Comparison of Methods for Encapsulating *Saccharomyces cerevisiae* in Bacterial Cellulose produced by *Komagataeibacter intermedius*

By
Natasha Denisa
17010055

Submitted to
i3L – Indonesia International Institute for Life Sciences
School of Life Sciences

In partial fulfillment of the requirements for the Bachelor of Science in
Bio Technology

Thesis Advisor: Katherine, Ph.D.

Jakarta, Indonesia
2021

Certificate of Approval

INSTITUT BIO SCIENTIA INTERNASIONAL INDONESIA

Jl. Pulomas Barat Kav. 88 Jakarta Timur 13210 Indonesia
+6221 295 67888, +6221 295 67899, +6221 296 17296
www.i3l.ac.id

Thesis Committee Meeting Report

Note: Thesis committee meetings can start without the student being present, in this case Thesis Advisor will review student's progress with committee members. At the end of the meeting, the student may opt for the Thesis Advisor to leave the room and talk alone with committee members.

The most senior member of the committee (other than the Thesis Advisor) usually serves as the "chair", and should fill out the required information after discussion with the committee.

Committee evaluation of progress (check):

- ☐ The student is on trajectory for completion of the final thesis project.
- ☐ Concern regarding trajectory or thesis project (Student and Thesis Advisor must meet with Head of Study Program and Director of Academic Affairs).

The above named student is declared:

- ☑ Passed.
- ☐ Pass with revision.
- ☐ Fail.

Supervisor's Approval	03 August 2021	Student's Approval
Approved		**Approved**
Katherine, S.T., Ph.D.		**Natasha Denisa**

Names and signature of other committee members present:

1	Field Supervisor	: Putu Virgina Partha Devanthi, S.Si., M.Si., Ph.D.	Approved
2	Lead Assessor	: Solmaz Aslanzadeh, B.Sc., M.Sc., Ph.D.	Approved
3	Assessor 2	: apt. Pietradewi Hartrianti, M.Farm., Ph.D.	Approved

Summary of committee recommendations:

The committee agrees that the student is in the final phase and that completion of the following allows the student to write their thesis report and graduate.

This document has been verified by system, no signature needed.
Scan the QR code to verify the document validity.

Statement of Originality

FR-i3L-5.3.4.4 Rev.0

Statement of Originality

submitted to

Indonesia International Institute for Life Sciences (i3L)

I, **Natasha Denisa**, do herewith declare that the material contained in my thesis entitled:

"Comparison of Methods for Encapsulating *S.cerevisiae* in Bacterial Cellulose Produced by *K.intermedius*"

is original work performed by me under the guidance and advise of my Thesis Advisor,

Katherine, S.T., Ph.D. I have read and do understand the definition and information on use of source and citation style published by i3L. By signing this statement I unequivocally assert that the aforementioned thesis conforms to published information.

i3L has my permission to submit and electronic copy of my thesis to a commercial document screening service with my name included. If you check NO, your name will be removed prior to submission of the document for screening.

☑ Yes
☐ No

Name of student: Natasha Denisa Student ID: 17010055

Study Program: Biotechnology

Signature: _____ Date: 3 August 2021

COPYRIGHT NOTICE

Abstract

Bacterial cellulose (BC) is a three-dimensional structure made up of nanofibrils that are created by aerobic bacteria such as *K.intermedius* which is able to give a high yield of BC despite it being a relatively new strain. BC is well known for its high crystallinity and water retention which are favorable for encapsulating compounds. BC are also known to have wound healing properties but it lacks antimicrobial activity. Probiotic in this case *S.cerevisiae*, is able to be encapsulated into BC to add antimicrobial activity without triggering antibiotic resistance. *S.cerevisiae* is a well-known probiotic that is able to have an antimicrobial effect against *S.aureus* and *P.aeruginosa* which are both common pathogenic bacteria that are found in wounds.

This study aims to find the most effective method of encapsulating *S.cerevisiae* into BC that is produced by *K.intermedius* as well as testing the antimicrobial activity of the encapsulated *S.cerevisiae*. The encapsulation methods to be tested are adsorption-incubation, injection-incubation and co-culture. The method that yields the highest probiotic loading number will be chosen and is tested for the antimicrobial activity through the Kirby-Bauer test as well as the time kill assay. The results however showed that method of encapsulation does not have an effect on the probiotic loading number hence the most practical method, injection-incubation was chosen instead. *S.cerevisae* probiotic BC produced through injection-incubation method was able to exhibit antimicrobial activity towards *S.aureus* and *P.aeruginosa*. In the Kirby-Bauer test it is able to show that it is more effective against *S.aureus* meanwhile in the time kill assay it has a larger bactericidal activity against *P.aeruginosa*.

Keywords: Bacterial cellulose, encapsulation method, *S.cerevisiae*, *K.intermedius*, antimicrobial activity.

Acknowledgements

First and foremost, I would like to thank my supervisors Ms. Katherine and Ms. Gina for all the guidance they have provided all throughout the process of this thesis. I would also like to extend my thanks to all of the lab technicians, especially Mr. Novan who has allowed us to work in the lab as well as continuously supply our materials despite being busy with the Covid lab.

Secondly, I would like to thank my lab partner as well as assistants Michael, Evelyn and Alung for without them I would not be able to finish all the lab work from this thesis. It was a comfort to not work alone on a project that could be said as one of the most important projects of our university life. I am glad I was able to do this project with you all and I am proud of all of us for pushing through and gaining great results. I will never forget the amount of hard work that we all put into this project.

Thirdly, I would like to thank my friends and family. Thank you for believing in me when I don't believe in myself. Thank you as well for always being supportive and understanding. Lastly, thank you for listening to me ramble about my project even though you don't understand much of what I am talking about. I appreciate you all for caring.

Lastly, I would like to thank myself for pulling through. It was hard, there were ups and downs but I am proud of myself to be able to push through and get things done. I would like to give credit to BTS (yes, the band, mind your business.), Ghibli studios and my dogs for keeping me sane as well as giving me a supply of serotonin and comfort midst all the chaos.

If you are reading this as a student who are about to write their thesis, know that it will be hard but I believe you will get through it just fine. Listen to your advisors and just keep going. No storms last forever. All the best of luck on your journey!

Sincerely,

Natasha

Table of Contents

List of Figures

List of Tables

List of Abbreviations

BC	**Bacterial Cellulose**
MRSB	**De Man Rogosa and Sharpe Broth**
PDB	**Potato Dextrose Broth**
NB	**Nutrient Broth**
MHB	**Mueller Hinton Broth**
MHA + 2% Glu	**Mueller Hinton Agar + 2% Glucose**
SC	***S.cerevisiae***
SA	***S.aureus***
PA	***P.aeruginosa***
A-I	**Adsorption-Incubation**
I-I	**Injection-Incubation**
C-C	**Co-Culture**
SEM	**Scanning Electron Microscopy**

CHAPTER 1

1.1 Introduction

Bacterial cellulose (BC) is a three-dimensional structure made up of nanofibrils produced by aerobic bacteria such as *Acetobacter xylinum* (Silvia et al., 2017), *Gluconacetobacter xylinus* (Silvia et al., 2017), and *Komagataeibacter intermedius* (Lin et al., 2016). *K.intermedius* is first isolated from commercial vinegar. *K.intermedius* has a higher yield of BC when compared to *Komagataeibacter xylinus;* formerly known as *Gluconacetobacter xylinus,* which is another common strain that is used to produce BC (Fernandez et al., 2019). Fernandez *et al*.'s study proves that using *K.intermedius* to produce BC has the potential to be used industrially due to its high yield.

BC has been widely studied and used in the pharmaceutical, food and biomedical industries. BC is well known for its high-water retention, high crystallinity as well as great mechanical strength (Manoukian et al., 2018). It also has qualities such as being non-toxic, pure, permeable to liquid, biodegradable and biocompatible which is appealing to the biomedical industries in application for wound healing and local drug delivery. With those characteristics BC is able to act as a scaffold that is able to protect the wound from the outside environment and secondary infections as well as help the wound healing process by inserting compounds such as drugs, antimicrobial agents and even cells into its matrix (Portela et al., 2019).

Antibiotics are usually added into wound dressings to kill pathogenic bacteria at the site of wound (Negut et al., 2018). Repeated usage of products containing antibiotics could develop antibiotic resistance in the long run. Antibiotic resistance could complicate the treatments to otherwise simple bacterial infections, in this case the physician would need to look for another antibiotic that could work to cure the bacterial infection. This would take more time and is risky for the patient as the pathogenic bacteria will continue to proliferate inside the body. According to the World Health Organization antibiotic resistance is one of the biggest health threats globally. Per year there are at

least 700,000 deaths that are caused by antibiotic resistance (Willyard, 2017). To avoid antibiotic resistance the incorporation of probiotics has been suggested instead. There is evidence that probiotics are able to reduce the adverse effects of using antibiotics such as destroying the normal microbiota (Reid, 2006). The most well-known probiotics are strains from *Lactobacillus*, *Bifidobacterium,* some lactic acid bacterias and non-lactic acid bacterias such as *Saccharomyces cerevisiae* (Kechagia et al.,2013). *Saccharomyces cerevisiae,* otherwise known as yeast, has potential of usage in the medical field as a probiotic, even though it is well known for its use in the food industry. Yeast strains have also been extensively studied on their effect on gut health (Chzeruka et al., 2007). There are no studies done yet on whole viable yeast's probiotic effect on cutaneous wound healing.

Non-encapsulated probiotics show a decrease in viability and health benefiting effects when it is released to the environment of a wounded tissue (Saarela et al., 2000). Encapsulation is able to protect the probiotic from external factors such as acidity, oxygen as well as cell injury. By protecting it from external factors it is able to enhance its viability. Other than viability, encapsulation is also able to increase the probiotic's stability due to better binding with the fibrils inside the BC matrix (Huq et al.,2013).

The probiotic of interest *Saccharomyces cerevisiae* is a well-known probiotic but its knowledge on wound healing as well as encapsulation are still limited, which shows that this study is novel and is able to contribute to the field of study. *K.intermedius* adds to the contribution as well as novelty as most BC in the field of study are produced by *K.xylinus*. Using *Saccharomyces cerevisiae* as the probiotic and *K.intermedius* as the BC producing bacteria makes this topic novel and of significance in the field of research.

1.2 Research Question

The research question to be addressed in this study is as follows:

- Which encapsulation method is able to give the highest amount of *S.cerevisiae* loaded inside the BC?
- Would encapsulated *S.cerevisiae* exhibit antimicrobial activity ?

1.3 Research Objectives

The objective of this research is as follows:

- To determine the best way to encapsulate *S.cerevisiae* in bacterial cellulose.
- To measure encapsulated *S.cerevisiae*'s antimicrobial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa.*

1.4 Hypothesis

Based on the information gathered through literature review, the hypothesis is as follows:

- Injection-incubation method is the most effective method to encapsulate *Saccharomyces cerevisiae* and is able to exhibit an antimicrobial activity.
- Encapsulated *Saccharomyces cerevisiae* is able to exhibit an antimicrobial activity.

1.5 Scope of Research

The scope of research for this study is divided into several parts. The first part focuses on the optimization of adsorption-incubation (A-I) and injection-incubation (I-I) methods through modifications of the initial cell loading as well as the method of adsorption and injection. The optimized method with the best encapsulation results will be compared with the co-culture method (C-C) which is not able to be optimized. The best encapsulation method obtained from this round of comparison will proceed to be used to create probiotic BC which will be tested for its antimicrobial activity. The overall flow chart of this study will be shown in figure 1.1.

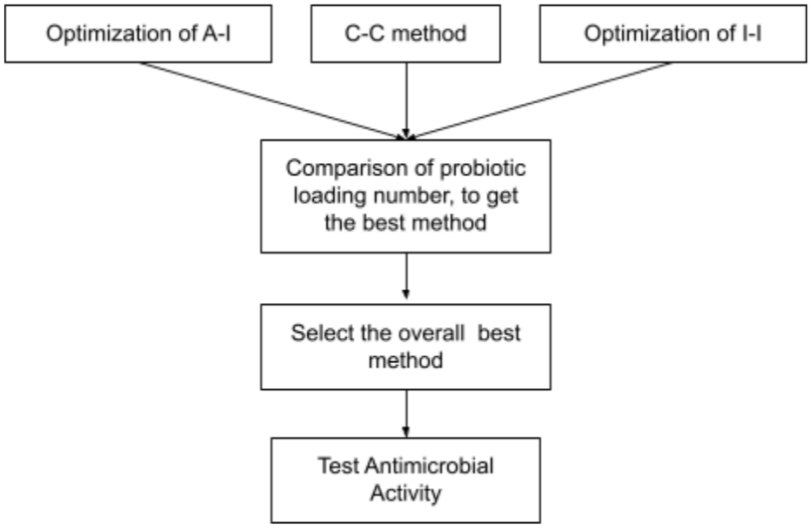


Figure 1.1 Flowchart of the overall study

CHAPTER 2 - Literature Review

2.1 Bacterial Cellulose for Wound Healing

Cellulose is a natural polymer that is able to be produced by different types of organisms such as bacteria and plants. *Komagataeibacter* strains produce BC to maintain high oxygenation for the colonies that are near the surface, protecting the colonies from drying as well as radiation (Portela et al, 2019). As mentioned in the introduction, *K.intermedius* is able to produce a high yield of BC. Not to mention, it also has a higher degree of crystallinity as well as a more homogenous size distribution when compared to BC that was produced by *K.xylinus* (Fernandez et al., 2019). These aspects are desirable to be able to encapsulate a compound. BC's porous structure allows the incorporation of compounds or even probiotics into the BC.

When BC is compared with plant cellulose (PC), BC is chemically pure as it does not contain any hemicellulose, pectin or lignin which makes processing of the BC to be more efficient industrially as there is no need to remove those impurities. This purity factor of BC is also responsible for its biocompatible and nontoxic properties. BC also has thinner fibrils that range from 40-80nm in diameter, which is 100x smaller than PC. This allows BC to have favorable characteristics such as elasticity, resistance, flexibility, higher surface area, high water holding capacity as well as high adsorption (Sabio et al., 2021; Portela et al.,2019). BC is also moist and hydrophilic, which provides a suitable environment for wound healing. The structure of BC itself is able to facilitate cell migration and accelerate granulation. In addition, BC's flexibility and elasticity allows it to conform following the wound's shape and protect it from outside factors (Khalid et al.,2017). Therefore, it can be concluded that BC is a suitable scaffold for wound dressing as well as wound healing. However, BC on its own is not able to help wound healing as effectively as when it is combined with another component as it lacks antimicrobial activity. For this reason, studies have been done to add antimicrobial agents into BC. A study done by Lemnaru (Popa) et al. in 2020 added antibiotics (bacitracin and amoxicillin) to add

antimicrobial activity to the BC. Addition of these antibiotics on the BC was tested against *E.coli* and *S.aureus* and it showed that BC loaded with bacitracin was able to inhibit cell growth meanwhile both bacitracin and amoxicillin were able to lower the growth rates of cells. However, repeated usage of antibiotics can lead to antibiotic resistance.

