LACTIC ACID PRODUCTION BY IMMOBILIZED *RHIZOPUS SP*. THROUGH SIMULTANEOUS SACCHARIFICATION AND FERMENTATION PROCESS USING BANANA PEEL AND BEET PULP

BY

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ABSTRACT

The industrial demand for lactic acid (LA) has increased significantly due to their functional properties in different fields. LA production by *Rhizopus sp*. using various substrates have been studied. But, the problem of low production yield is still unsolved due to the following issues: finding a suitable low-cost substrate and controlling the fungal morphology during fermentation to maintain high oxygen concentration. Therefore, this project's main aims are to improve the LA yield by selecting a better substrate between banana peel (BaP) and beet pulp (BeP) for higher LA yield; to optimize the *Rhizopus sp.* immobilization process for a maximum weight of fungal attachment on the loofah using potato dextrose, and to increase the LA yield through semi-continuous simultaneous saccharification and fermentation (SSF) in air pulsed airlift reactor (ALR) (using the selected substrate with the optimum immobilization condition). For the first part of this research, two fermentation experiments with different BaP and BeP concentrations (40 to 100 g/L) in a shake flask were carried out to determine the best substrate for higher LA yield. The results showed that the produced LA yield from BaP (0.044 g/g) was 3-fold higher than that of BeP (0.0146 g/g). For the second part of the study, optimization of *Rhizopus sp*. immobilization conditions (potato dextrose (0.02 to 1.5 M), agitation speed (0 to 150 rpm), and incubation time (24 to 72 h)) onto a loofah sponge were carried based on a face-centered central composite design (FCCCD) by Design-Expert software v11.1.2.0 with the attached biomass weight as the response for each parameter. Then, a scanning electron microscopy (SEM) test was conducted to evaluate the fungal attachment and loofah structure after the immobilization. Based on the parameters proposed by FCCCD, the maximum biomass weight of 0.252 g was achieved onto 0.3 g of loofah at dextrose concentration, agitation speed, and incubation time of 0.45 M, 100 rpm, and 24 h, respectively. SEM results showed a good fungal attachment with no degradation to the loofah fibers. In the third part of the research, three SSF experiments with different air pulsation frequencies (0.0384 to 0.1667 s^{-1}) were conducted in loofah immobilized ALR using 60 g/L BaP for 8 days. After that, the immobilized loofah from each experiment was examined using SEM to elucidate the effect of different pulsation frequencies on the fungal morphology. From SSF results, the 0.0384 s^{-1} was the best frequency to increase the LA yield to 0.091 g/g after 3 days of fermentation. It is 2.1-fold higher in comparison with the free-cell non-pulsed shake flask culture in the previous study. Unlike the frequency at 0.0384 s^{-1} the SEM images showed that at 0.1667 s^{-1} frequency the airlift flow was restricted by the irregular formation and the trapped BaP particles between hypha may lead to oxygen transfer limitation in the ALR. The results may improve LA productions by proposing an easy, fast, and efficient method for fungal immobilization on loofah using potato dextrose. In addition, the uncontrolled fungal growth problem inside submerged cultures can be resolved by applying a simple air pulsing system. Further studies on the fermentation conditions for BaP and BeP for better fungal utilization and LA production, the substrate consumption rate and culture composition during the SSF process are also recommended for future research.

