinElmer Spectrum™ Fourier Transform-Infrared Spectroscopy (FT-IR). Firstly, the biocellulose and KBC was placed on the filter paper followed by placing it in the desiccator overnight for drying. The Spectrum Two™ FT-IR was then prepared according to the manual instruction and the instruments were wiped with Kimwipes and absolute ethanol before being used. The background sample was then scanned priorly to measure the absorbance of the atmosphere. Afterward, the dried biocellulose was placed on the disks and the gauge pressure of 60-80 was applied before scanning. The scanning was then performed under the following settings: 20 times scans, the wavenumber of 500-4000 $cm^{-1}$ and 4 $cm^{-1}$ resolution. The data was observed using PerkinElmer Spectrum IR Software Ver 10.6.2.

### 3.2.5 Antimicrobial assay of keratin and tamanu oil

#### 3.2.5.1 Antimicrobial assay of keratin extract, green, and black tamanu oil.

The antimicrobial property of the keratin extract, green, and black tamanu oil was assessed through the Kirby-Bauer disk diffusion test. The black and green tamanu oil was obtained from previous research at i3L. The samples were prepared by diluting each batch of keratin extract to a concentration of 10mg/ml through the addition of type III deionized water whereas the pure tamanu oil was diluted to 2% concentration by mixing it with type III deionized water, span 80, and tween 80 using homogenizer. The samples were priorly exposed to UV light for 15 minutes to sterilize the sample before being used. The MH agar that was used in this experiment was produced by dissolving bacteriological agar powder (17g/L) with the MH broth powder (21g/L) a day before the experiment. The bacteria used, *Staphylococcus aureus* and *Pediococcus aeruginosa* were cultured in the MH broth and the absorbance was adjusted to 0.8-1.0 using a spectrophotometer. Afterward, 100µL of bacteria were inoculated into the agar and spread evenly on the agar using a glass spreader. The paper disks were then dipped into each sample and placed in the plates in triplicate. 5µg/ml of ciprofloxacin was used as a positive control whereas a paper disk without any addition as a negative control. The plates were incubated for 24 hours at 37℃ incubators, and the zone of inhibition was measured after 18-24 hours of incubation by recording the un growth area around the discs using vernier calipers.

### 3.2.6 Statistical analysis

The % swelling and % erosion index were statistically analyzed one-way ANOVA in GraphPAd Prism software for determining the significance of the 3 times point data.

# CHAPTER IV: RESULT

## 4.1 Biocellulose production and characterization

The capability of bio cellulose to absorb and release water was evaluated using swelling and erosion tests respectively. The recorded data of the swelling and erosion test is demonstrated in *Table 4.1* illustrated in a graph in *Figure 4.1* The weight of the biocellulose was increased in respect to the time in which the 1-minute immersion group had the lowest % swelling of 170.22%. The water-absorption ability of biocellulose starts to inflate to 186.77% and 206.22% after 10 minutes and 100 minutes of immersion from its initial weight respectively. On the other hand, the highest % erosion was observed in the 100-minute bio cellulose group with a % erosion of 18.57% followed by 10 and 1 minutes groups respectively. The % yield of biocellulose extraction was calculated to determine the effectiveness of biocellulose production. It was calculated that *K. intermeidius* exhibited a BC yield of 1.48mg/ml of MRS media. The sterility test of extraction was performed on nutrient agar with 24 hours of incubation. The result shows an absence of microbial growth on the agar, which indicates that the biocellulose produced is successfully sterilized and free from microorganisms.

**Table 4.1** The changes of bio-cellulose weight after swelling and erosion after immersion in PBS and subsequent drying

| Biocellulose Subgroup | Avg. ΔW/Wo of the swelling test (mg) | Avg. ΔW/Wo of the erosion test (mg) | % Swelling | % Erosion |
|---|---|---|---|---|
| 1 minutes | 1.70 | 0.085 | 170.22 ± 11.22 | 8.52 ± 4.2 |
| 10 minutes | 1.867755991 | 0.1699346405 | 186.77 ± 23.88 | 16.99 ± 3.38 |
| 100 minutes | 2.062663399 | 0.1857142857 | 206.26 ± 0.39 | 18.57 ± 3.78 |

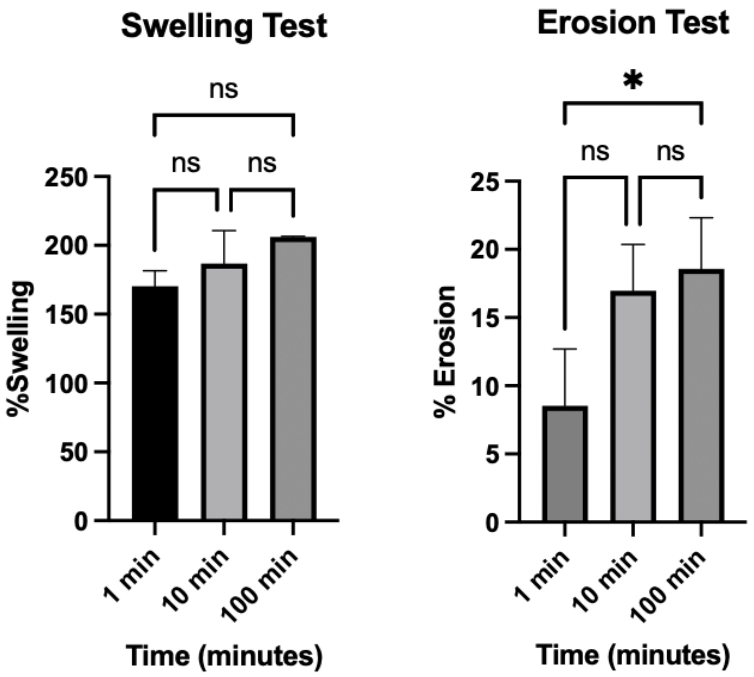



**Figure 4.1** The graphs represent the % swelling and % erosion value of biocellulose in triplicate with respect to time.