2.2 Common Skin Pathogens' Antibiotic resistance and Probiotic's potential

Most pathogenic bacteria are able to develop resistance towards antimicrobial agents. The main mechanisms of antimicrobial resistance are limiting the uptake of a drug, modification of the drug target, inactivation of a drug, and active efflux of a drug. Not all bacteria are susceptible or resistant towards a particular antimicrobial agent. The level of antimicrobial resistance varies within different bacteria (C Reygaert, 2018). The factors that have contributed to the antimicrobial resistance problem are, increased consumption of antimicrobial drugs, improper prescription of antimicrobial therapy and the overuse of common antimicrobial agents. Physicians tend to overuse as well as improperly prescribe antimicrobial drugs due to the low cost as well as low toxicity of these drugs (Griffith et al., 2012). The improper prescription may also be due to the prescription of a broad-spectrum antibiotic that is unnecessary and/or ineffective towards the organism(s) that caused the infection (Yu, 2011).

Some of the most common skin pathogens are *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Both pathogens have a notorious ability to become antimicrobial resistant. *S.aureus*' antibiotic resistance is often acquired through horizontal gene transfer from external sources. Factors such as mutations as well as antibiotic selection also play a role in antibiotic resistance. Infections caused by *S.aureus* have reached an epidemic scale globally, specifically infections caused by methicillin resistant S.aureus strains (MRSA). This pathogen is also commonly found in wounds that could lead to a secondary infection (Chambers & DeLeo, 2009).

P.aeruginosa is another pathogenic bacteria that is well known to have a remarkable ability to resist antibiotics. They resist antibiotics through resistance mechanisms such as biofilm production

and formation of multidrug tolerant cells (Pang et al.,2019). The spread of the multidrug tolerant strains has been a public health threat. *P.aeruginosa* are able to cause severe secondary infections in healthcare settings and spread its ability of antimicrobial resistance *in-vivo* (Horcajada et al., 2019). Wounds provide favorable environments for bacteria to be able to grow. It provides a warm, moist and nutritious environment for the bacteria. The amount and diversity of the microorganism that is able to infect the wound depends on the type, depth and location of the wound as well as the host's immune response (Bowler et al., 2001). Both *S.aureus* and *P.aeruginosa* are among the organisms that are most commonly isolated from severe wounds (Almeida et al., 2014). Unfortunately, there is a lack of therapeutic alternatives to treat their antibiotic resistant strains.

The use of probiotics could be an option to treat antibiotic resistant strains. Probiotics are well known for their health benefits and its ability to fight off pathogens. Probiotics produce antimicrobial factors that are able to serve as an alternative to antibiotic treatments towards topical infections as well as chronic wounds. The desirable traits that probiotic has include acid/bile tolerance for oral administration, adhesion towards mucosal and epithelial membranes that enables modulation of immune responses and its ability to exude antimicrobial activity towards pathogenic bacteria. The exact mechanism of action of probiotics has still yet to be discovered. Studies have proposed mechanisms such as being able to produce bacteriocin, lowering the gut pH, competing for nutrients that simulates immunomodulation, inducing phagocytosis and modifying T-cell responses (Kechagia et al., 2013).

2.3 Yeast's potential as a probiotic that assists wound healing

Saccharomyces cerevisiae (S.cerevisiae) is one of the most well studied as well as used in industrial settings. 20 species of the *Saccharomyces* genus are of biotechnological importance as it is applied in alcoholic fermentations, single cell proteins, recombinant protein, vitamin production as well as biological control (Webster & Weber, 2007). Out of all the species under Saccharomyces, *S.cerevisiae* is the most significant due to its economic impact. Every year, S.cerevisiae is used to

produce 30 million tons of wine, 60 million tons of beer, 800.000 million ton of single cell protein and 600.000 million ton of baker's yeast (Pretorius et al., 2003).

A strain of *S.cerevisiae*; *S.cerevisiae boulardii* (*S.boulardii*) is extensively studied for its potential as a probiotic that is able to treat diarrhea and colitis (Kelesidis & Pothoulakis, 2012). *S.boulardii*'s probiotic mechanism includes binding or neutralizing toxins that are produced from pathogenic bacteria, reducing inflammation and inducing the secretion of IgAs (Palma et al., 2015). When *S.cerevisiae* is compared with *S.boulardii,* they are both genetically similar with the phenotypic differences of acidity and heat tolerance (Sen & Mansell, 2020). *S.boulardii* is able to tolerate high temperatures; its optimum temperature being 37ºC as opposed to S.cerevisiae with 30ºC as well as acidity which allows it to survive in the gastric environment (Palma et al., 2015). With its similarity in genetic makeup it can be hypothesized that *S.cerevisiae* could have a similar mechanism of being a probiotic.

Some strains of *S.cerevisiae* are able to have antimicrobial and probiotic properties (Nayak, 2011). Anti-bacterial capability of *S. cerevisiae* are suspected to be due to production of extracellular protease, secretion of inhibitory proteins, stimulation of immunoglobulin A, elimination of secreted toxins, production of killer toxins and production of sulfur dioxide (Fakruddin et al., 2017). *S.cerevisiae* are able to produce toxins that are able to kill pathogenic bacteria in food (Younis et al.,2017).

There are no studies on *S.cerevisiae* as a whole cell on its effect as a probiotic specifically for wound healing. However, there are studies that look into the antimicrobial and probiotic abilities in treating gut diseases. Food grade yeast are found to be able to excrete anti-inflammatory factors during colitis in mice (Foligné, 2010). *Saccharomyces cerevisiae* was able to inhibit *Bacillus subtilis, B. cereus, Escherichia. coli, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella typhimurium, Salmonella typhi, Staphylococcus aureus, Yersinia enterocolitica*, and *Candida albicans* during intestinal tract infection (Fakruddin et al., 2017). Two amongst all the bacteria mentioned; *Staphylococcus aureus* and *Pseudomonas aeruginosa* are common skin pathogens found in wounds.

In the context of wound healing, it has been found that live yeast cell derivative is able to help wound healing. It is to be noted that live yeast cell derivatives are not whole cells of yeast, instead it is an alcoholic extract of *S.cerevisiae* (Bentley, 1990). The extract was well known to be able to increase oxygen uptake as well as induce the production of collagen. The live yeast cell derivative is able to help wound healing by inducing angiogenesis in chick embryo yolk sac membrane assay and rabbit cornea assay (Bentley, 1990). Angiogenesis is able to facilitate cell epithelization towards the wound. A more recent study done in 2018 by Gruenstein et al. shows that live yeast cell derivative is able to enhance the closure of wound on diabetic mice by inducing angiogenesis, formation of granulation tissue as well as epithelization of cells. If *S.cerevisiae* whole cells are able to also have similar effects as its derivative it would be beneficial towards wound healing.

2.4 Encapsulation Methods

Encapsulation is the process in which material(s) are coated or entrapped within another material. The coating or entrapping material protects the active material encapsulated from external environmental stresses such as acidity, oxygen and aids it to pass through a barrier. Encapsulation isolates the cells from the adverse environment which is able to reduce cell injury (Huq et al., 2013). In this study the coating or entrapping material is BC and the probiotic *S.cerevisiae* is the active material encapsulated. BC has hydroxyl groups on the surface which facilitates coating of a compound through chemical bonding. Unfortunately, the bonds formed during coating are weak hence it is prone to shed easily. Meanwhile, during encapsulation the compound is entrapped inside the matrix of the BC which allows for a more secure adherence as compared to coating (Sabio et al.,2021).

There are three types of encapsulation methods that have been done to encapsulate cells into bacterial cellulose; adsorption-incubation, co-culture method and injection-incubation method. The adsorption-incubation method takes the advantage of the mechanism of microbial cells being able to adhere to porous or non-porous surfaces. This method is also considered to be the easiest as well as cost effective (Żywicka et al, 2019). Initially the adsorption-incubation method did not include the incubation portion, it was only added after an optimization of the method done by D.N. Nguyen et al.

The method was optimized because adsorption was not able to give a high biomass concentration per unit volume. After the addition of the incubation step it increased the biomass concentration per unit volume but still remains to be a simple method for encapsulation (D.N. Nguyen et al., 2009).

Adsorption-incubation method also have been used by Żywicka *et al*. to encapsulate *S.cerevisiae* (spherical shaped), *L.delbrueckii* (rod shaped) and *Y.lipolytica* (hyphae form) to see the immobilization pattern of microorganisms with different cell shapes and size. The microorganisms are incubated for 5 hours at the adsorption stage, this yielded the adsorption efficiency of 52% for *S.cerevisiae,* 51% *Y.lipolytica* and 61% for *L.delbrueckii*. The incubation stage was done for 48 hours which is the maximum for all of the microorganisms. The efficiency of the incubation is recorded to be 34% for *S.cerevisiae*, 33% for *Y. lipolytica* and 51% for *L. delbrueckii*. This study found that using BC that was grown for a short period of time (3 days) yielded the highest adsorption and incubation results as it has more surface area (Żywicka *et al*.,2019).

The second method of incubation is the co-culturing method. This method is done by growing the BC along with the microorganism of interest that will be encapsulated. The co-culturing method was done by Sabio *et al*. to grow probiotics of *Lactobacillus* strain into BC that is produced by *K.xylinus*. To remove the *K.xylinus* that is entrapped in the BC, the BC was moved into a media that creates an anaerobic environment that is more suitable for the probiotic to proliferate. This resulted in the probiotic to proliferate and take more space inside of the BC which was shown through gram staining and imaging through dark field microscopy. In Sabio et al.'s study, it was found that the adsorption-incubation method was able to encapsulate 10^{10} CFU of probiotic per gram of BC. When compared to the amount that was encapsulated through the co-culture method that yielded 10^{14} CFU per mg of cellulose, adsorption-incubation method clearly has lower encapsulation efficiency. The probiotic BC obtained from the adsorption-incubation method was only able to show little to none antimicrobial activity. This led to the conclusion of the probiotic only being able to give antibacterial when it is encapsulated inside the BC and not adsorbed (Sabio et al., 2021). Another study also used the co-culturing method to incorporate probiotic into BC and the results showed that agitated co-culture is

able to encapsulate more probiotics as compared to other methods tested; adsorption-incubation and static co-culture (Fijałkowski et al.,2016).

The third method of encapsulation is the injection-incubation method. This method is the simplest as well as the least time consuming. It has been done to encapsulate *S.cerevisiae* into BC for ethanol fermentation. The result of the immobilization was shown by SEM observation, the picture that was shown in the paper showed that *S.cerevisiae* was encapsulated securely between the fibrils of the BC. This study did not count the amount of *S.cerevisiae* that is encapsulated in the BC after the injection. However, they were able to find that injecting BC that was grown at a static condition was more suitable for encapsulation because the SEM showed that the yeast was held securely between the fibrils and it maintained the structure of the yeast as well (Yao et al., 2011).

Between the three methods for encapsulation it seems that adsorption-incubation is the most common method to encapsulate cells into BC as there has been more previous studies available. When all the three methods are compared, co-culture seems to be the one able to encapsulate more of the probiotic which is constant among two studies. Despite its effectiveness as an encapsulation method there isn't any study that has attempted to encapsulate *S.cerevisiae* using the co-culture method. The injection-incubation method could be the most suitable for *S.cerevisiae* encapsulation despite its lack of study as compared to adsorption-incubation and co-culturing methods. Injection-incubation is able to give a shortcut of entry into the BC as compared to the other two methods (adsorption-incubation and co-culture) where the *S.cerevisiae* need to localize itself into the BC while it grows inside the media. Not to mention, during co-culture it needs to compete with *K.intermedius* for space in the BC. This could be harder for *S.cerevisiae* as it has a larger size (5-10 µm in diameter) compared to bacteria (0.2 - 2µm in diameter). It must be noted that there is no type of immobilization method that is suitable for all cell types (Górecka & Jastrzębska, 2011).

CHAPTER 3 - Material and Methods

The material and methods are split into several sections: preparation of microorganisms (section 3.1), preparation of BC (section 3.2), encapsulation methods (section 3.3), antimicrobial assay (section 3.4), analysis and characterization (section 3.5) and lastly the statistical analysis (section 3.6). Section 3.1 and 3.2 explains how the microorganisms are grown and how the BC was made and treated prior to encapsulation. Section 3.3 explains the methods used to encapsulate *S.cerevisiae* into the BC and section 3.4 explains the method to measure the antimicrobial activity of the encapsulated *S.cerevisiae*. Section 3.5 and 3.6 explains what is measured and how it was analyzed from the encapsulation as well as the antimicrobial assay.

3.1 Preparation of Microorganism

3.1.1 Preparation of Microorganisms

Komagataeibacter intermedius was isolated from Kombucha provided by PT. Tujju Bio Indonesia. Powder culture of *Saccharomyces cerevisiae* was obtained from SIGMA (Yeast from S.cerevisiae, SIGMA). The cultures are grown in 100 ml of MRS broth (MRS,Merck) on a 250 ml erlenmeyer and 200 ml of potato dextrose broth (PDB) (Potato Dextrose Broth, OXOID) on a 250 ml erlenmeyer respectively. Both cultures were incubated statically at 30ºC for 3 days.

S.aureus and *P.aeruginosa* cultures were obtained from the i3L lab in the form of streaked agar plates. Both cultures were grown on TSA agar. Single colonies from each agar plate were taken using a sterilized loop and are cultured into two separate 100 ml of nutrient broth (Nutrient Broth, OXOID) in a 250 ml erlenmeyer. Both cultures were incubated statically at 37ºC for 3 days.

3.2 Preparation of Bacterial Cellulose

3.2.1 Production of Bacterial Cellulose

Each well in a 24 well plate (BIOLOGIX) was added with 100 µl of 5 x 10^7 CFU/ml *K.intermedius* from the starter culture and 2 ml of MRSB. The well plates were incubated at 30°C for four days until

a bacterial cellulose layer was formed. The bacterial cellulose produced will be used for the encapsulation methods (A-I, I-I and CC) as well as the antimicrobial assay (time kill assay and Kirby bauer).

3.2.2 Sterilization of Bacterial Cellulose

This method was done to remove any bacteria that is inside the BC before encapsulation. BC harvested from the 24 well plates will be rinsed using sterilized water to remove excess media. To sterilize, the BC was submerged in 100 ml of 1M NaOH solution (NaOH, MERCK) for 24 H at 30°C. Acetic acid (Acetic Acid, MERCK) was then added into the NaOH solution containing the BC to neutralize the BC's pH. The pH was measured using a pH meter (Starter300, OHAUS), to make sure it stays between the range of pH 6.5-7. Lastly, the BC was rinsed with sterile water to remove excess NaOH and acetic acid (Savitskaya et al.,2019). Sterilization of BC was done for BC that will undergo adsorption-incubation and injection-incubation method.