خلاصة البحث

زاد الطلب الصناعي على حمض اللاكتيك (LA) بشكل كبير بسبب خصائصه الوظيفية في مختلف المجالات. تمت دراسة إنتاج LA بواسطة *Rhizopus sp.* باستخدام مواد أولية مختلفة من قبل. ومع ذلك، فإن مشكلة انخفاض الإنتاج لا تزال دون حل بسبب القضايا التالية: العثور على مادة أولية مناسبة منخفضة التكلفة والتحكم في التشكل الفطري أثناء التخمير للحفاظ على تركيز أكسجين العالي. لذلك، فإن الأهداف الرئيسية لهذا المشروع هي تحسين إنتاجية LA عن طريق: اختيار أفضل مادة أولية بين قشر الموز (BaP) ولب البنجر (BeP) للحصول على مردود أعلى من LA، تحسين عملية تثبيت *Rhizopus sp.* للحصول على أقصى وزن للارتباط الفطري على اللوف باستخدام دكستروز البطاطس، واستخدام المادة الأولية المختارة مع ظروف التثبيت المثلى لزيادة مردود LA من خلال عملية التخمير والتسكير المتزامن شبه المستمر (SSF)في مفاعل الرفع الهوائي النابض (ALR). للجزء الأول من هذا البحث، تم إجراء تجربتي تخمير بتركيزات مختلفة من BaP وBeP (40 إلى 100 جم/لتر) بطريقة اهتزاز القارورة لتحديد أفضل مادة أولية للحصول على إنتاجية أعلى من LA. أظهرت النتائج أن مردود LA الناتج من BaP (0.044 جم/جم) كان أعلى بثلاثة أضعاف من BeP (0.0146 جم/جم). بالنسبة للجزء الثاني من الدراسة، تم تحسين ظروف تثبيت *Rhizopus sp.* على إسفنجة اللوف (دكستروز البطاطس (0.02 إلى 1.5 محلول مولي)، وسرعة اهتزاز (من 0 إلى 150 دورة في الدقيقة)، ووقت الحضانة (24 إلى 72 ساعة)) باستخدام تصميم مركب مركزي الوجه (FCCCD) بواسطة برنامج Design-Expert v11.1.2.0. مع تسجيل جَوَاب واحد لكل عامِل وهو وزن الكتلة الحيوية المثبتة. بعد ذلك، تم إجراء اختبار المسح المجهري الإلكتروني (SEM) لتقييم الارتباط الفطري وبنية اللوف بعد التثبيت. بناءً على المعلمات المقترحة من قبل FCCCD، تم تحقيق أقصى وزن للكتلة الحيوية قدره 0.252 جم على 0.3 جم من اللوف بتركيز دكستروز البطاطس، سرعة التحريض، ووقت الحضانة البالغ 0.45 محلول مولي، 100 دورة في الدقيقة، و24 ساعة، على التوالي. أظهرت نتائج SEM ارتباطًا فطريًا جيدًا مع عدم وجود تحلل لألياف اللوف. في الجزء الثالث من البحث، أجريت ثلاث تجارب SSF بترددات نبض هواء مختلفة (0.0384 إلى 0.1667 بالثانية) في فطر مثبت باللوف في ALR باستخدام 60 جم/لتر BaP لمدة 8 أيام. بعد ذلك، تم اختبار الفطر المثبت باللوف من كل تجربة باستخدام SEM توضيح تأثير ترددات النبض المختلفة على التشكل الفطري. من نتائج SSF، تردد 0.0384 بالثانية كان هو الأفضل لزيادة إنتاجية LA إلى 0.091 جم/جم بعد ثلاثة أيام من التخمير. و كانت الإنتاجية أعلى بمقدار 2.1 ضعف مقارنة بتجربة اهتزاز القارورة غير النبضية ذات الخلايا الحرة في الدراسة السابقة. على عكس عندما كان التردد 0.0384 بالثانية، أظهرت صور SEM عند تردد 0.1667 بالثانية أنه تم تقييد تدفق الهواء بالتكوين غير المنتظم وجزيئات BaP المحتبسة بين الخُوْط الفطرية مما يؤدي إلى تقييد نقل الأكسجين في ALR. قد تحسن هذه النتائج إنتاج LA من خلال اقتراح طريقة سهلة سريعة وفعالة لتثبيت الفطريات على اللوف باستخدام دكستروز البطاطس. بالإضافة، مشكلة النمو الفطري غير المنضبط داخل الزراعات المغمورة يكن حلها عن طريق تطبيق نظام نبضات هواء بسيط. بالإضافة إلى ذلك، يوصى بإجراء مزيد من الدراسات للبحث في المستقبل حول ظروف التخمير لـ BaP وBeP من أجل استخدام فطري وإنتاج LA أفضل و معدل استهلاك المادة الأولية وتركيب المسْتَنْبَتٌ أثناء عملية SSF.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science in Engineering

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DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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In the name of Allah, the Most Gracious and Most Merciful

Firstly, praises to the Sole Creator for giving me good health, patience, knowledge, strength and determination in my effort to complete my master thesis.