The statistical analysis through one-way ANOVA demonstrated a non-significant difference in the % swelling percentage of each time stamp, while in the erosion test, a significant difference was observed between 1 min and 100 mins groups.

## 4.2 Characterization of Keratin Extract

### 4.2.1 SDS-PAGE

The results of SDS analysis for keratin extracted using the Shindai method are presented in *Figure 4.2*. The fifth lane was the 10-250kDa of protein ladder whereas the other lane was keratin extracts from a different batch. The molecular weight of human hair keratin is ranging from 44 to 56 kDa for acidic keratin (type I) and 53 to 66 kDa for basic keratin (type II) (Moll et al., 2008). Based on the visible band observed, all the keratin extract has both type I and type II human hair keratin. This proves that the Shindai method of keratin extraction and the subsequent dialysis process was successful.

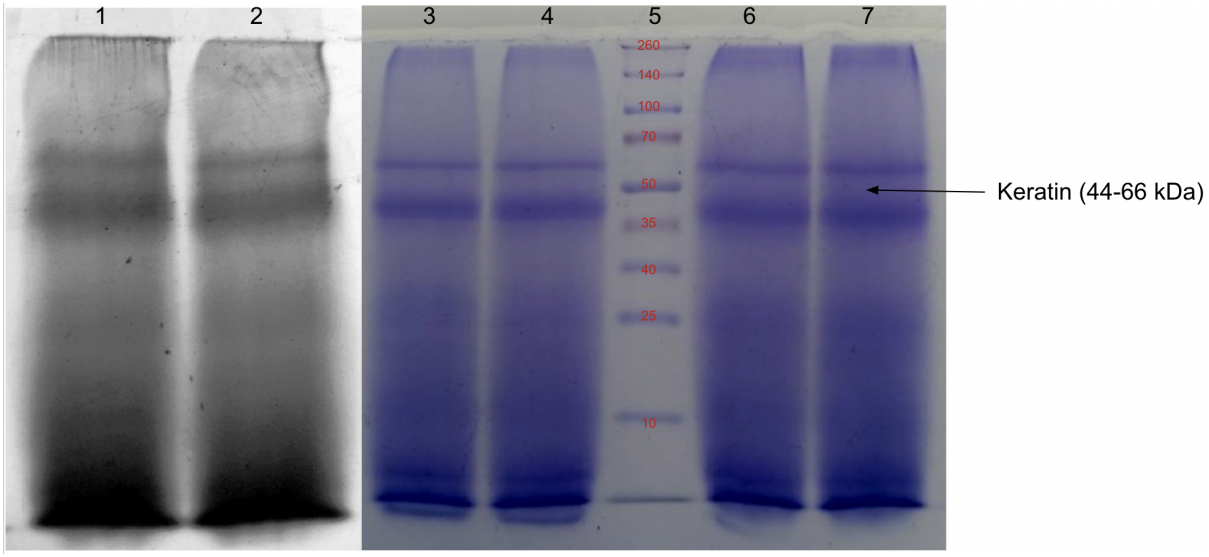
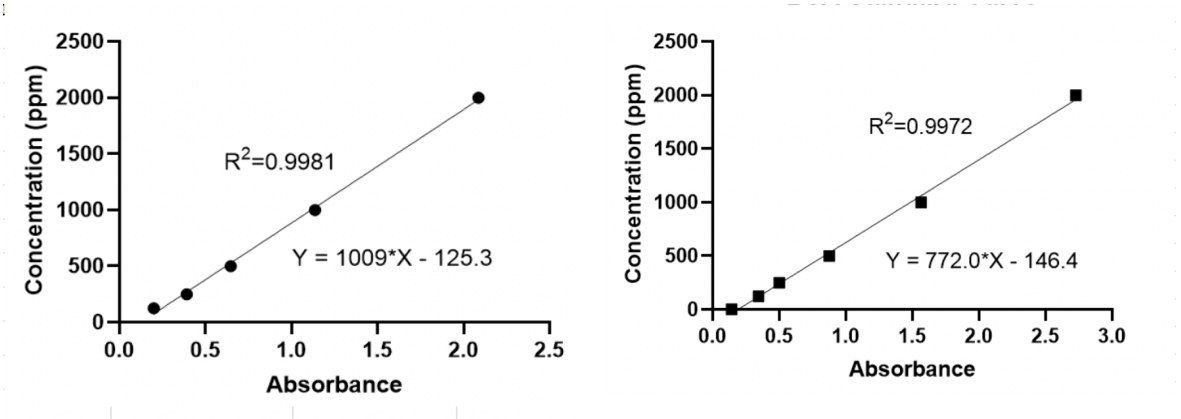



**Figure 4.2**. Keratin extract characterization using SDS-PAGE. Lane 1 was the keratin 1st batch, lane 2 was the keratin 2nd batch, Lanes 3 & 4 were the keratin 4th batch in duplicate, lane 5 was the protein ladder, and Lanes 6 & 7 were the keratin 5th batch in duplicate.