3.3 Encapsulation Methods

3.3.1 Co-culture (CC) Encapsulation

An equal amount (100 µl) of $5x10^6$ CFU/ml *K.intermedius* and $5x10^7$ CFU/ml *S.cerevisiae* culture were added to the wells of a 24 well plate along with 1.8 ml of MRSB in each well. The 24 well plates were incubated for 4 days at 30°C to allow the BC to grow while simultaneously encapsulating the probiotic into the BC (Sabio et al., 2021). After incubation, an anaerobic environment was created by adding 1 ml of mineral oil on top of the cultures. This was done to remove any *K.intermedius* from the BC as well as the liquid culture. Giving the desired result of a probiotic BC only containing *S.cerevisiae*. Before the addition of the mineral oil, the BC was gently sunk into the media using a sterilized spatula. This prevents the BC from getting in contact with the oil which is hard to rinse off. The culture was incubated for another 2 days at 30°C statically under these anaerobic conditions. The amount of *S.cerevisiae* encapsulated inside the BC will be measured using the method described in section 3.5.4.

3.3.2 Optimization of Adsorption-incubation (A-I) Method

Firstly optimization was done for the adsorption-incubation method by varying the initial cell concentration to be encapsulated inside the BC as well as the mode of adsorption. The two modes of adsorption were static or shaking at 120 rpm. The cell concentrations used were 1 x10^5, 1 x 10^7 and 1 x 10^9 CFU/ml of *S.cerevisiae.*

The experimental design of the method can be seen in Table 1.1. During shaking adsorption it was incubated at 30ºC on an orbital shaker incubator (Orbital Shaker Incubator, MRC) with a speed of 120 rpm for one day. Meanwhile, during static adsorption it was incubated statically inside a 30ºC incubator for one day. The adsorption stage allows the *S.cerevisiae* cells to adhere to the BC's surface.

During the incubation stage, the adsorbed BC was moved into individual 100 ml erlenmeyer flasks filled with 50 ml of PDB to allow the *S.cerevisiae* to proliferate inside the BC. It was incubated at 30ºC under static condition for two days (Nguyen et al., 2009). The amount of *S.cerevisiae* encapsulated inside the BC will be measured using the method described in section 3.5.4.

Table 1.1 Experimental design of Adsorption-Incubation method (SH: shaking adsorption; ST: static adsorption; 5: initial cell loading of 10^5 CFU/ml *S.cerevisiae*; 7: initial cell loading of 10^7 CFU/ml *S.cerevisiae*; 9: initial cell loading of 10^9 CFU/ml *S.cerevisiae*)

Method of adsorption/Initial cell loading	1 x 10^5 CFU/ml *S.cerevisiae*	1 x 10^7 CFU/ml *S.cerevisiae*	1 x 10^9 CFU/ml *S.cerevisiae*
Shaking Adsorption	SH-5	SH-7	SH-9
Static Adsorption	ST-5	ST-7	ST-9

3.3.3 Optimization of Injection-incubation (I-I) Method

Firstly optimization was done for the injection-incubation method by varying the initial cell concentration to be encapsulated inside the BC as well as the site of injection whether it was single

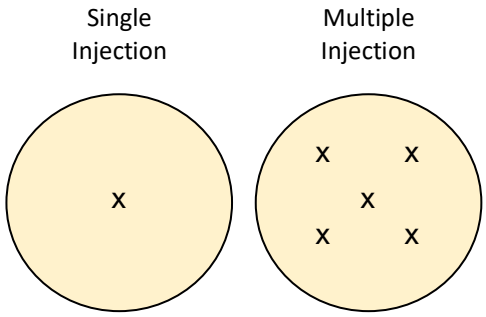
or multiple (5 sites). The cell concentrations used were 1 x10^5, 1 x 10^7 and 1 x 10^9 CFU/ml of *S.cerevisiae.*

The experimental design of the method can be seen in table 1.2. Sterilized BC were injected with the three different concentrations of cells as well as different injection methods which were done in triplicates. A single site injection was done by injecting the *S.cerevisiae* culture into the middle of the BC. Meanwhile, multiple sites will be injected in five different sites. The diagram of the injection sites can be seen in figure 1.2. *S.cerevisiae* cultures are all injected into the BC using a sterile syringe (Sterile Syringe 1 ml, BIOLOGIX).

The volume of injection depended on the wet weight of the BC, it followed the BC's water capacity of 0.8 ml/g BC. The push given from the insertion using syringe helped to push the liquid culture of *S.cerevisiae* to spread all throughout the porous structure of the BC. The excess culture was then rinsed off using deionized water. The injected BC was put into a 100 ml erlenmeyer with 50 ml of PDB to allow the *S.cerevisiae* to proliferate inside the BC, this was incubated statically at 30ºC for 2 days (Yao et al., 2011). The amount of *S.cerevisiae* encapsulated inside the BC will be measured using the method described in section 3.5.4.

Table 1.2 Experimental design of Injection-Incubation method (SI: single injection; MI: Multiple injection; 5: initial cell loading of 10^5 CFU/ml *S.cerevisiae*; 7: initial cell loading of 10^7 CFU/ml *S.cerevisiae*; 9: initial cell loading of 10^9 CFU/ml *S.cerevisiae*)

Method of injection/Initial cell loading	1 x 10^5 CFU/ml *S.cerevisiae*	1 x 10^7 CFU/ml *S.cerevisiae*	1 x 10^9 CFU/ml *S.cerevisiae*
Single injection	SI-5	SI-7	SI-9
Multiple injection	MI-5	MI-7	MI-9



Note: x annotates the injection sites

Figure 1.2 Diagram of injection sites on the BC for single and multiple injection

3.4 Antimicrobial Activity Assays

3.4.1 Kirby Bauer Method

Kirby Baeur method was used to determine the antimicrobial activity of the probiotic BC after encapsulation. This test was done against the pathogenic bacteria *S. aureus* and *P. aeruginosa.* The list of the samples and controls can be found in table 1.3.

Firstly, 1 X 10^8 CFU/ml *S.aureus* and *P.aeruginosa* were spread plated onto separate Mueller Hinton Agar (MHA) with 2% glucose and was left to dry and settle for 30 minutes. Probiotic BC from each encapsulation method; A-I and I-I, was placed on the agar plates that were already spread with *S.aureus* and *P.aeruginosa.* For the controls, filter paper was cut out to the same size as the probiotic BC and was soaked on their respective solutions; *S.cerevisiae* culture and antibiotic solutions. The discs were allowed to soak in the solutions for 30 minutes. The soaked filter paper discs are then placed on their respective agar plates that were already spread with *S.aureus* and *P.aeruginosa.* All measurements were done in triplicates.

The plates were incubated at 37°C for 24 hours. After 24 hours, the plates were observed to measure the clear zone formed around the probiotic BC and filter paper discs. The clear zone's area was calculated through ImageJ software; the method is detailed in section 3.5.5. The measurements are all reported in millimeters square.

Table 1.3 List of Samples and Controls for Kirby Bauer Test.

Sample	Control
S.cerevisiae probiotic BC + *S.aureus*	*S.cerevisiae* only + *S.aureus*
S.cerevisiae probiotic BC + *P.aeruginosa*	*S.cerevisiae* only + *P.aeruginosa*
	S.aureus + BC only
	P.aeruginosa + BC only
	S.aureus + Chloramphenicol (1 mg/ml)
	P.aeruginosa + Polymyxin B (1 mg/ml)
	S.aureus only
	P.aeruginosa only

3.4.2 Time kill assay

Time kill assay was done to assess the bactericidal or bacteriostatic activity of the probiotic BC. This assay was done against the pathogenic bacteria *S. aureus* and *P. aeruginosa.* The list of samples as well as controls can be seen in table 1.4. Firstly, 100 µl of the pathogenic bacteria with the CFU of $1x10^5$ CFU/ml were incubated with the probiotic BC inside a 50 ml centrifuge test tube filled with 5 ml of Mueller Hinton Broth with addition of 2% glucose (MHB) (Sabio et al.). The tubes were incubated at a 37°C incubator for a maximum of 24 hours. The amount of pathogenic bacteria in the media will be measured after incubation of 1H, 6H and 24H (Savitskaya et al.,2019).

Miles-Misra plating was done to obtain the CFU of pathogenic bacteria after each incubation period (after 1h, 6h and 24h). 100µl aliquots were taken from the culture after incubation. The aliquots of the samples and controls were then diluted 12 times with the factor of 10 with PBS solution (Phosphate buffered saline tablet, SIGMA) as the diluent. The amount of dilution can increase as needed.

Agar plates used for miles misra were divided into four quadrants and labeled with the desired dilution to be dropped on the respective quadrants before use. 5 µl of the concentrations 10^{-1} to 10^{-12} were dropped in triplicates onto MacConkey Bile Agar (MCBA) for *P.aeruginosa* cultures and Baird Parker Agar (BPA) for *S.aureus* cultures. All of the agar plates containing *S.aureus* and *P.aeruginosa* were incubated in a 37°C incubator for one to two days. After incubation, the plates were counted using a plate counter. Quadrants that have drops with ≤ 20 single colonies were counted. The CFU values obtained were converted to CFU/ml and compared to the initial value to determine its antimicrobial effect.

If the CFU/ml value stayed the same as the initial, the antimicrobial activity was deemed to be bacteriostatic. Meanwhile, if the CFU/ml value dropped by 3 magnitudes of order it can be considered as bactericidal (Gallant-Behm et al., 2005). The CFU/ml values of the sample and the control were compared by doing ANOVA method. If a significant difference is indicated then it shows that the encapsulated probiotic is able to have an antimicrobial effect against the pathogen.

Table 1.4 Experimental Design for Time Kill Assay (SA: *S. aureus*; PA: *P.aeruginosa*; SC: *S.cerevisiae*; SC BC: *S.cerevisiae* probiotic BC; BC: bacterial cellulose only)

Pathogenic Bacteria/Sample	*S.cerevisiae* BC	*S.cerevisiae* only	BC only	Pathogen only
S.aureus	SA + SC BC	SA + SC	SA + BC	SA only
P.aeruginosa	PA + SC BC	PA + SC	PA + BC	PA only

3.5 Analysis and Characterization Method

3.5.1 Cell count through spread plate method

Cell count by spread plate method was used to obtain initial CFU/ml value for *K.intermedius* before making the BC. 1 ml of the *K.intermedius* starter culture was taken and was diluted with the factor of 10 using PBS solution (Phosphate buffered saline tablet, SIGMA) as the diluent. Concentrations 10^{-3} to 10^{-7} were spread onto MRSA agar plates and were left to incubate for 1-2 days at 30°C. After incubation the plates were counted using a plate counter. Plates with more than 300 colonies are deemed TNTC (too numerous to count).

3.5.2 *S.cerevisiae* Cell Count using Haemocytometer

Haemocytometer was used to obtain the CFU of *S.cerevisiae* before and after encapsulation inside the BC. The haemocytometer (Haemocytometer, Assistent) was prepared by cleaning the slide as well as cover slip with ethanol. Solution for the count was prepared by adding 100 µl of the *S.cerevisiae* culture or digested probiotic BC liquid that contains *S.cerevisiae* with 100 µl of trypan blue dye (Trypan Blue Stain (0.4%), Gibco). Both of the solutions were mixed by resuspending and were left to incubate for 1 minute. After incubation was done, 10 µl of the solution was pipetted onto the haemocytometer slide and it was covered with the coverslip. The slide was then observed using a light microscope under 40x magnification.

S.cerevisiae cells were counted using a manual tally counter. Budding cells were counted as two cells or more if the bud is 50% of the mother cell's size. Only viable cells are counted which are distinguished by the white color of the cells as they were able to flush out the trypan blue dye. Cells that are colored dark blue are not counted as they are deemed unviable.

3.5.3 Standard Curve for Pathogenic Bacterias

Standard curve was made to be able to count the initial number of pathogenic bacteria to be used for the time-kill assay and Kirby Bauer test. Standard curve was made for both *S.aureus* and *P.aeruginosa* separately. 100 µl of *S.aureus* and *P.aeruginosa* culture respectively from 100 ml of nutrient broth with the CFU of $1x10^{10}$ CFU/ml was taken and diluted 12 times by the factor of 10 using

PBS as the diluent. Each dilution was then measured for their optical density. Optical density (OD) measurements of the culture were done using a UV-vis Spectrophotometer (NanoQuant infinite M200, TECAN) at the absorbance of 600 nm. Spread plating was also done alongside the 0D600 measurement to obtain the CFU of the cultures. The CFU were then plotted against the absorbance values to form a standard curve. The standard curve graph for both pathogenic bacteria can be seen in appendix 1.1 and 1.2.

3.5.4 Probiotic Loading

Probiotic loading was done to assess which encapsulation method was able to encapsulate the most amount of *S.cerevisiae* into the BC before incubation and after incubation. Measurement was done before and after incubation to show if *S.cerevisiae* is able to grow inside the BC during the incubation period.

Firstly, the probiotic BC's diameter, thickness and wet weight was measured using a vernier caliper and an electronic balance (Mettler Toledo) respectively. The probiotic BC was then digested using a 10% cellulase solution that was made by adding 500µl of cellulase into 5 ml of PBS. The probiotic BC-cellulase solution was incubated for one day. The liquid obtained after the digestion was used to count the CFU of *S.cerevisiae* present inside the BC. The cells are then counted using a haemocytometer. The haemocytometer count method is detailed at section 3.5.2. The data from probiotic loading will be shown as CFU/g BC.

3.5.5 ImageJ Analysis

ImageJ software was used to calculate the area of inhibition for the Kirby Bauer test results. First the image is loaded into the software and the scale is set. After setting the scale the zone of inhibition is traced using the free hand tool to select the area to be calculated. The area is then reported as mm^2.

3.6 Statistical analysis

For statistical analysis, one-way ANOVA, two-way ANOVA, Post Hoc analysis (Tukey HSD) and unpaired T-test was performed in Microsoft Excel to compare the means of the sample group to the control groups in this experiment. One-way ANOVA was used to was used to compare three or more groups of means that has one independent variables, it was used to analyze the results for probiotic loading number (CFU/g) during comparison of the three encapsulation methods. Two-way ANOVA was used to compare three or more groups of means that has two independent variables, it was used to analyze the results for probiotic loading number (CFU/g) during optimization of A-I and I-I method. Post Hoc Analysis was done as a follow up to the two-way ANOVA test that yields significant difference in data means. T-test was performed to compare two groups of means, it was used to analyze the results from the probiotic loading number (CFU/g) Time kill test (CFU/ml) and Kirby Bauer test (mm^2). For all the statistical analysis, the P-value was taken as the result. The difference is deemed to have a significant difference if the P-value is less than 0.05.

Chapter 4 - Results

The probiotic loading number (log CFU/g of BC) before and after incubation for the optimized method of Adsorption-Incubation and Injection-Incubation methods is evaluated to obtain the optimized parameter for each method. The probiotic loading number of the optimized adsorption-incubation and injection-incubation method is then compared to the unoptimized co-culture method to decide the best method to encapsulate *S.cerevisiae* into BC. In the antibiotic assay the area of inhibition of the samples and the control for the Kirby Bauer method are analyzed to see if the probiotic BC has antimicrobial activity. Meanwhile in the time kill assay the log CFU/ml of the pathogen was analyzed to determine the type of antimicrobial activity that is exhibited by the probiotic BC.