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LIST OF ABBREVIATIONS

Adeq	Adequate
Adj	Adjusted
ALR	Airlift reactor
ANOVA	Analysis of variance
BaP	Banana peel
BeP	Beet pulp
DO	Dissolved oxygen
FCCCD	Face-centered central composite design
HMF	Hydroxymethylfurfural
LA	Lactic acid
LDH	Lactate dehydrogenase
mg	Milligram
mL	Milliliter
MnP	Manganese peroxidase
NAD	Nicotinamide adenine dinucleotide
nm	Nanometer
PDA	Potato dextrose agar
PLA	Polylactic acid
Pred	Predicted
PU	Polyurethane
PVA	Polyvinyl alcohol
R. oryzae	*Rhizopus oryzae*
rpm	Revolutions per minute

RSM	Response surface methodology
SMI	Small and medium industry
SPW	Solid pineapple waste
SEM	Scanning electron microscopy
SHF	Separate hydrolysis and fermentation
SSF	Simultaneous saccharification and fermentation
STR	Stirred tank reactor
vvm	Volumetric flow rate per unit volume of culture medium
XOS	Xylooligosaccharides

LIST OF SYMBOLS

^{o}C	Degree Celsius
t	Time
$\left(C_p\right)_t$	Concentration of LA produced at any time
$\left(C_s\right)_i$	Initial amount of substrate
$\left(V_w\right)$	Working volume
$\left(A_l\right)$	Loofah sponge area

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF STUDY

Lactic acid (LA) is an essential organic acid with diverse applications in cosmetics, chemicals, food, pharmaceutical, chemical feedstocks, and etc (Ahmad et al., 2020). LA can be produced either by chemical synthesis or microbial synthesis (Guo et al., 2010). Most studies have focused on microbial producers because the raw materials for chemical synthesis are limited (Zhang et al., 2007).

Fermentations with the fungus *Rhizopus sp.* are often preferred to the bacterial systems because of the nutrition complexity and the challenging downstream process problems associated with bacterial fermentation, such as difficulty in separation and recovery process (Zhang et al., 2015). In the past decades, LA production through fermentation by *Rhizopus sp.* has been extensively studied. Besides using *Rhizopus* for lactic acid production, low-value lignocellulosic substrates have also received good attention as *Rhizopus* is capable of performing simultaneous saccharification and fermentation using lignocellulosic materials (Zain et al., 2020). Some of the lignocellulosic substrates that have been used for LA production by *Rhizopus* fungus are cassava starch (Trakarnpaiboon et al., 2018; Yuwa-Amompitak & Chookietwattana, 2014), solid pineapple waste (Aziman et al., 2015; Zain et al., 2020), potato pulp (Peng & Feng, 2014), sugar bagasse (Ranjit & Srividya, 2016), corn cob (Bai et al., 2008; Guo et al., 2010; Miura et al., 2004; Ruengruglikit & Hang, 2003), and wheat straw (Maas et al., 2006; Saito et al., 2012; Vially et al., 2010).

High cellulose and hemicellulose compounds in banana peel (BaP) and beet pulp (BeP) (Anhwange et al., 2009; Foster et al., 2001; Kabenge et al., 2018), and their wide availability in Malaysia and Turkey make them the chosen substrates for this study. These lignocellulosic materials had never been used as substrates for LA production by *Rhizopus sp,* and this is the first comparative study on the yield of lactic acid produced by *Rhizopus sp*. using BaP and BeP.