### 4.2.2 Bicinchoninic acid (BCA) ASSAY.

The extracted keratin concentration was determined by using the BCA standard curve. The standard curve of the BCA assay was generated by plotting the absorbance against the concentration of BSA solution and it is shown in *Figure 4.3.* As shown below, both BSA standard curves had an $R^2$ of 0.9981 and 0.9972, which indicates an excellent linear equation to be used as a standard. The concentration of extracted keratin from human hair was determined using the linear equation of y=1009x - 125.3 and y= 772.0x - 146.4. The absorbance value of the sample at 562nm and the measured concentration of each diluted keratin sample are demonstrated in *Table 4.2.*




*(a)* *(b)*

**Figure 4.3**. The standard curve for concentration measurement (a) keratin extraction batch 1-3 (b) keratin extraction batch 4-5

The keratin solution of 1st, and 3rd batches with a dilution factor of 10 are not available as the sample is too concentrated. The human hair keratin extracted through the Shindai method had a concentration ranging from 13.4mg/ml to 49.48mg/ml.

***Table 4.2.*** *The concentration of extracted keratin calculated from BCA assay*

| No. Keratin Batch | Dilution factor (DF) | Avg. (Abs-blank) | Keratin Concentration (ppm) | Keratin Conc X DF (ppm) | Final Concentration (mg/ml) |
|---|---|---|---|---|---|
| 1 | 10 | Overflow | Overflow | Overflow | Overflow |
| | 20 | 2.3398 | 2235.55 | 44711.16 | 44.71±2.16 |
| | 30 | 1.6428 | 1532.31 | 45969.56 | 45.97±0.4 |
| | 40 | 1.3501 | 1236.98 | 49479.38 | 49.48±0.63 |
| 2 | 10 | 2.0247 | 1917.62 | 19176.22 | 19.28±0.54 |
| | 20 | 1.2009 | 1086.40 | 21728.16 | 21.73±0.95 |
| | 30 | 0.5884 | 468.46 | 14053.88 | 14.05±2.87 |
| | 40 | 0.6220 | 502.33 | 20093.23 | 20.09±1.09 |
| 3 | 10 | Overflow | Overflow | Overflow | Overflow |
| | 20 | 1.6257 | 1515.03 | 30300.62 | 30.3±2.5 |
| | 30 | 0.9575 | 840.81 | 25224.52 | 25.22±0.84 |
| | 40 | 0.9309 | 814.04 | 32561.81 | 32.56±1.12 |
| 4 | 10 | 2.3353 | 1656.45 | 16564.51 | 16.56±0.42 |
| | 20 | 1.4217 | 951.20 | 19024.07 | 19.02±0.26 |
| | 30 | 0.9998 | 625.44 | 18763.3 | 18.76±0.70 |
| | 40 | 0.7862 | 460.5721333 | 18422.88 | 18.42±0.50 |
| 5 | 10 | 1.9252 | 1339.90 | 13399.05 | 13.4±1.53 |
| | 20 | 1.1339 | 728.99 | 14579.93 | 14.58±0.35 |
| | 30 | 0.8753 | 529.33 | 15879.94 | 15.88±0.30 |
| | 40 | 0.6512 | 356.37 | 14255.11 | 14.30±0.86 |

**4.3 Antimicrobial assay through Kirby-Bauer Method**

The antimicrobial activity of the keratin solution, green, and black tamanu oil is determined by measuring the zone of inhibition against *S. aureus* and *P. aeruginosa.* An inhibition zone was absent in the keratin sample as well as 2% of green and black tamanu oil against *S. aureus*. Meanwhile, the 100% green and black tamanu oil formed a zone of inhibition of 1.33cm and 1.39cm respectively, which indicated that the bacteria are sensitive to black and green tamanu oil although their sensitivity was lower than the positive control with 1.57cm. For *P. aeruginosa,* no inhibition zone was observed in all the samples except the positive control with 1.14cm of inhibition. Thus, the *P. aeruginosa* used in the experiment was not susceptible to both of the tamanu oil.

**Table 4.3**. The zone of inhibition of antimicrobial assay of keratin extract and tamanu oil from Kirby-Bauer assay

| *S. aureus* | | | *P. aeruginosa* | | |
|---|---|---|---|---|---|
| Sample | Concentration | Avg. diameter zone of inhibition (cm) | Sample | Concentration | Avg. diameter zone of inhibition (cm) |
| Ciprofloxacin (+) | 5mg/ml | 1.57 ± 0.05 | Ciprofloxacin (+) | 5mg/ml | 1.14 ± 0.21 |
| Negative control | - | 0 | Negative control | - | 0 |
| Keratin extract | 10mg/ml | 0 | Keratin extract | 10mg/ml | 0 |
| Green tamanu oil | 100% | 1.33 ± 0.04 | Green tamanu oil | 100% | 0 |
| | 2% | 0 | | 2% | 0 |
| Black tamanu oil | 100% | 1.39 ± 0.04 | Black tamanu oil | 100% | 0 |
| | 2% | 0 | | 2% | 0 |