4.1 Probiotic Loading Number of Optimized A-I and I-I

4.1.1 Optimization of The Method of Adsorption and Initial Probiotic Loading in Adsorption Incubation Method

Probiotic loading number was measured to show the amount of *S.cerevisiae* cells present in the BC before and after incubation. Measurement taken before incubation was done to observe if *S.cerevisiae* is able to enter the BC right after encapsulation. Measurement taken after the incubation was done to observe if *S.cerevisiae* is able to grow inside the BC. The results are shown in log CFU/g of BC.

The results shown in figure 2.1 shows that before incubation. When the results among initial cell concentrations were compared using two - way ANOVA it yielded the P-value of 0.047 which was deemed significant. This means that initial cell concentration does have an effect on probiotic loading before incubation. A post hoc test was done and it showed that the result from the group that was initially loaded with 1 x 10^5 CFU/ml *S.cerevisiae* has a significant difference with results from the other

two cell concentrations, this is indicated using the alphabets above the bars in figure 2.1. The post hoc results can be seen in table 2.1 at the appendix.

Results among shaking and static adsorption were also compared using two-way ANOVA and it yielded the P-value of 0.732 which was statistically not significant. This means that the method of adsorption has no effect on the probiotic loading number. Nevertheless, this indicates that *S.cerevisiae* was able to enter the BC during adsorption.

The highest probiotic loading number was achieved by static adsorption with an initial cell concentration of 1 x 10^9 giving the value of 7.796 log CFU/g of BC. The lowest probiotic loading number was achieved by shaking adsorption with an initial cell concentration of 1 x 10^5 giving the value of 5.197 log CFU/g of BC.

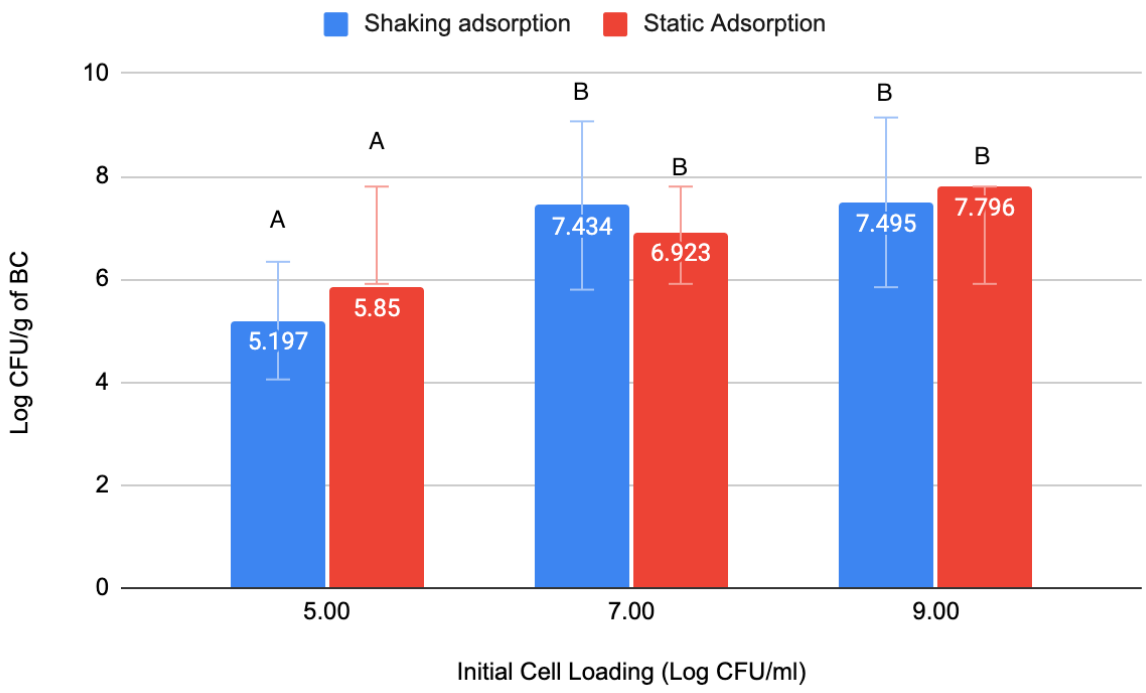


Figure 2.1 The effect of method of adsorption and initial cell loading on probiotic loading number of *S.cerevisiae* after adsorption step in A-I method. The sets of data that share the same alphabet are not significantly different. (P-value > 0.05)

The results shown in figure 2.2 shows that after incubation. When the results among initial cell concentrations were compared using two-way ANOVA it yielded the P-value of 0.165 which was

deemed not significant. Results among shaking and static adsorption were also compared using two-way ANOVA and it yielded the P-value of 0.102 which was statistically not significant. This indicates that both the method of adsorption and the initial cell loading does not affect the probiotic loading number after incubation. The amount of *S.cerevisiae* inside the BC has a maximum amount of approximately 7 log CFU/g of BC.

All three initial cell loading groups have approximately 10^7 CFU of *S.cerevisiae* per gram of BC. The highest probiotic loading number was achieved by shaking adsorption with an initial cell concentration of 1 x 10^9 giving the value of 7.893 Log CFU/g of BC. The lowest probiotic loading number was achieved by static adsorption with an initial cell concentration of 1 x 10^7 giving the value of 7.06 log CFU/g of BC.

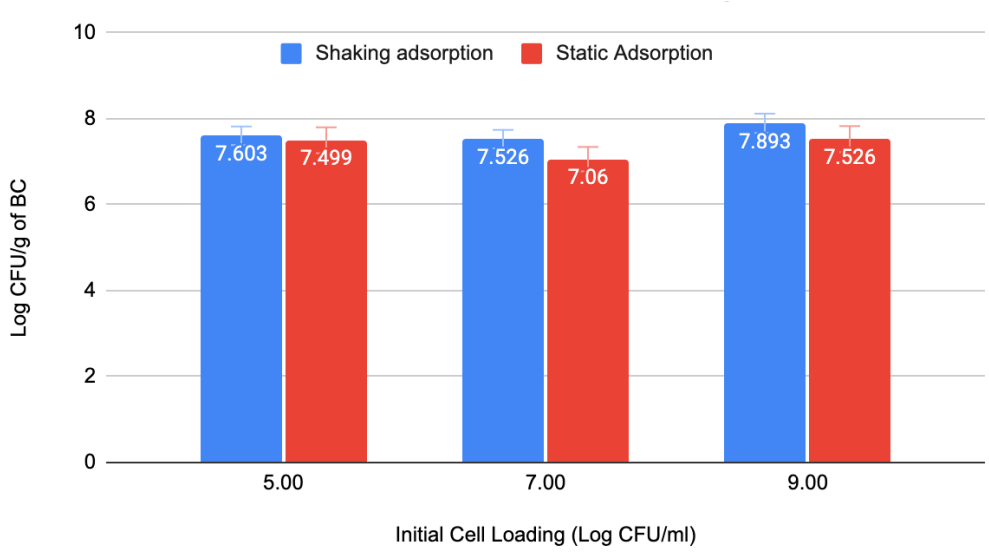


Figure 2.2 The effect of method of adsorption and initial cell loading on probiotic loading number of *S.cerevisiae* after incubation step in A-I method.

4.1.2 Optimization of The Method of Injection and Initial Cell Loading in Injection Incubation Method

Figure 2.3 shows how method of injection and initial probiotic concentration affects PLN in BC before incubation step. When the results among initial cell concentrations were compared using ANOVA it yielded the P-value of 0.012 which was deemed significant. This means that initial cell

concentration does have an effect on probiotic loading before incubation. A post hoc test was done and it showed that the result from the group that was initially loaded with 1 x 10^5 CFU/ml *S.cerevisiae* has a significant difference with results from the other two cell concentrations. The post hoc results can be seen in table 2.2 at the appendix.

Results among single site and multiple site injection were also compared using ANOVA and it yielded the P-value of 0.117 which was statistically not significant. This indicates that the method of injection does not have an effect on the probiotic loading number before incubation. Nevertheless, it shows that *S.cerevisiae* was able to enter the BC regardless of the initial amount of cell loading and the different method of injection.

The highest probiotic loading number was achieved by single site injection with an initial cell concentration of 1 x 10^9 giving the value of 7.706 log CFU/g of BC. The lowest probiotic loading number was achieved by multiple site injection with an initial cell concentration of 1 x 10^5 giving the value of 5.562 log CFU/g of BC.

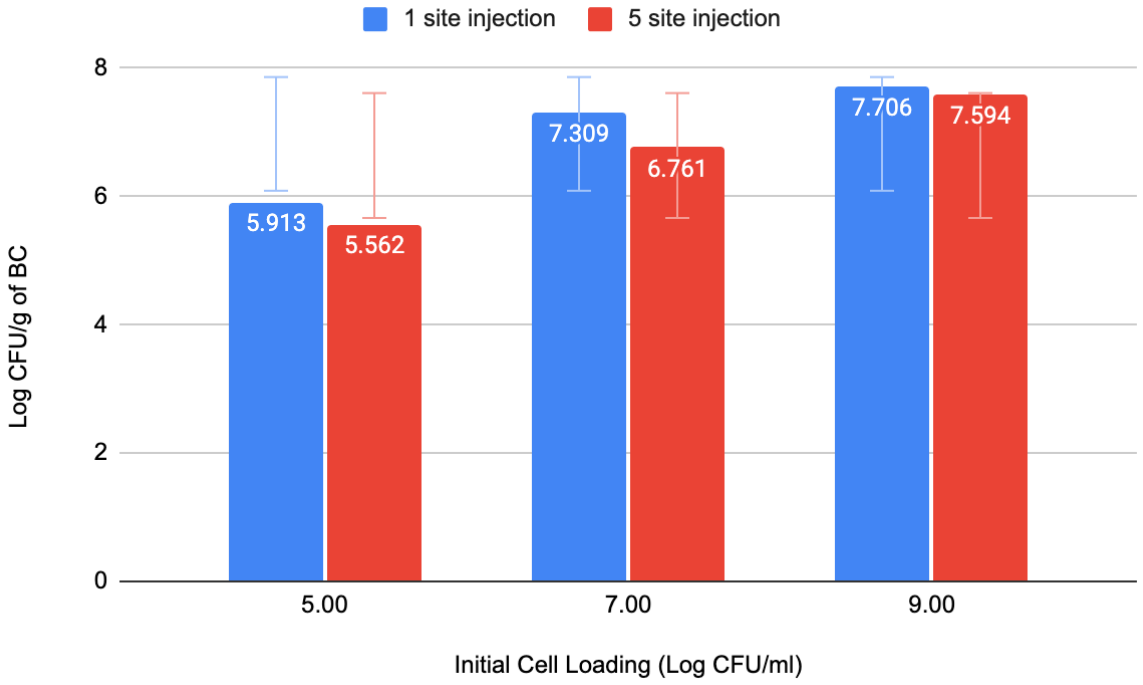


Figure 2.3 The effect of method of injection and initial cell loading on probiotic loading number of *S.cerevisiae* after injection step in I-I method. The sets of data that share the same alphabet are not significantly different (P-value > 0.05).

Figure 2.4 shows how method of injection and initial probiotic concentration affects PLN in BC after incubation step. When the results among initial cell concentrations for after incubation were compared using two-way ANOVA it yielded the P-value of 0.108 which was deemed not significant. Results among single and multiple injections were also compared using two-way ANOVA and it yielded the P-value of 0.12 which was statistically not significant. This indicates that regardless of the method of injection and the initial cell loading, the amount of *S.cerevisiae* inside the BC is similar, which is at an approximate number of 10^7 CFU/g of BC.

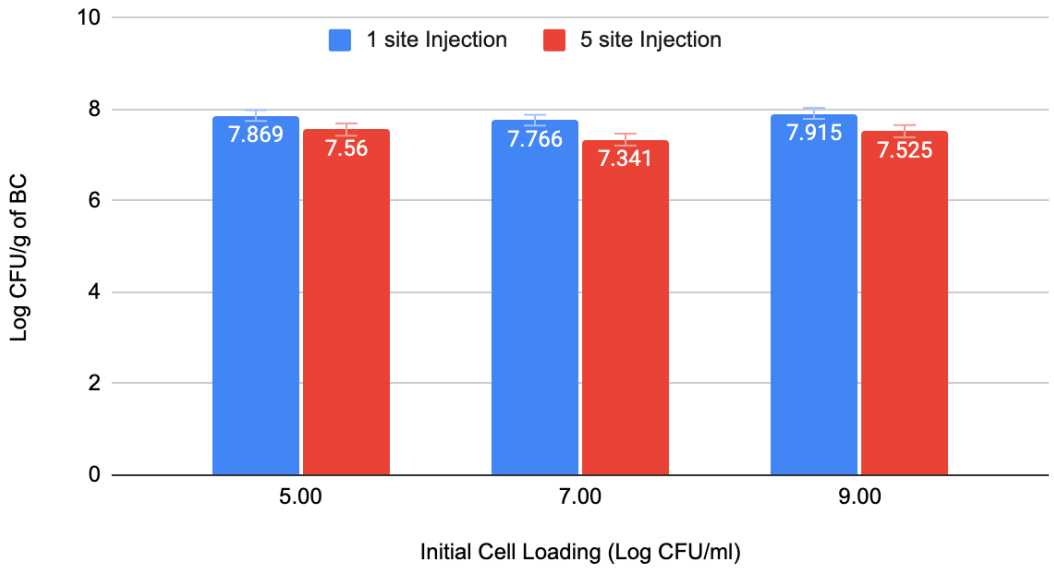


Figure 2.4 The effect of method of injection and initial cell loading on probiotic loading number of *S.cerevisiae* after incubation step in I-I method.

4.1.3 Effect of Incubation on Optimized A-I and I-I Methods

The effect of incubation shows the comparison of log CFU/g of BC of *S.cerevisiae* inside the BC right after the encapsulation and after the incubation period. Through this comparison we are able to see if *S.cerevisiae* is able to grow inside the BC after encapsulation.

The results shown in figure 3.1 were obtained from the average CFU/g of static and shaking adsorption that was converted into log CFU/g of BC. As seen from the result of BC that was adsorbed with 1 x 10^5 CFU/ml *S.cerevisiae,* there is an increase of the log CFU after incubation with the value being 5.9 before incubation and 7.554 after incubation. The results from BC that were adsorbed with 1 x 10^7 and 1 x 10^9 CFU/ml *S.cerevisiae* did not show any significant changes between after adsorption and after incubation. The value for both stayed around 7 log CFU after adsorption and after incubation. The results of the log CFU after and before incubation were compared with T-test, giving the P-value of 0.31 which was deemed not significant.