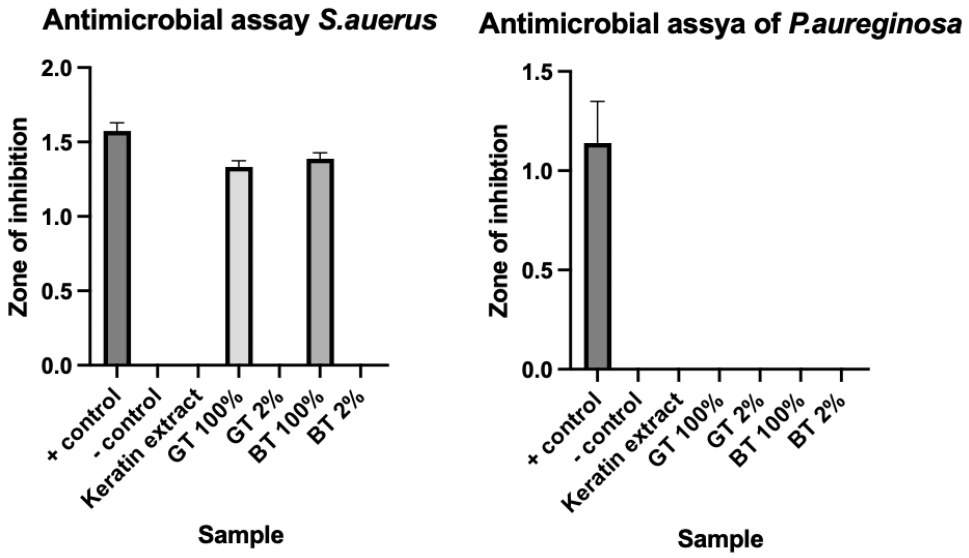
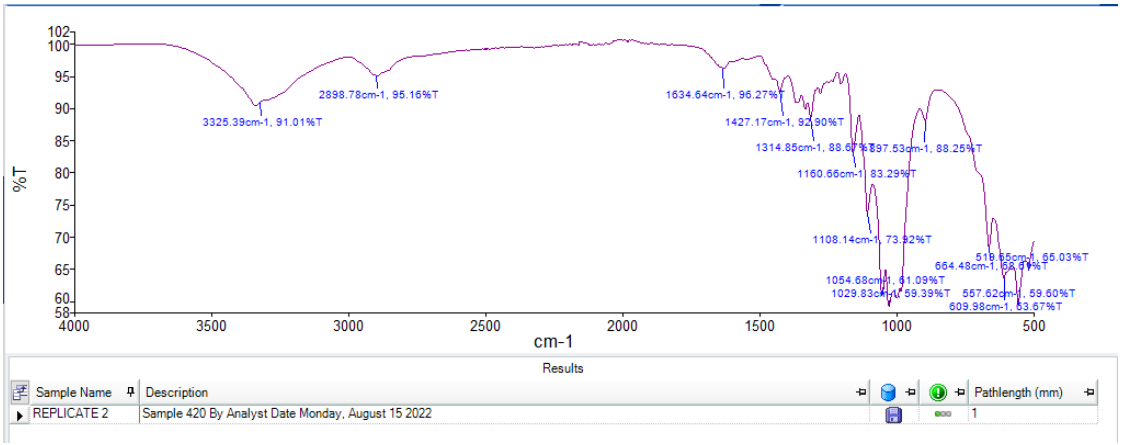



**Figure 4.4.** The graph represents the antimicrobial activity of ciprofloxacin 5mg/ml, keratin extract green & black tamanu oil, and negative control

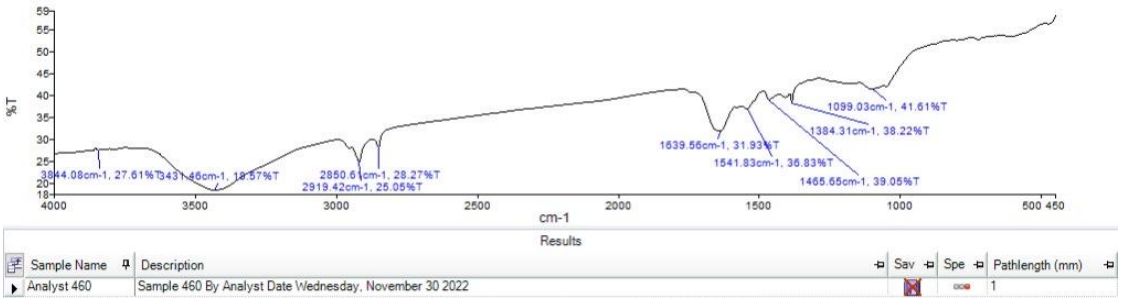
**4.4 Fourier Transform-Infrared (FT-IR)**

The FT-IR was performed on keratin solution, biocellulose, and KBC hydrogel to determine the functional groups and chemical bonds present in the samples. The FT-IR analysis is shown in *Figure 4.5*. The Bio-cellulose spectra (A) displayed a peak at 3325.39 cm-1 which refers to O-H stretching. Other than the hydroxyl group, other peaks were also found including 2898.78 $cm^{-1}$ referring to C-H stretching vibration, 1314.85 $cm^{-1}$ referring to CH2 bending, 1054.68 $cm^{-1}$ referring to C-O stretching and 1160.66 cm-1 indicating C-O-C stretching. For the keratin pellet spectrum (B), the strong peaks detected at 1639.55 cm-1 and 1541.83 cm-1 indicate the presence of amide I and amide II in the keratin solution (Coates et al., 2016). The presence of those peaks indicate that the keratin extraction was successful. Most of the peaks found in the BC hydrogel and keratin solution such as O-H stretching, CH2 bending, C-O stretching, and amide I were also presented in the KBC spectrum (C). However, the amide II band, C-O-C stretching, and C-H stretching are absent from this spectrum.

A)




B)




C)

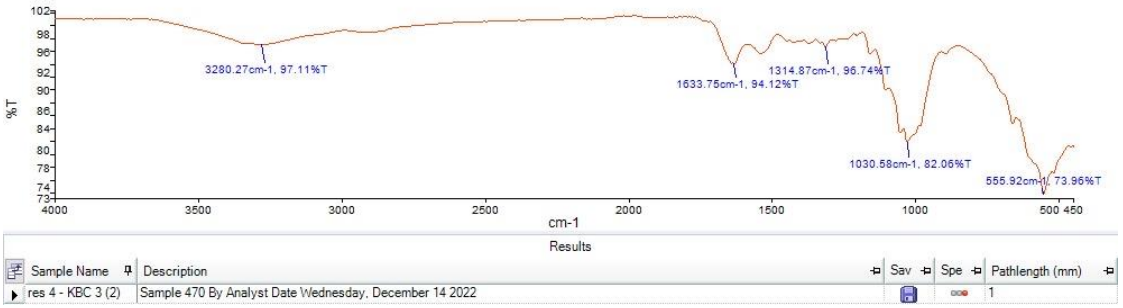



**Figure 4.5**. FTIR Spectra of BC (A), Keratin pellet (B), and KBC (C). The x-axis stands for the wavelength ($cm^{-1}$) while the y-axis stands for the % transmittance.

# CHAPTER V: DATA ANALYSIS & DISCUSSION

## 5.1 Production and characterization of the bacterial cellulose

The biocellulose production using *K. intermedius* reached a maximum yield of 1.42mg/ml using MRS media with a pH of 6.0. This production yield was similar to the other experiments conducted by Fernandes et al., (2019), in which *K. intermedius* JF2 exhibited a maximum yield of 1.6mg/ml of biocellulose using the HS media supplemented with mannitol and a pH of 5.5-6.0. The slight difference in the production yield may be attributed to the different strains and additional carbon source used as it was found that *K. intermedius* can utilize the mannitol. Moreover, another experiment conducted by Lin et al., (2016), found that *K. intermedius* FST 213-1 exhibited a production yield of 1.2mg/ml using an HS medium with a pH of 8.0. The lower yield may be caused by the different pH levels used for the culture as it was found that the range of pH value for BC production from *K. intermedius* is ranging from 4.0-7.0.

For the first characterization of BC, the swelling and erosion test were conducted to examine the biocellulose's water absorption and retention ability respectively. This water absorption evaluation is essential in wound dressing as it would represent the ability of the biocellulose to uptake wound exudate.(Bodea et al., 2017). With the high water uptake abilities, biocellulose could be able to absorb wound exudate, preventing the accumulation of fluids produced by the wound and altering the wound maceration to occur (Portela et al., 2019). The high % swelling also indicates that the BC can uptake a high amount of keratin which means that more keratin could be released into the target area (Ngadaonye et al., 2013; De Lima Fontes., 2018). From *Figure 4.1*, the % swelling of the biocellulose increased as the time increased which means that the BC still can absorb the PBS solution after 100 minutes. Even though the percentage increased throughout the time, no significant difference was observed between each time, which indicates that even at 1 minute of immersion, the BC has a good water uptake ability. Meanwhile, the robusticity and retention ability of the biocellulose were evaluated through an erosion test (Wang, 2020). The value of erosion percentage represents the disintegration rate of the biocellulose after being regularly swelled and dried. The disintegration may be demonstrated by the loss of weight and deformation of biocellulose. Based on *Figure 4.1*, The erosion index was elevated as time increased which means that the % erosion is proportional to the amount of water absorbed. This result corresponds with the previous studies as higher water intake causes huge expansion at the swelled state of hydrogels, leading to greater surface stress (Wang, 2020). Even though some parts of the biocellulose disintegrated, the erosion rate of the BC may still be considered as acceptable as it is below 100%

(Febriani, 2021). With all being said, the BC produced by K. intermedius had good physicochemical properties, proved by high % swelling and low % erosion value.

## 5.2. Characterization of keratin extract

### 5.2.1. SDS-PAGE of Keratin Extract

As the most common size-based separation and protein analysis method, SDS-PAGE has been utilized in various applications to identify a protein based on its molecular mass, examine the molecular mass of a known protein, and analyze the subunit composition of known protein (Pavlova, et al., 2018; Carter, et al., 2013). In this experiment, the solution isolated from human hair was examined for the presence of keratins using the SDS-PAGE, which showed two protein fractions at 44 to 66kDa in *Figure 4.2*. These bands are consistent with the literature, which states that type I acidic keratin is present in the protein band ranges from 44 to 56kDa, and type II basic keratin is present in the protein band ranges from 53 to 66kDa (Kakkar et al., 2014). Both of the α keratins are strictly interdependent for assembly into 10nm filaments, initially by forming dimers with one acidic and basic chain which then form protofilaments and finally into Intermediate filaments (IF) (Mohamed, J.M. et al., 2021).

### 5.2.2. Determination of keratin concentration by BCA assay

After characterization using SDS-PAGE, a quantitative method called BCA assay was utilized to calculate the concentration of keratin. A BCA assay was utilized in this study due to its sensitivity, simplicity, repeatability, and reproducibility (Cortes Rios et al., 2020). BCA assay is based on the reduction of $Cu^{2+}$ to $Cu^{+}$ by the amino acid and peptide bond from the protein samples under alkaline conditions. As a result of that reaction, the $Cu^{+}$ can react with the BCA to form a copper ion-bicinchoninic acid (Cu1-BCA2) chromophore complex and resulting in an intense purple-colored solution. The amount of chromophore complex formed that is associated with protein concentration could be calculated by measuring the absorbance at 562nm (Walker, n.d.). In this experiment, the concentration of extracted keratin from 100g of human hair is ranging from 13.4mg/ml to 49.48mg/ml. This yield mainly corresponds with the previous study conducted by Valkov et al., 2020 in which they successfully yield a maximum concentration of 28mg/ml of keratin using the Shindai method after 72h of immersion. The low yield of extracted keratin in batches 2, 4, and 5 may be caused by the variation of keratin concentration contained in the human hair samples. The quality of the keratin contained in the human hair may be related to the ages of the subjects. An investigation performed by Giesen et al., (2011) revealed that the aging process can decrease the expression of

two members of the acidic keratin family which may correspond to the keratin contained in human hair. Other than that, the lower concentration of keratin is might due to an incomplete mixing of the keratin solution before being transferred to the well plate for BCA assay. The keratin has also been stored for a long period before being analyzed which may also lead to degradation of the keratin protein. However, the range of the concentration extracted is still acceptable since the target keratin concentration that will be used in the K-BC hydrogels production is 10mg/ml.