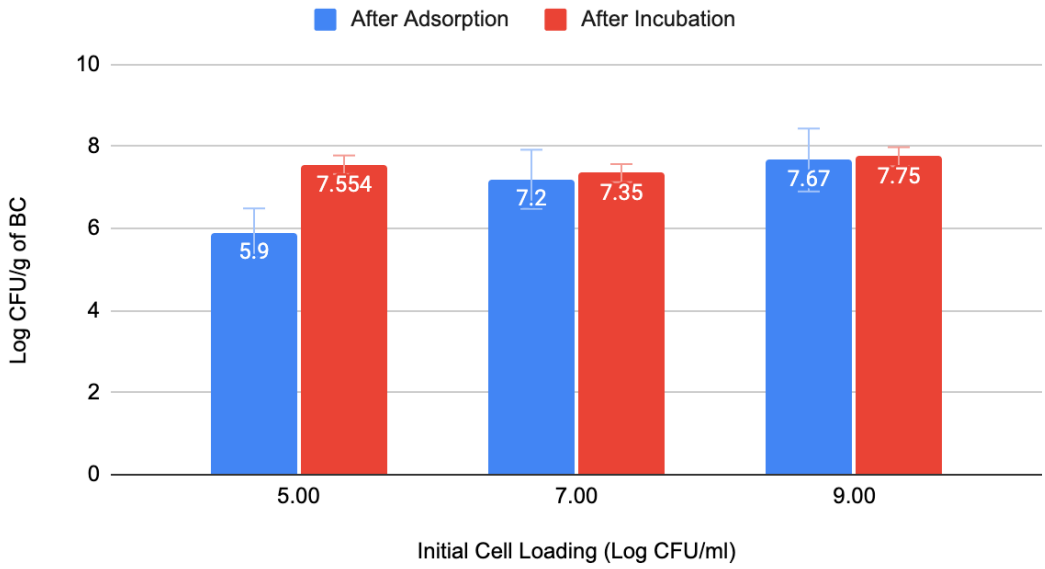


Figure 3.1 Effect of incubation and initial cell concentration in adsorption-incubation method

The results shown in figure 3.2 were obtained from the average CFU/g of single site and multiple site injection that was converted into log CFU/g of BC. As seen from the result of BC that was adsorbed with 1 x 10^5 CFU/ml *S.cerevisiae,* there is an increase of the log CFU after incubation with the value being 7.81 and 5.77 before incubation. This was consistent with the results obtained with

the adsorption-incubation method. The results from BC that were adsorbed with 1 x 10^7 and 1 x 10^9 CFU/ml *S.cerevisiae* did not show any significant changes between after adsorption and after incubation. The value for both stayed around 7 log CFU for both after adsorption and after incubation. This was consistent with the results obtained with the adsorption-incubation method. The results of the log CFU after and before incubation were compared with T-test, giving the P-value of 0.202 which was deemed not statistically significant.

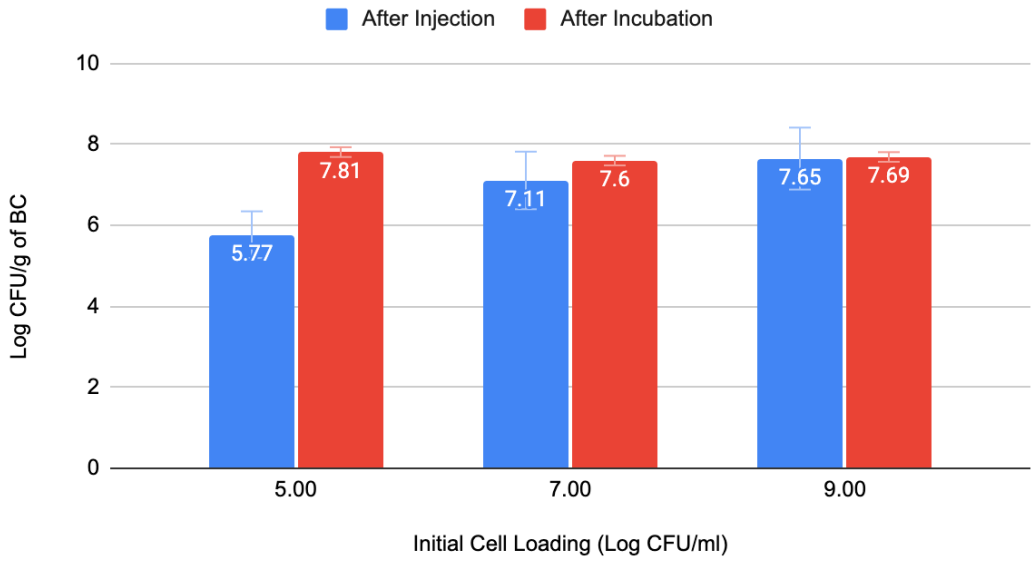


Figure 3.2 Effect of incubation and initial cell concentration in injection-incubation method

Both optimized encapsulation methods show that there were no significant differences between before and after incubation. However, the results show that *S.cerevisiae* is able to grow inside the BC indicated by the results obtained with BC that are initially encapsulated with the initial cell concentration of 1 x 10^5 CFU/ml *S.cerevisiae.*

4.2. Comparison of Optimized Encapsulation Methods with Co-Culture Method

After optimization of the Adsorption-Incubation and Injection-Incubation methods, shaking adsorption-incubation with initial loading of 1 x 10^9 CFU/ml and single site injection-incubation with 1 x 10^9 CFU/ml was able to give the highest probiotic loading number. Now, the optimized encapsulation methods are compared with the probiotic loading number of the co-culture method that is not able to be optimized.

As seen in figure 4.1 when the optimized injection-incubation and adsorption-incubation was compared with the co-culture method, the co-culture method has the lowest probiotic loading number with 7.531 log CFU per gram of BC. One-way ANOVA was used to compare the probiotic loading number amongst the three methods and it gave the P-value of 1 which is deemed not significant. This further suggests that the method of encapsulation does not affect the probiotic loading number.

With C-C having the lowest amount of log CFU/g of BC and being the least practical method compared to the other methods of encapsulation, it was removed from the candidate for encapsulation method to be tested with the antimicrobial assay. C-C was also not able to be optimized due to the limitations of the method itself. In addition, there was no significant difference between the result of A-I and I-I method. Hence, single injection with incubation with the initial cell concentration 1 x 10^9 CFU/ml was chosen to be the most effective method of encapsulation due to practicality and simplicity of the method. The Injection-incubation method will be used to create probiotic BC to be tested with the antimicrobial assay. The antimicrobial assay includes the Kirby Bauer test and time kill assay.

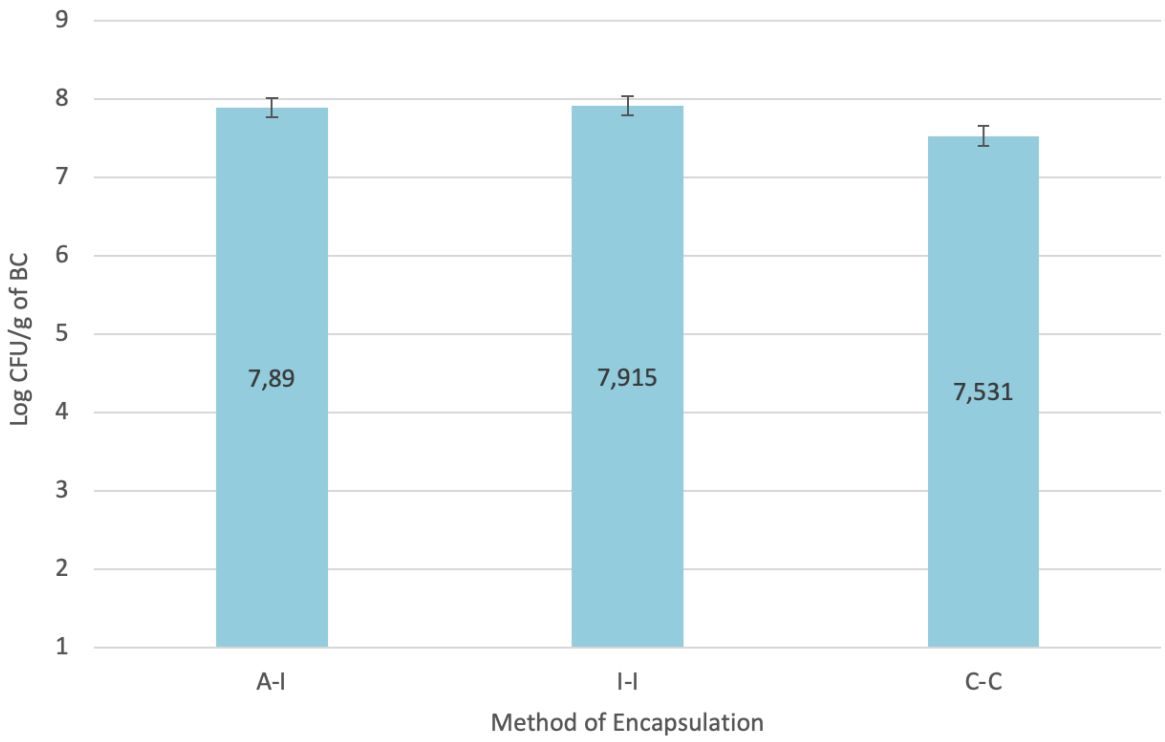


Figure 4.1 Comparison of Probiotic Loading Number (Log CFU/g of BC) between adsorption-incubation, Injection-incubation and co-culture method

4.3 Antimicrobial Activity Assay Results

4.3.1 Kirby-bauer Test

Agar plates used for the Kirby-Bauer test were incubated at 37ºC for 1 day before it was observed for the clear zone or growth around the disc. The area of inhibition was calculated by image J software. The area of inhibition calculated was the clear zone and growth of *S.cerevisiae* observed surrounding the filter paper disc or the probiotic BC.

The area of inhibition created by the *S.cerevisiae* probiotic BC when against *S.aureus* was 325.61 mm^2 which is larger as compared to unencapsulated *S.cerevisiae* when against *S.aureus* with an area of 72.26 mm^2 as seen on figure 5.1. This shows that encapsulated probiotics have more antimicrobial activity. The difference between *S.cerevisiae* probiotic BC and unencapsulated *S.cerevisiae* area of inhibition was tested using T-test and the results are statistically significant with the P-value of 0.0035.

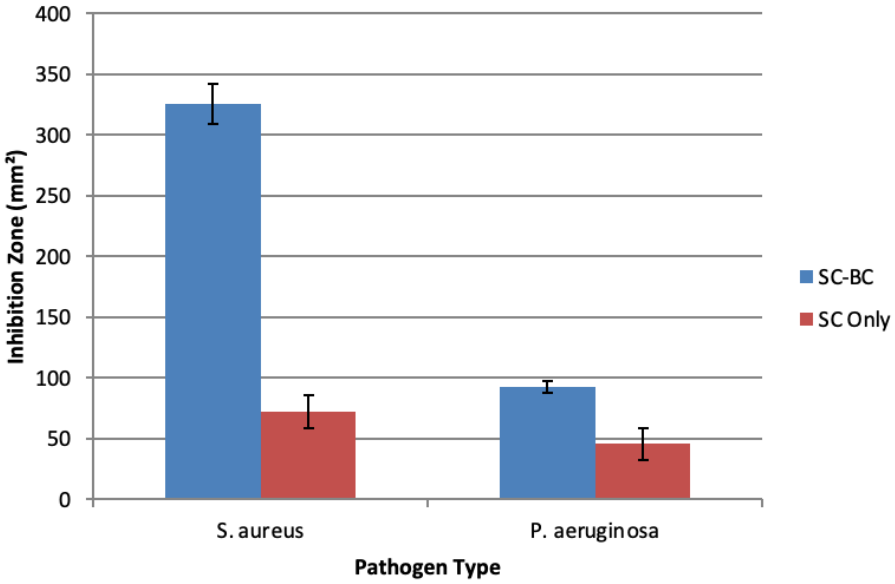


Figure 5.1 Inhibition zone of created by *S.cerevisiae* probiotic BC (SC-BC) and *S.cerevisiae* only control (SC only) against *S.aureus* and *P.aeruginosa*

As seen on figure 5.1, the area of inhibition created by the *S.cerevisiae* probiotic BC when against *P.aeruginosa* was 92.7 mm^2 which is larger as compared to unencapsulated *S.cerevisiae* against *P.aeruginosa* with an area of 45.92 mm^2. This shows that encapsulated probiotics have more antimicrobial activity. The difference between *S.cerevisiae* probiotic BC and unencapsulated *S.cerevisiae* area of inhibition is statistically significant with the P-value of 0.0249.

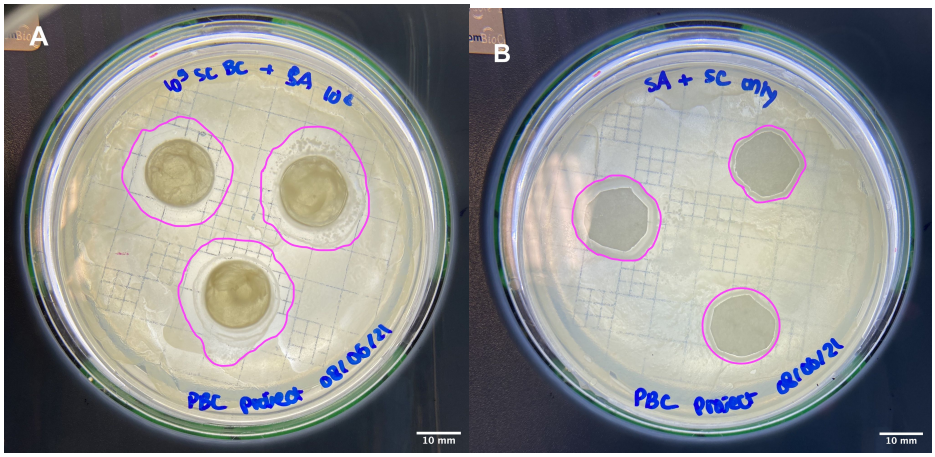


Figure 5.2 Kirby Bauer Test agar plates against *S.aureus* A) 1 x 10^7 CFU/g *S.cerevisiae* probiotic BC against 1 x 10^8 CFU/ml *S.aureus.* B). Filter paper soaked with 1 x 10^7 CFU/ml *S.cerevisiae* against 1 x 10^8 CFU/ml *S.aureus.* Pink outline around the BC and filter paper shows the zone of inhibition.