**5.3 Antimicrobial assay of keratin extract, black, and green tamanu oil.**

Biocellulose has many favorable aspects to be used in wound healing due to its physicochemical properties of high water absorption and retention capability as well as high biocompatibility. However, biocellulose lacks active compounds and antimicrobial properties to promote tissue regeneration, thus the addition of bioactive compounds which are keratin and tamanu oil in this experiment may significantly increase the wound healing process (Bodea et al., 2017). The antimicrobial assay of keratin extract and black and green tamanu oil was performed through Kirby-Bauer disk assay to evaluate the antimicrobial activity against *S. aureus* and *P. aeruginosa*. These two bacteria were chosen since they are pathogenic bacteria that are commonly found in the skin and readily cause skin affection and colonies on ulcers ( DeLeon et al., 2014). In the first assay which is against *S. aureus*, both pure black and green tamanu oil displayed a zone of inhibition indicating that the samples possess an antibacterial against *S. aureus*. However, no inhibition zone was found in both 2% of black, and green tamanu oil. This result does not align with the previous research as tamanu oil possesses calophyllolide and xanthone that exhibits antibacterial and anti-inflammatory properties (Nguyen et al., 2017). It was found that calophyllolide combined with other neoflavonoid compounds is effective to inhibit the growth of *S. aureus* (Raharivelomanana et al., 2018). The absence of antimicrobial activity could be attributed to the concentration of tamanu oil being too low to display an antibacterial effect as the pure itself only shows a small diameter of inhibition. In contrast, the antimicrobial assay of keratin showed no zone of inhibition. Even though keratin may possess antimicrobial peptides (AMPs), a previous study conducted by Tran et al., (2016) showed that keratin could not effectively inhibit the growth of *E. coli*, *S. aureus*, and VRE. However, the absence of antimicrobial activity of keratin did not interfere with the experiment as the purpose of combining biocellulose with the keratin is due to the keratin's ability to improve fibroblast attachment and proliferation. Keratin is also involved in cell processes such as cell adhesion, migration, and differentiation which are important for complete wound healing (Konop et al., 2021). Moreover, a previous study revealed that the combination of keratin with biocellulose dramatically enhances wound closures (Sadeghi et al., 2020). With all being said,

the incorporation of keratin into the biocellulose still may provide a beneficial impact on the wound healing process, although, in this experiment, the effectiveness of KBC hydrogels to promote wound closures was not conducted.

For the antimicrobial assay against *P. aeruginosa*, no inhibition area was observed in all samples except in 5µg/ml of ciprofloxacin. The absence of antimicrobial activity of tamanu oil may be due to the defense mechanism of *P. aeruginosa* as this bacteria is known to possess many mechanisms for antimicrobial resistance including intrinsic antibiotic resistance, and antibiotic-inactivating enzymes. The ability of *P. aeruginosa* to form a biofilm also contributes to the defense mechanism which was found in this experiment, indicated by the presence of the green-color film on the agar surface (appendix 4.) (Wilson, & Pandey, 2018). These results are aligned with the previous experiment conducted by Tran et al., (2018) in which they found that commercial tamanu oil only exhibits antibacterial activity against *S. aureus* but not against *P. aeruginosa*.

**5.4 FT-IR analysis of KBC- hydrogels**

The FT-IR measurement was utilized to examine the molecular structure of the BC, and keratin, and also validate the encapsulation of keratin to the biocellulose hydrogels. A typical molecular structure of biocellulose is $(C_6H_{10}O_5)n$, which consists of O-H stretching, C-O-C stretching, C-H stretching, C-O stretching, and $CH_2$ bending which corresponds to our results (Voon et al., 2018). The keratin FTIR measurement also aligns with the literature as it shows identical peaks of amide I (C-N and C=O stretching) and amide II (stretching C-N, C-C stretching, and N-H bending) (Kakkar et al., 2014). Therefore, it can be concluded that the extraction of keratin from human hair and biocellulose from K. intermedius was successfully performed. This keratin was then used for KBC production by immersing the BC with 10mg/ml of keratin for 24 hours. The FTIR analysis of KBC shows an identical spectrum as biocellulose and keratin solution peaks combined, proving that the keratin was incorporated into the BC hydrogels. However, the absence of amide II peak in the BC was observed in the KBC spectrum. This result may be attributed to the weak amide II signal in the liquid keratin solution which probably causes the peak indistinguishable and looks insignificant (Coates, 2006).

In addition to further research, the human hair utilized for the keratin extraction should be prepared from a similar subject to minimize the variation of the result as the keratin content may be affected by the age and the types of the subjects. Another recommendation is to have a longer erosion and swelling test period on purpose to examine the maximum capability of hydrogels to uptake water and maintain their structure.

# CHAPTER VI: SELF-REFLECTION

The opportunity to be a part of this research as an intern in i3L enhanced the author's knowledge and expanded his expertise. Being able to be directly involved in the high level of research has given the author numerous experiences to learn, advance, and develop as a biotechnology student as well as a future biotechnologist. Over the four-month internship, the author gained into how to manage works inside the projects professionally as even though it is conducted at i3L, the author needs to be accountable for his works to the field supervisor and DIKTI as the external institution and also for further research. The author also learned how to cooperate with the other research team members during the internship period. Other than that, the author gained a massive amount of information from the research assistant on how to work efficiently and effectively which improved the author's time management skills. The opportunities to work independently as an intern in this research project also equipped him to have critical thinking to overcome the problem and challenges he faced which is a beneficial foundation to become a future biotechnologist.