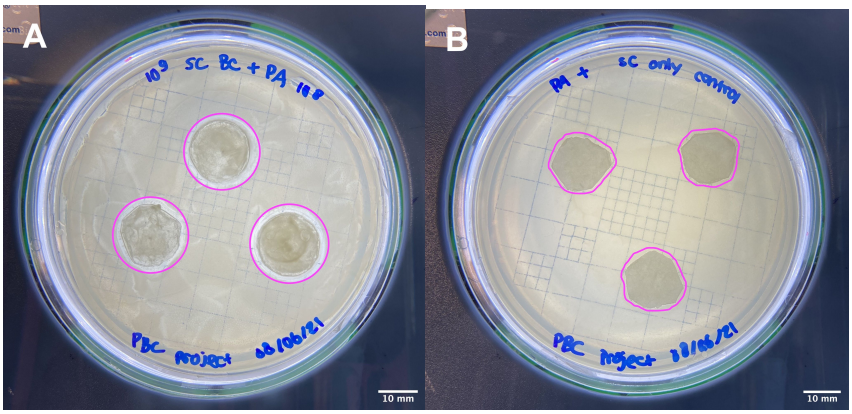


Figure 5.3 Kirby Bauer Test agar plates against *P.aeruginosa* A) 1 x 10^7 CFU/g *S.cerevisiae* probiotic BC against 1 x 10^8 CFU/ml *P.aeruginosa.* B). Filter paper soaked with 1 x 10^7 CFU/ml *S.cerevisiae* against 1 x 10^8 CFU/ml *P.aeruginosa.* Pink outline around the BC and filter paper shows the zone of inhibition.

As seen in figure 5.2 B and 5.3 B the area of inhibition for *S.cerevisiae* only control against *S.aureus* and *P.aeruginosa* shows a growth of the *S.cerevisiae* instead of a clear zone that was exhibited by the *S.cerevisiae* probiotic BC in figure 5.2 A and 5.3 A. This shows that *S.cerevisiae* is able to have different ways of exhibiting antimicrobial activity at different circumstances.

In conclusion, the results showed that encapsulated *S.cerevisiae* was able to give more antimicrobial activity compared to unencapsulated *S.cerevisiae*. This was consistent between the two pathogens as well as the time kill test results. *S.cerevisiae* probiotic BC was more effective against *S.aureus* as compared to *P.aeruginosa*.

4.3.2 Time kill assay

Time kill assay was done to see *S.cerevisiae* probiotic BC's antimicrobial activity towards pathogens, namely *S.aureus* and *P.aeruginosa*. It is also done to see if the antimicrobial activity is bacteriostatic or bactericidal. Results were obtained by counting the CFU/ml of pathogens present in the solution after the incubation period.

Figure 6.1 shows that after incubation of 1 hour the amount of *S.aureus* has reached approximately 10 log CFU/ml for all samples. After incubation of 6 hours the amount of *S.aureus* has started to decrease for the group treated with the *S.cerevisiae* probiotic BC. The *S.aureus* values dropped from 9.86 log CFU/ml to 7.07 log CFU/ml after 6 hours. Meanwhile, the other groups are still experiencing an increase in the amount of *S.aureus* at 6 hours.

After incubation of 24 hours there is a decrease in the amount of *S.aureus* in the group treated with *S.cerevisiae* probiotic BC and unencapsulated *S.cerevisiae*. The *S.aureus* value decreased from 7.07 log CFU/ml to 5.42 log CFU/ml for the group treated with S.cerevisiae probiotic BC. The *S.aureus* value decreased from 14.9 log CFU/ml to 11.72 log CFU/ml for the group treated with *S.cerevisiae* only. At this point encapsulated *S.cerevisiae* and unencapsulated *S.cerevisiae* can be considered to have a bactericidal effect as it is able to reduce more than 3 magnitudes of power. When the results of the group treated with *S.cerevisiae* BC and *S.cerevisiae* only were compared using T-test it gave a P-value of 0.0001 which deems it statistically significant. This means that the encapsulated *S.cerevisiae* is more effective compared to unencapsulated *S.cerevisiae* only control.

The values for the BC only and SA only group kept rising starting from 1 hour to 24 hour incubation. The BC only group started with 9.78 log CFU/ml and ended up with 18.15 log CFU/ml of *S.aureus*. The SA only group started with 9.82 log CFU/ml and ended up with 18.48 log CFU/ml of *S.aureus*. This shows that the BC only group did not have any antimicrobial activity.

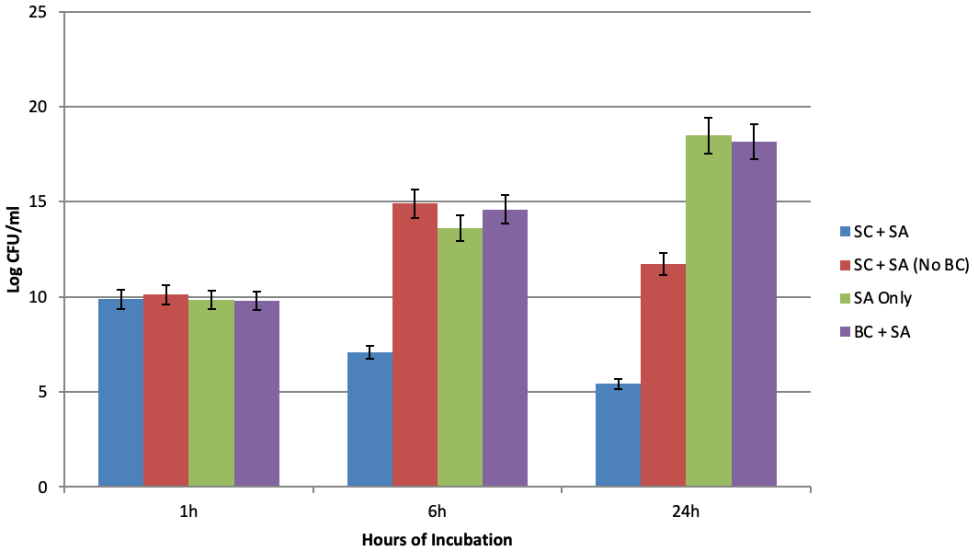


Figure 6.1 The effect of incubation time of *S.cerevisiae* Probiotic BC and time kill test control groups against the amount of *S.aureus.* (SC+SA: *S.cerevisiae* probiotic BC + *S.aureus*; SC + SA (no BC): unencapsulated *S.cerevisiae* + *S.aureus*; SA only: *S.aureus* only control; BC + SA: BC only control + *S.aureus*)

Figure 6.2 shows that after incubation of 1 hour the amount of *P.aeruginosa* has reached approximately 8 log CFU/ml for all samples except for the unencapsulated *S.cerevisiae* sample with a value of 10 log CFU/ml. After incubation of 6 hours the amount of *P.aeruginosa* has started to decrease for the group treated with the *S.cerevisiae* probiotic BC as well as the group treated with unencapsulated *S.cerevisiae*. The *P.aeruginosa* values dropped from 8.42 log CFU/ml to 4.3 log CFU/ml after 6 hours for groups treated with *S.cerevisiae* probiotic BC. Meanwhile, the amount of *P.aeruginosa* dropped from 10 log CFU/ml to 6.6 log CFU/ml after 6 hours for the group treated with unencapsulated *S.cerevisiae*. On the other hand, the other groups are still experiencing an increase of *P.aeruginosa* numbers at 6 hours. At this point encapsulated *S.cerevisiae* and unencapsulated *S.cerevisiae* can be considered to have a bactericidal effect as it is able to reduce more than 3 magnitudes of power. When the results of the group treated with *S.cerevisiae* BC and *S.cerevisiae* only were compared using T-test it gave a P-value of 0.0001 which deems it statistically significant.

This means that the encapsulated *S.cerevisiae* is more effective compared to unencapsulated *S.cerevisiae* only control.

After incubation of 24 hours there is a continued decrease in the amount of *P.aeruginosa* in the group treated with *S.cerevisiae* probiotic BC. The *P.aeruginosa* value decreased from 4.3 log CFU/ml to less than 2 log CFU/ml for the group treated with *S.cerevisiae* probiotic BC. Meanwhile, the *P.aeruginosa* value increased from 6.6 log CFU/ml to 14.32 log CFU/ml for the group treated with unencapsulated *S.cerevisiae*.

The values for the BC only and PA only group kept rising starting from 1 hour to 24 hour incubation. The BC only group started with 8.84 log CFU/ml and ended up with 11.86 log CFU/ml of *P.aeruginosa*. The PA only group started with 8.78 log CFU/ml and ended up with 17.12 log CFU/ml of *P.aeruginosa*.

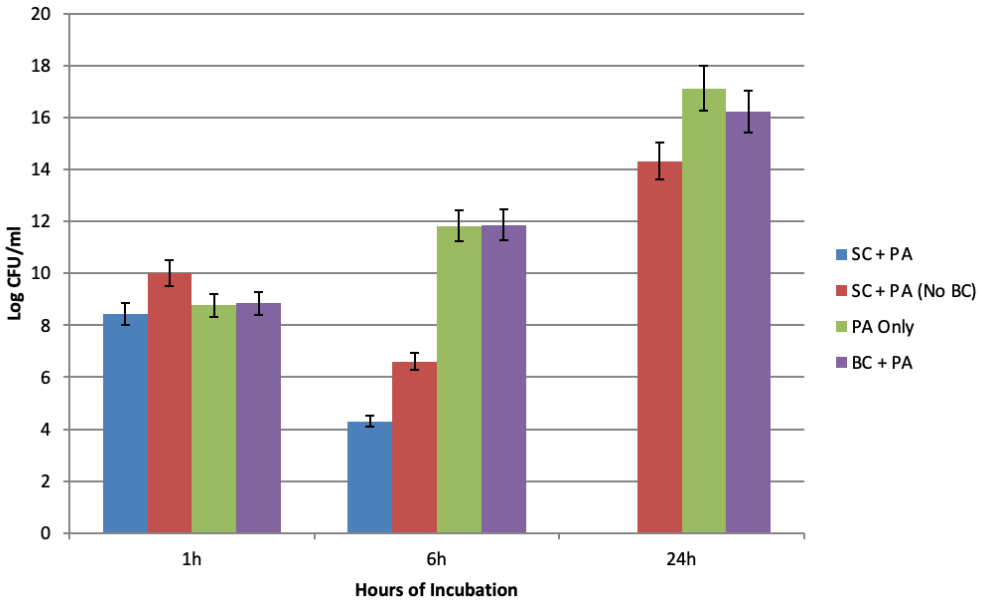


Figure 6.2 The effect of incubation time of *S.cerevisiae* Probiotic BC and time kill test control groups against the amount of *P.aeruginosa.* (SC+PA: *S.cerevisiae* probiotic BC + *P.aeruginosa*; SC + SA (no BC): unencapsulated *S.cerevisiae* + *P.aeruginosa*; PA only: *P.aeruginosa* only control; BC + PA: BC only control + *P.aeruginosa*)

In conclusion, encapsulated and unencapsulated *S.cerevisiae* has a bactericidal effect against *S.aureus* and *P.aeruginosa*. Although, *S. cerevisiae* encapsulated inside BC can be concluded to be more effective than unencapsulated *S. cerevisiae* against *S.aureus* and *P.aeruginosa.* When compared between the two pathogens *S.cerevisiae* probiotic BC seem to be more effective towards *P.aeruginosa* as compared to *S.aureus*. Lastly, the BC only control does not exhibit any antimicrobial activity against both pathogens.

Chapter 5 - Discussion

5.1 Probiotic Loading Number of Optimized A-I and I-I Methods

The probiotic loading number was taken two separate times; before incubation and after incubation. As mentioned in the methods, measurement taken before incubation was done to observe if *S.cerevisiae* is able to enter the BC right after encapsulation and measurement taken after the incubation was done to observe if *S.cerevisiae* is able to grow inside the BC. When the results for before and after incubation are compared in section 4.1.3 (Effect of Incubation), it shows that *S.cerevisiae* is able to enter and grow inside the BC. It was most apparent with the group that was initially loaded with 1 x 10^5 CFU/ml of *S.cerevisiae,* there was an increase from approximately 5 to 7 Log CFU/g of BC which was consistent in both encapsulation methods.

Despite the differences in method, initial cell loading and variables in each method all the BC ended up with 10^7 CFU of *S.cerevisiae* inside the BC. A minimal concentration for a product to be called a probiotic is 10^6 CFU/g for *S.cerevisiae* (Kechagia et al., 2013). With the result being 10^7 CFU/g of BC for both encapsulation methods, the probiotic BC produced in this study is still in the range of being considered a probiotic product.

There are no significant differences between initial cell loading, encapsulation methods as well as between the variables for each method; mode of adsorption for A-I and mode of injection for I-I. This suggests that BC produced by *K.intermedius* has the maximum capacity of 10^7 CFU *S.cerevisiae* inside the BC. It also confirms that the limiting factor for probiotic loading number is not the method of encapsulation. The limiting factor would most likely be the properties of BC itself as well as the large size of *S.cerevisiae* cells (5 - 10 µm).

The size of pores in BC produced by *K.xylinus* is maximum 10 µm (Gao et al., 2011) and *S.cerevisiae* have a maximum size of 10 µm which means, *S.cerevisiae* is able to fit into the pores of a *K.xylinus* BC and is able to enter well inside the BC. A previous study by Żywicka *et al*. which used adsorption-incubation as the method of encapsulation of *S.cerevisiae* into BC produced by *K.xylinus*

was able to have an encapsulation efficiency of 52% which is one of the lowest amongst other microorganism that they tried to encapsulate into BC. The low encapsulation efficiency was explained to be due to the big size of the *S.cerevisiae* itself (Żywicka *et al*.,2019). Another previous study did the Injection-incubation method to encapsulate *S.cerevisiae* and it was also done using BC that was produced by *K.xylinus*, unfortunately this study did not mention how much *S.cerevisiae* is inside the BC (Yao et al., 2011). Nevertheless, this shows that *S.cerevisiae* is able to enter *K.xylinus* BC.

There is a possibility that *K.intermedius* BC has smaller pores as compared to *K.xylinus*' BC. The pore size of a *K.intermedius* BC is unknown as producing BC from *K.intermedius* is still a relatively new method. Hence, it is possible that *S.cerevisiae* has a difficulty in entering *K.intermedius* BC which is why it reaches a maximum of 10^7 CFU/g of BC despite changing the amount of the initial cell loading and method of encapsulation.

The pre-incubation probiotic loading number shows that the cells were able to enter the BC but it was not able to tell exactly where the cells are located in or on the BC. There is a possibility that the *S.cerevisiae* cells are not fully inside the BC instead it could be encapsulated somewhere near the surface of the BC. This can only be confirmed through SEM visualization to see where the cells are located in the BC.

5.2 Comparison of Optimized Encapsulation Methods with Co-Culture Method

When I-I is compared with A-I, I-I can be considered to be the most practical as it takes less time and materials for the encapsulation itself. A-I takes a maximum of 4 days for the encapsulation meanwhile I-I only takes 3 days. A-I also requires more resources such as PBS and flasks due to the adsorption stage. As seen in figure 4.1 I-I has the highest log CFU/g of BC value with 7.915 even though the difference between method was not significant.

When I-I method is compared to C-C, I-I's method is straightforward and there is no need to worry about the interaction of *S.cerevisiae* with *K.intermedius* who is responsible for producing the BC. The interaction between these two microbes is an important factor to consider in the C-C method

because in the beginning both of these microbes will be present in the media while the BC is forming. A concern is that both microorganisms consume glucose as their carbon source and most importantly *K.intermedius* uses glucose to create BC (Kayikci & Nielsen, 2015; Delmer & Amor, 1995). If the glucose runs out then the *K.intermedius* is not able to create BC and the *S.cerevisiae* will not be able to grow well inside the media.

Furthermore, optimization was not possible for the C-C method due to limitations such as media volume, risk of spillage and nutrient supply for the microbes. There is not much that is able to be done with C-C because the BC is grown together with the probiotic. If the initial cell loading was increased then there may be a nutrient shortage in the media due to more cells being inside. If the amount of the media was increased it would have a high chance of spillage and it might affect the dimension of the BC. If the container was changed to accommodate a bigger volume of media, then the dimension of the BC would differ from the other two methods (A-I and I-I), making dimension of the BC a variable in the experiment. At the end a dead end was faced with this method which led to its elimination.

Since it was mentioned above that the method of encapsulation was not the limiting factor for encapsulation, the most convenient method with the highest probiotic loading number was chosen to proceed for the antimicrobial test. The method chosen was single injection-incubation with the initial cell concentration of 1×10^9.

5.3 Kirby-bauer test

The area of inhibition was taken as the result in the Kirby Bauer test due to the irregular shape of the zone of inhibition. Since the area of inhibition was measured using ImageJ software it also offers more accuracy compared to measuring the diameter using a ruler/vernier caliper. *S.cerevisiae* probiotic BC is able to show a clear zone of inhibition around the BC. The clear area of inhibition created by the *S.cerevisiae* probiotic BC against *S.aureus* is 22.19% larger than the area of inhibition of the unencapsulated *S.cerevisiae* which indicates that *S.cerevisiae* probiotic BC is more effective

against *S.aureus* than unencapsulated *S.cerevisiae.* The clear area of inhibition of the *S.cerevisiae* probiotic BC for *P.aeruginosa* is 49,53% larger than the area of inhibition of the unencapsulated *S.cerevisiae* which indicates that probiotic BC is more effective against *P.aeruginosa* than unencapsulated *S.cerevisiae*. Overall, the probiotic BC as well as the unencapsulated *S.cerevisiae* was more effective towards inhibiting *S.aureus* as compared to *P.aeruginosa*

When tested using the Kirby-Bauer method in a previous study, unencapsulated *S.cerevisiae* itself was found to have antimicrobial activity against *P.aeruginosa* and *S.aureus* with 5 mm and 8 mm diameter of inhibition zones respectively (Younis et al.,2017). Results obtained in this study are according to previous studies in which the probiotic BC as well as the unencapsulated *S.cerevisiae* had more effect against *S.aureus* compared to *P.aeruginosa*.

The unencapsulated *S.cerevisiae* grew outwards from the disc which prevented the growth of the pathogen surrounding it which indicates that *S.cerevisiae* has multiple ways of exhibiting antimicrobial activity. The growth was also considered as an inhibition zone during the measurement of results. In nature with the diverse microbial community it was found that all microbes were able to survive by competing with other microorganisms which lead to natural selection. Nutrition is usually the main motivation of microbial competition. This was first studied by Jacques Monod, who was able to show the relationship between nutrient concentrations and bacterial growth (Hibbing et al., 2009). Growth by competition could be the mechanism of inhibition exhibited by unencapsulated *S.cerevisiae* as it was able to grow and inhibit the growth of the pathogen surrounding it. Another possible reason for *S.cerevisiae* being able to grow outwards from the filter paper is because the *S.cerevisiae* is not bound on to the paper as well as it is entrapped inside the BC.

The zone of inhibition; both clear zone and growth was able to show it has an antibacterial activity but it was not able to determine what is the mode of inhibition (bacteriostatic or bactericidal). Hence, to further strengthen the result for antimicrobial activity a time-kill assay is performed.

5.4 Time-kill assay

Time-kill test is a method that is able to determine the nature of the antimicrobial activity as the antimicrobial agent interacts dynamically with the pathogen. It can be determined whether it has a bactericidal or bacteriostatic effect through this method. The time-kill test results also are able to reveal the effect of length of incubation and concentration of the antimicrobial agent on the pathogen (Gallant-Behm et al., 2005).

The results show that *S.cerevisiae* probiotic BC has a bactericidal effect against both *S.aureus* and *P.aeruginosa* as it is able to reduce the amount of pathogen more than 3 magnitudes of power (Gallant et al., 2005). The antimicrobial effect of the *S.cerevisiae* BC was considered to be bactericidal against *S.aureus* after 24 hour of incubation. Meanwhile, the antimicrobial effect of the *S.cerevisiae* BC was considered to be bactericidal against *P.aeruginosa* after 6 hours of incubation. The result shows that probiotic BC is more effective against *P.aeruginosa* as compared to *S.aureus*. Which was the opposite of the Kirby Bauer test results.

There isn't any study yet that has used the time-kill assay to observe the antimicrobial activity of *S.cerevisiae*. The difference in the media used in between Kirby Bauer and time kill assay could have an effect of the mechanism of action as well as interaction between *S.cerevisiae* and *P.aeruginosa*. The production of biofilm by the pathogen could also affect the interaction between the pathogen and *S.cerevisiae*. In a solid media (during Kirby Bauer) biofilm could be more stable as it anchors to the agar's solid surface meanwhile in liquid media (during time kill test) the biofilm is not as stable as there is no anchor. Biofilm production is able to protect the pathogen cells from antimicrobial treatments as it provides limited diffusion of antimicrobial agents (Gingichashvili et al., 2020).

Time kill assay has been done for a probiotic BC with *B.subtilis* as the probiotic. The *B.subtilis* probiotic BC was able to exhibit a 100% bactericidal effect after incubation of 10 hours for *S.aureus* and 24 hours for *P.aeruginosa*. This study also included a BC only control which indicates that there

was no inhibition done by the BC only samples. Instead, the amount of pathogen in the BC only samples doubled in its CFU/ml value (Savitskaya et al.,2019). This was in line with the results obtained in this study's BC only control in which there was no inhibition. By 24 hours of incubation the BC only group for *P.aeruginosa* started with 8.84 log CFU/ml and ended up with 11.86 log CFU/ml and for *S.aureus* it started with 9.78 log CFU/ml and ended up with 18.15 log CFU/ml. It can be concluded that BC by itself has no antimicrobial activity. As mentioned in the literature review, BC on its own lacks antimicrobial activity hence a study done by Lemnaru (Popa) et al. in 2020 tried to add antibiotics (bacitracin and amoxicillin) to add antimicrobial activity to the BC. The BC was later tested against *E.coli* and *S.aureus* and it showed that BC loaded with bacitracin was able to inhibit cell growth meanwhile both bacitracin and amoxicillin were able to lower the growth rates of cells (Lemnaru (Popa) et al., 2020).

S.cerevisiae when introduced unencapsulated into the pathogenic solution was also able to exhibit bactericidal effect as well although not as effective as encapsulated. When the results of the unencapsulated *S.cerevisiae* were compared to *S.cerevisiae* Probiotic BC using T-test there was a statistically significant difference with a P-value of 0.0001 for both pathogens. This indicates that encapsulated *S.cerevisiae* is more effective against the pathogens as compared to when unencapsulated. Encapsulated cells are protected from the adverse environment which reduces cell injury (Huq et al., 2013). A previous study also mentioned that non-encapsulated probiotics show a decrease in viability and health benefiting effects when it is released to the environment of a wounded tissue (Saarela et al., 2000). By reducing chances of cell injury and protecting the cells from the outside environment it is able to have higher viability. This higher viability is important as the cells need to be viable to be able to exert its antimicrobial activity. Hence it is in line with the result obtained in this study where encapsulated *S.cerevisiae* is more effective compared to unencapsulated.

Chapter 6 - Conclusion and Recommendations

In conclusion, the method of encapsulation does not affect the amount of *S.cerevisiae* that is encapsulated inside bacterial cellulose that is produced by *K.intermedius*. Hence, single injection incubation with 1 x 10^9 CFU/ml initial cell loading, as the most practical method with the highest probiotic loading number was chosen. On the other hand the limiting factor for the amount of *S.cerevisiae* inside the BC is most likely due to the characteristic of the BC itself. Further studies can be done to investigate the porosity of the *K.intermedius* to further confirm this conclusion. SEM results could also give more support towards the conclusion of *S.cerevisiae*'s size as well as *K.intermedius*' BC pore size being the limiting factor for amount of probiotic loading. This was initially planned to be done but due to time constraints as well as an unconducive situation to travel for sending the sample it was not possible to be done.

S.cerevisiae probiotic BC was able to exhibit antibacterial activity when tested against *S.aureus* and *P.aeruginosa* using the Kirby Bauer method. *S.cerevisiae* probiotic BC was proven to have a bactericidal effect when tested with the time-kill assay. A study on S. cerevisiae's mechanism of action towards pathogenic bacteria could be studied to further understand how its antimicrobial activity works.

Lastly, for future recommendation in-vivo research is still needed to confirm its antimicrobial effect during wound healing. As the final aim is to produce a wound healing product. A storage testing study could be done to see the probiotic BC's viability during storage. As it is important that the probiotic stays viable so it is able to exert its antimicrobial activity.

References:

Almeida, G., dos Santos, M., Lima, N., Cidral, T., Melo, M., & Lima, K. (2014). Prevalence and factors associated with wound colonization by Staphylococcus spp. and Staphylococcus aureus in hospitalized patients in inland northeastern Brazil: a cross-sectional study. *BMC Infectious Diseases*, *14*(1). doi: 10.1186/1471-2334-14-328

Bowler, P., Duerden, B., & Armstrong, D. (2001). Wound Microbiology and Associated Approaches to Wound Management. *Clinical Microbiology Reviews*, *14*(2), 244-269. doi: 10.1128/cmr.14.2.244-269.2001

C Reygaert, W. (2018). An overview of the antimicrobial resistance mechanisms of bacteria. *AIMS Microbiology*, *4*(3), 482-501. doi: 10.3934/microbiol.2018.3.482

Chambers, H., & DeLeo, F. (2009). Waves of resistance: Staphylococcus aureus in the antibiotic era. *Nature Reviews Microbiology*, *7*(9), 629-641. doi: 10.1038/nrmicro2200

Delmer, D., & Amor, Y. (1995). Cellulose biosynthesis. *The Plant Cell*, *7*(7), 987-1000. doi: 10.1105/tpc.7.7.987

D.N., Nguyen & Ton, Minh Nguyet & Le, Van. (2009). Optimization of Saccharomyces cerevisiae immobilization in bacterial cellulose by 'adsorption- Incubation' method. International Food Research Journal, 16, 59-64

Fakruddin, M., Hossain, M., & Ahmed, M. (2017). Antimicrobial and antioxidant activities of Saccharomyces cerevisiae IFST062013, a potential probiotic. *BMC Complementary And Alternative Medicine*, *17*(1). doi: 10.1186/s12906-017-1591-9

Fernández, J., Morena, A., Valenzuela, S., Pastor, F., Díaz, P., & Martínez, J. (2019). Microbial Cellulose from a Komagataeibacter intermedius Strain Isolated from Commercial Wine Vinegar. Journal Of Polymers And The Environment, 27(5), 956-967. doi: 10.1007/s10924-019-01403-4

Fijałkowski, K., Peitler, D., Rakoczy, R., & Żywicka, A. (2016). Survival of probiotic lactic acid bacteria immobilized in different forms of bacterial cellulose in simulated gastric juices and

bile salt solution. LWT - Food Science and Technology, 68, 322–328. doi:10.1016/j.lwt.2015.12.038

Foligné, B. (2010). Probiotic yeasts: Anti-inflammatory potential of various non-pathogenic strains in experimental colitis in mice. *World Journal Of Gastroenterology*, *16*(17), 2134. doi: 10.3748/wjg.v16.i17.2134

Gallant-Behm, C., Yin, H., Liu, S., Heggers, J., Langford, R., & Olson, M. et al. (2005). Comparison of in vitro disc diffusion and time kill-kinetic assays for the evaluation of antimicrobial wound dressing efficacy. Wound Repair And Regeneration, 13(4), 412-421. doi: 10.1111/j.1067-1927.2005.130409.x

Gao, C., Wan, Y., Yang, C., Dai, K., Tang, T., Luo, H., & Wang, J. (2011). Preparation and characterization of bacterial cellulose sponge with hierarchical pore structure as tissue engineering scaffold. *Journal of porous materials*, *18*(2), 139-145.

Gingichashvili, S., Feuerstein, O., & Steinberg, D. (2020). Topography and Expansion Patterns at the Biofilm-Agar Interface in Bacillus subtilis Biofilms. *Microorganisms*, *9*(1), 84. doi: 10.3390/microorganisms9010084

Górecka, E., & Jastrzębska, M. (2011). Immobilization techniques and biopolymer carriers. Biotechnology and Food Science, 75(1), 65-86.

Griffith, M., Postelnick, M., & Scheetz, M. (2012). Antimicrobial stewardship programs: methods of operation and suggested outcomes. *Expert Review Of Anti-Infective Therapy*, *10*(1), 63-73. doi: 10.1586/eri.11.153

Gruenstein, E., Schlemm, D., Bethi, M., & Keller, S. (2018). The early signaling pathway of live yeast cell derivative in THP-1 monocytes. *Cell Calcium*, *73*, 112-120. doi: 10.1016/j.ceca.2018.04.008

Hibbing, M. E., Fuqua, C., Parsek, M. R., & Peterson, S. B. (2009). Bacterial competition: surviving and thriving in the microbial jungle. Nature Reviews Microbiology, 8(1), 15–25. doi:10.1038/nrmicro2259

Horcajada, J., Montero, M., Oliver, A., Sorlí, L., Luque, S., & Gómez-Zorrilla, S. et al. (2019). Epidemiology and Treatment of Multidrug-Resistant and Extensively Drug-ResistantPseudomonas aeruginosaInfections. *Clinical Microbiology Reviews*, *32*(4). doi: 10.1128/cmr.00031-19

Huq, T., Khan, A., Khan, R. A., Riedl, B., & Lacroix, M. (2013). Encapsulation of Probiotic Bacteria in Biopolymeric System. Critical Reviews in Food Science and Nutrition, 53(9), 909–916. doi:10.1080/10408398.2011.573152

Kayikci, Ö., & Nielsen, J. (2015). Glucose repression in Saccharomyces cerevisiae. *FEMS Yeast Research*, *15*(6), fov068. doi: 10.1093/femsyr/fov068

Kechagia, M., Basoulis, D., Konstantopoulou, S., Dimitriadi, D., Gyftopoulou, K., Skarmoutsou, N., & Fakiri, E. (2013). Health Benefits of Probiotics: A Review. *ISRN Nutrition*, *2013*, 1-7. doi: 10.5402/2013/481651

Kelesidis, T., & Pothoulakis, C. (2012). Efficacy and safety of the probiotic Saccharomyces boulardii for the prevention and therapy of gastrointestinal disorders. *Therapeutic advances in gastroenterology*, *5*(2), 111-125.