The intern's capabilities and weaknesses are revealed during the internship period as a result of the challenges and obstacles encountered. During the initial period of the internship, the intern found it harder to work professionally every time, however throughout the period, the author learned to work in a timely manner and efficiently, especially after the supervisor gave him the responsibility to work independently. Besides the author's inner desires to overcome his weakness, the BRIGHT sessions held continuously by i3l have also extremely contributed as the BRIGHT session taught the author to have more initiative to ask for input from others as well as professionally commit to the work given. The i3L has also crucially contributed to the completion of this internship project as the knowledge learned at the university has helped in understanding and working in a laboratory. Several courses such as bioprocessing laboratory, microbiology laboratory, analytical instrumentation, and bioassay, and also molecular and cellular biology laboratory had given the author a strong foundation for conducting the works as well as analyzing and characterizing the results obtained.

All in all, the internship period was a phenomenal experience for the intern to sharpen his skills and gain new ones, which is crucial for the working world. Besides that, it has also brought benefits for the research project as the intern helped a lot with the production and extraction of the products that would be essential for further research.

# CHAPTER VI: CONCLUSION AND RECOMMENDATION

In conclusion, the biocellulose was produced from *K. intermedius* bacteria successfully based on the FTIR analysis and high yield percentages. The BC formed also showed good physicochemical properties of water absorption and retention capability, indicated by high % swelling and % erosion value. The keratin extraction process was also successfully done using the Shindai method in which high keratin concentration was obtained from human hair evidenced by a bold band on the SDS-PAGE and BCA assay. After both BC and keratin extract are harvested, keratin extract was incorporated into the biocellulose to generate keratin-bio cellulose (KBC) through immersion. In the FTIR test, the KBC spectrum showed a similar spectrum as BC and keratin extract combined which indicated the successful encapsulation of keratin. However, the absence of amide II in the KBC may be caused by a weak signal which leads to an undetectable peak. During the antimicrobial assay, both black and green tamanu oil had an antimicrobial effect on the *S. aureus* but not on *P. aeruginosa*, indicated that tamanu oil can be utilized as an antimicrobial dressing of biocellulose for wound dressing. The incapability of tamanu oil against *P. aeruginosa* may be due to its defense mechanism and the ability of this bacteria to produce biofilm. Lastly, keratin extract did not show any zone of inhibition towards both of the bacteria as expected in this experiment.

In this experiment, the keratin concentration obtained from human hair varied between batches, and some were lower than expected. This may be due to the variation in the types and ages of the hair. Moreover, for further experiments, swelling and erosion tests with a more extended time period should be performed to examine the maximum capacity of water uptake and retention ability of the biocellulose. Another further recommendation is in the swelling and erosion test, the drying process should be performed in three or more repetitions to ensure there are no considerable changes in the weight of the dry BC which means the BC is completely dried.

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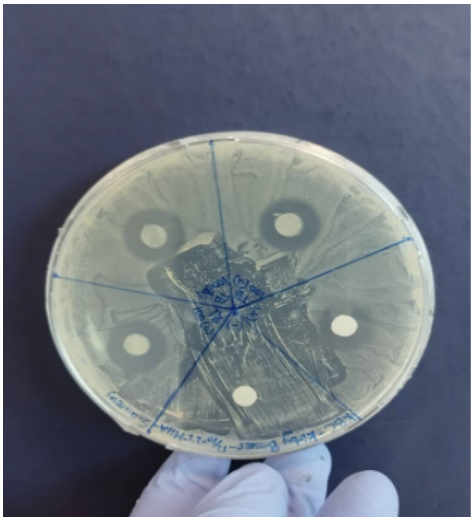
# APPENDICES

**Appendix 1.** Weight of the biocellulose after swelling and erosion.

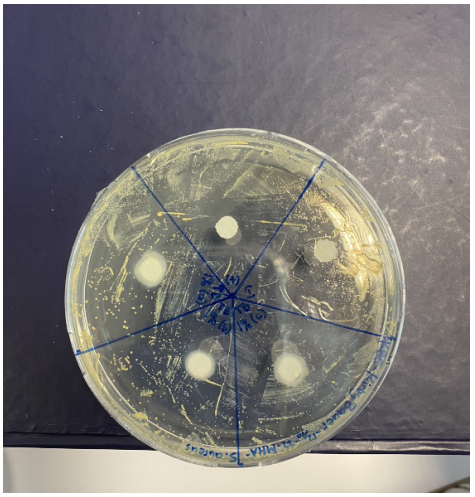
| Duration (min) | Initial Weight (mg) | | | Weight after swelling (mg) | | | Weight after erosion (mg) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| 1 | 1.7 | 1.6 | 1.7 | 4.4 | 4.5 | 4.6 | 1.5 | 1.5 | 1.8 |
| 10 | 1.7 | 1.8 | 2 | 5.2 | 5.3 | 5.2 | 1.7 | 1.5 | 1.5 |
| 100 | 1.5 | 1.6 | 1.7 | 4.6 | 4.9 | 5.2 | 1.4 | 1.4 | 1.3 |