Khalid, A., Ullah, H., Ul-Islam, M., Khan, R., Khan, S., Ahmad, F., ... & Wahid, F. (2017). Bacterial cellulose–TiO 2 nanocomposites promote healing and tissue regeneration in burn mice model. *RSC advances*, *7*(75), 47662-47668.

Lemnaru (Popa), G., Truşcă, R., Ilie, C., Țiplea, R., Ficai, D., & Oprea, O. et al. (2020). Antibacterial Activity of Bacterial Cellulose Loaded with Bacitracin and Amoxicillin: In Vitro Studies. Molecules, 25(18), 4069. doi: 10.3390/molecules25184069

Lin, S. P., Huang, Y. H., Hsu, K. D., Lai, Y. J., Chen, Y. K., & Cheng, K. C. (2016). Isolation and identification of cellulose-producing strain Komagataeibacter intermedius from fermented fruit juice. *Carbohydrate polymers*, *151*, 827-833.

Manoukian, O. S., Sardashti, N., Stedman, T., Gailiunas, K., Ojha, A., Penalosa, A., … Kumbar, S. G. (2018). Biomaterials for Tissue Engineering and Regenerative Medicine. Reference Module in Biomedical Sciences. doi:10.1016/b978-0-12-801238-3.64098-9

Negut, I., Grumezescu, V., & Grumezescu, A. (2018). Treatment Strategies for Infected Wounds. *Molecules*, *23*(9), 2392. doi: 10.3390/molecules23092392

Palma, M. L., Zamith-Miranda, D., Martins, F. S., Bozza, F. A., Nimrichter, L., Montero-Lomeli, M., … Douradinha, B. (2015). Probiotic Saccharomyces cerevisiae strains as biotherapeutic tools: is there room for improvement? Applied Microbiology and Biotechnology, 99(16), 6563–6570. doi:10.1007/s00253-015-6776-x

Pang, Z., Raudonis, R., Glick, B., Lin, T., & Cheng, Z. (2019). Antibiotic resistance in Pseudomonas aeruginosa: mechanisms and alternative therapeutic strategies. *Biotechnology Advances*, *37*(1), 177-192. doi: 10.1016/j.biotechadv.2018.11.013

Portela, R., Leal, C., Almeida, P., & Sobral, R. (2019). Bacterial cellulose: a versatile biopolymer for wound dressing applications. *Microbial Biotechnology*, *12*(4), 586-610. doi: 10.1111/1751-7915.13392

Pretorius, I. S., du Toit, M., and van Rensburg, P. (2003). Designer yeasts for the fermentation industry of the 21st century. *Food Technol. Biotechnol.* 41, 3–10.

Reid, G. (2006). Probiotics to Prevent the Need for, and Augment the Use of, Antibiotics. *Canadian Journal Of Infectious Diseases And Medical Microbiology*, *17*(5), 291-295. doi: 10.1155/2006/934626

Saarela, M., Mogensen, G., Fondén, R., Mättö, J., & Mattila-Sandholm, T. (2000). Probiotic bacteria: safety, functional and technological properties. *Journal of biotechnology*, *84*(3), 197-215.

Sabio, L., González, A., Ramírez-Rodríguez, G., Gutiérrez-Fernández, J., Bañuelo, O., & Olivares, M. et al. (2021). Probiotic cellulose: Antibiotic-free biomaterials with enhanced antibacterial activity. *Acta Biomaterialia*. doi: 10.1016/j.actbio.2021.01.039

Savitskaya, I., Shokatayeva, D., Kistaubayeva, A., Ignatova, L., & Digel, I. (2019). Antimicrobial and wound healing properties of a bacterial cellulose based material containing B. subtilis cells. Heliyon, 5(10), e02592. doi: 10.1016/j.heliyon.2019.e02592

Sen, S., & Mansell, T. (2020). Yeasts as probiotics: Mechanisms, outcomes, and future potential. *Fungal Genetics And Biology*, *137*, 103333. doi: 10.1016/j.fgb.2020.103333

Silva, S. S., Fernandes, E. M., Pina, S., Silva-Correia, J., Vieira, S., Oliveira, J. M., & Reis, R. L. (2017). 2.11 Polymers of Biological Origin ☆. Comprehensive Biomaterials II, 228–252. doi:10.1016/b978-0-12-803581-8.10134-1

Willyard, C. (2017). The drug-resistant bacteria that pose the greatest health threats. *Nature News*, *543*(7643), 15.

Webster, J., and Weber, R. (2007). *Introduction to Fungi*. Cambridge University Press. Available at: http://books.google.ca/books?id=HZLXFi-om-0C

Żywicka, A., Wenelska, K., Junka, A., Chodaczek, G., Szymczyk, P., & Fijałkowski, K. (2019). Immobilization pattern of morphologically different microorganisms on bacterial cellulose membranes. World Journal of Microbiology and Biotechnology, 35(1). doi:10.1007/s11274-018-2584-7

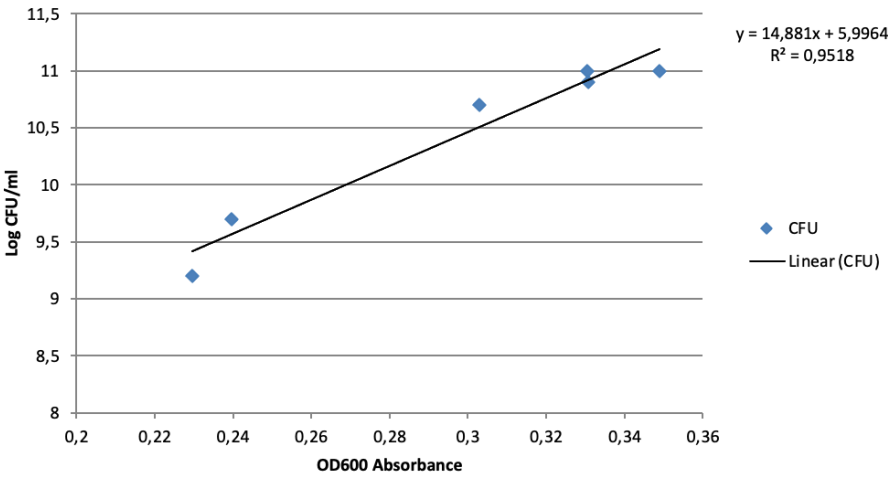
Yao, W., Wu, X., Zhu, J., Sun, B., Zhang, Y. Y., & Miller, C. (2011). Bacterial cellulose membrane – A new support carrier for yeast immobilization for ethanol fermentation. Process Biochemistry, 46(10), 2054–2058. doi:10.1016/j.procbio.2011.07.006

Younis, G., Awad, A., Dawod, R., & Yousef, N. (2017). Antimicrobial activity of yeasts against some pathogenic bacteria. *Veterinary World*, *10*(8), 979-983. doi: 10.14202/vetworld.2017.979-983

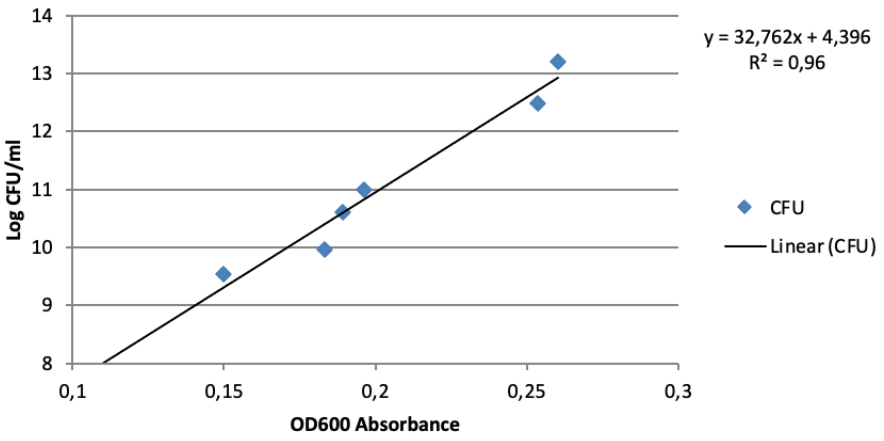
Yu, V. (2011). Guidelines for hospital-acquired pneumonia and health-care-associated pneumonia: a vulnerability, a pitfall, and a fatal flaw. *The Lancet Infectious Diseases*, *11*(3), 248-252. doi: 10.1016/s1473-3099(11)70005-6

Appendices

Pathogen Standard curve



Appendix 1.1 *S.aureus* Standard Curve



Appendix 1.2 *P.aeruginosa* Standard Curve

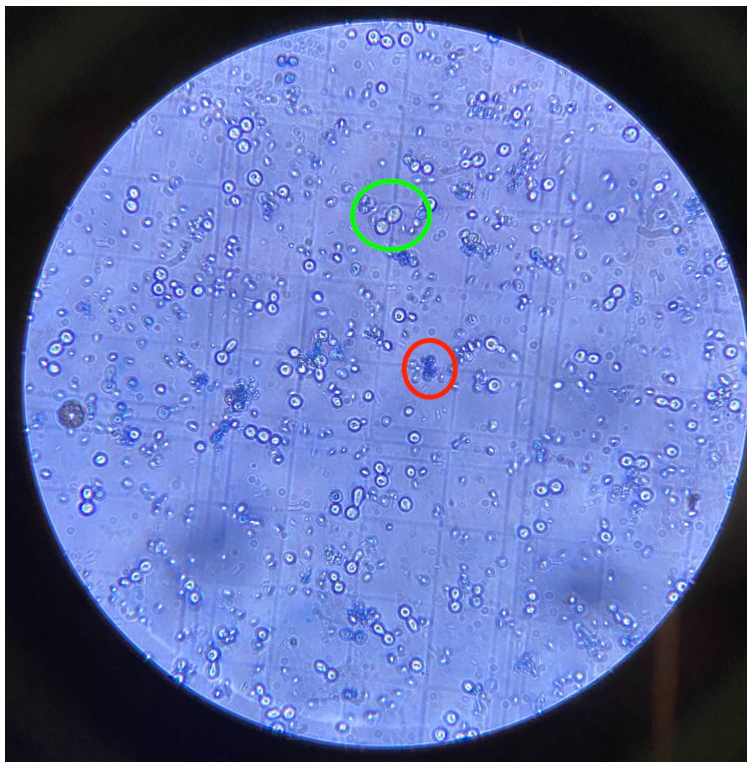
Table 2.1 Post Hoc analysis of Probiotic Loading Number for Adsorption-incubation before incubation (SC BC-5: *S.cerevisiae* Bacterial Cellulose with initial loading of 1 x 10^5 CFU/ml; SC BC-7: *S.cerevisiae* Bacterial Cellulose with initial loading of 1 x 10^7 CFU/ml; SC BC-9: *S.cerevisiae* Bacterial Cellulose with initial loading of 1 x 10^9 CFU/ml)

Sample	P-value	Significant?
SC BC-5 vs SC BC-7	0.026	Yes
SC BC-5 vs SC BC-9	0.011	Yes
SC BC-7 vs SC BC-9	0.278	No

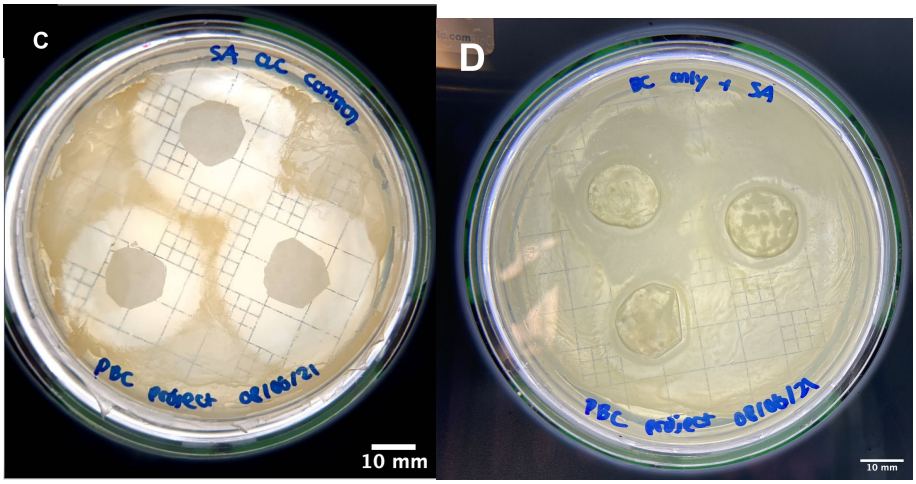
Table 2.2 Post Hoc analysis of Probiotic Loading Number for Injection-incubation before incubation (SC BC-5: *S.cerevisiae* Bacterial Cellulose with initial loading of 1 x 10^5 CFU/ml; SC BC-7: *S.cerevisiae* Bacterial Cellulose with initial loading of 1 x 10^7 CFU/ml; SC BC-9: *S.cerevisiae* Bacterial Cellulose with initial loading of 1 x 10^9 CFU/ml)

Sample	P-value	Significant?
SC BC-5 vs SC BC-7	0.034	Yes

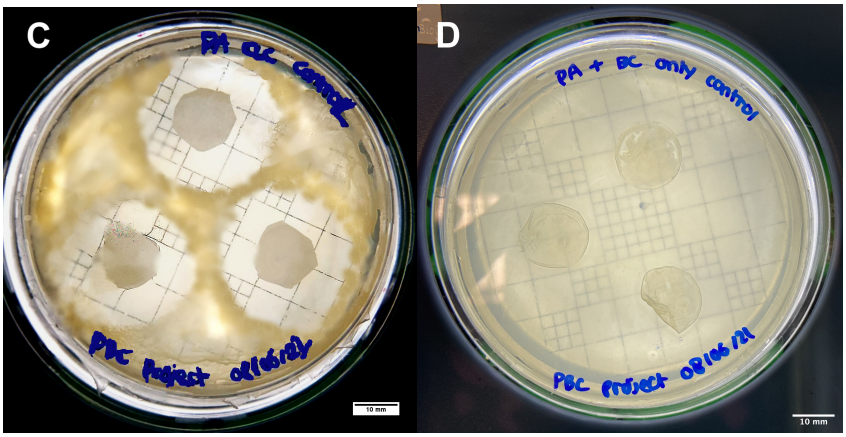
SC BC-5 vs SC BC-9	0.012	Yes
SC BC-7 vs SC BC-9	0.202	No



Appendix 1.3 *S.cerevisiae* cells in the Haemocytometer chamber under 40x magnification. Cells circled in green circle are counted as it is deemed viable meanwhile cells circled in red are not counted as its not viable.



Appendix 1.4 Positive and negative controls for kirby bauer test against *S.aureus* C) Filter paper soaked with 1 mg/ml Chloramphenicol against 1 x 10^8 CFU/ml *S.aureus.* D) BC only against 1 x 10^8 CFU/ml *S.aureus*



Appendix 1.5 Positive and and negative controls for kirby bauer test against *P.aeruginosa* C) Filter paper soaked with 1 mg/ml Polymyxin B against 1 x 10^8 CFU/ml *P.aeruginosa.* D) BC only against 1 x 10^8 CFU/ml *P.aeruginosa.*