**Appendix 2.** Sterility test of biocellulose on NA media

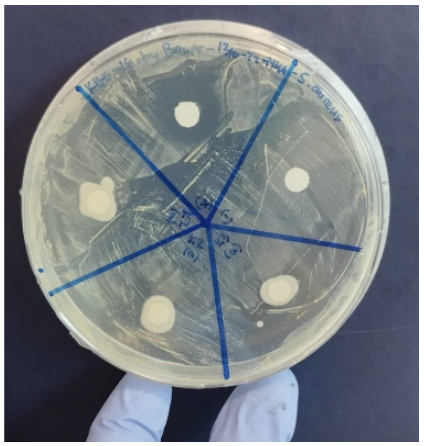
A



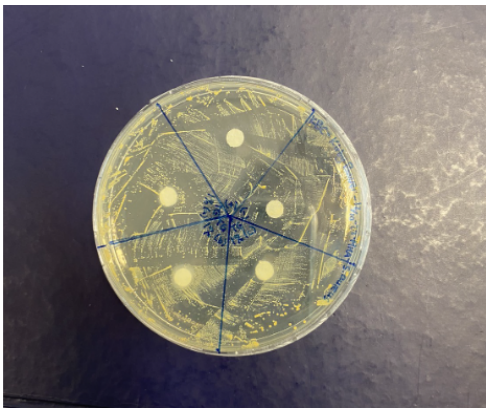
B



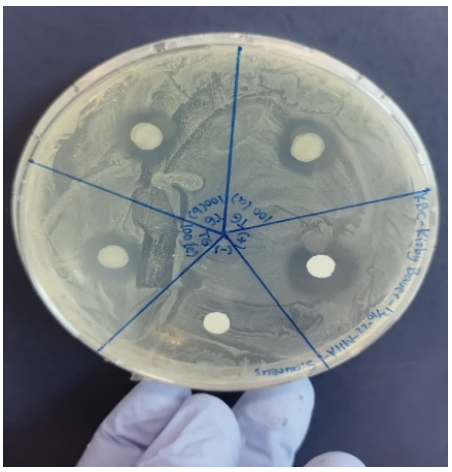
C



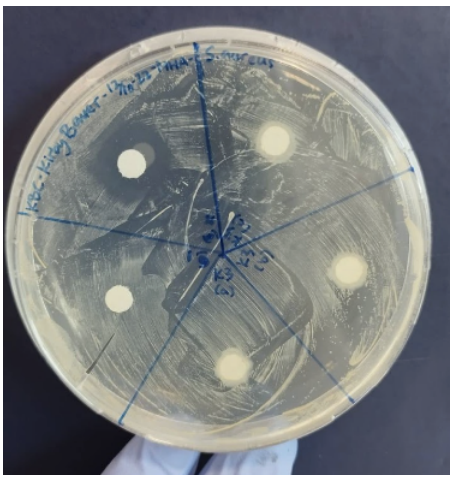
D



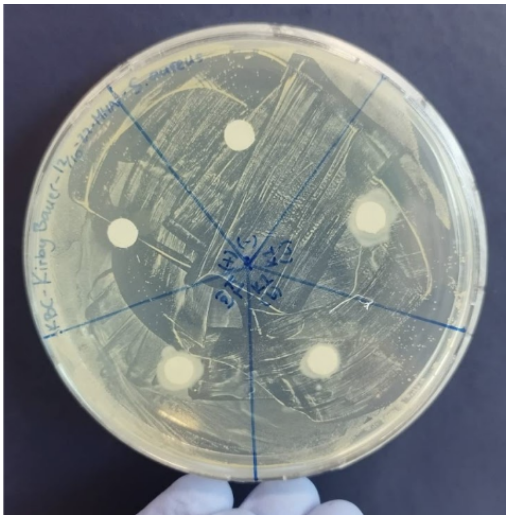
E



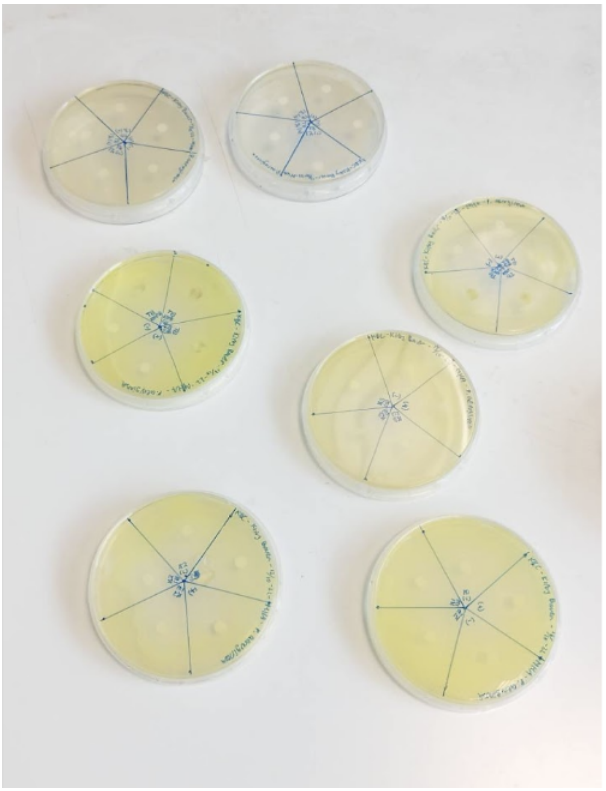
F



G



**Appendix 3**. Zone of inhibition of antimicrobial activity against *S. aureus* of (A) 100% *Calophyllum inophyllum* black oil, (B) 2% *Calophyllum inophyllum black* oil, (C) 10 mg/ml of keratin batch 3 (D) 2% *Calophyllum inophyllum* green oil, (E) 100% *Calophyllum inophyllum* green oil, and (F) 10 mg/ml of keratin batch 1, and (G) 10 mg/ml of keratin batch 2.



**Appendix 4**. Zone of inhibition of antibacterial activity against. *P. aeruginosa* of all samples

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