LA production by filamentous fungi like *Rhizopus* usually can be done through methods that include One-Step Fermentation (Shahri et al., 2020), Solid-State Fermentation (Aziman et al., 2015), Separate Hydrolysis and Fermentation (SHF) (Zhang et al., 2015) processes. The Simultaneous Saccharification and Fermentation (SSF) process has some advantages over the other methods in improving the LA concentration and yield while decreasing the overall production cost by converting the released sugars from the saccharification stage immediately into LA. This also increases productivity and minimizes processing time due to reducing product (glucose) inhibition on cellulose hydrolysis and reducing the osmotic pressure of cells caused by glucose accumulation (Zhang et al., 2015). On the other hand, the LA production by *Rhizopus* in submerged cultures results in excessive mycelium and fungal biomass growth, which seriously affects the metabolic rate, mass transfer, and product secretion. Also, fungal hypha can cause mass and oxygen transfer limitations by wrapping around impellers and causing blockages, spreading into nutrient and sampling feed lines, and causing an increase in broth viscosity (Moreira et al., 2003). They are the causes of reduced LA production yield as reported by Shahri et al., (2020). To mitigate this problem, *Rhizopus* was immobilized on a non-reactive, non-toxic, and highly porous (> 90%) loofah sponge made of the dry fruit of *Luffa cylindrica,* which has been successfully used as an immobilization matrix for ethanol production by yeast (Ahmadi et al., 2006). To immobilize *Rhizopus* on a loofah sponge, potato dextrose, a simple, abundantly available, and cheap substrate, was used instead of regular complex media.

However, *Rhizopus*' immobilization on the loofah sponge did not prevent the fungal hypha from growing extensively and resulted in oxygen starvation (Shahri et al., 2020). Therefore, an air pulsation system was added to the airlift reactor (ALR) to supply air at different frequencies (0.167-0.038 s^{-1}). Thereby, the fungus morphology can be controlled during the submerged fermentation process, and the oxygen starvation that reduces the LA productivity can be prevented. This fungal morphology control was successfully used in continuous manganese peroxidase (MnP) production by free pellets of *Phanerochaete chrysosporium* (Moreira et al., 2003).

1.2 PROBLEM STATEMENT AND ITS SIGNIFICANCE

Although *Rhizopus* fungi have been widely investigated for LA production, some significant technical challenges are associated with this biotechnological process. First, the great demand for fermentable sugars derived from low-cost materials to produce chemical products such as ethanol and lactic acid gave the lignocellulosic biomass considerable significance among other substrates. Despite the massive diversity of the agricultural wastes that can be considered rich carbohydrate sources, not all of them can be used by *Rhizopus* as a substrate for LA fermentation. Some wastes are preferable to others to be utilized by Rhizopus; some lignocellulosic materials release toxic elements such as lignin and carbohydrate degradation in a corn cob and stover hydrolysates to inhibit LA fermentation (Zhang et al., 2016). To find out the best substrate for LA fermentation by *Rhizopus sp.*, substrates need to be examined and compared in term of their LA production yield. This study investigated the LA fermentation yield by *Rhizopus sp.* using two different substrates, namely banana peel (BaP) and beet pulp (BeP).

Morphology control is crucial and challenging for a submerged fermentation system using filamentous fungi. Therefore, control of fungal morphology is required to achieve a high production rate and good processing performance. Various cell immobilization methods have been studied to control fungal morphology to achieve high cell density and production rates. Immobilizing whole cells provides a way to entrap a multi-step and cooperative enzyme system in the intact cell to improve usability and stability. Limited studies focused on maximizing the fungal attachment by using carbohydrate sources. One example, Seaby et al., (1988) found that adding oats as a carbohydrate source to a soya liquid culture medium in a ratio of 0.5 increased the weight of *R. oryzae* mycelia by more than 50%. However, no studies have been done on obtaining a high cell density by using dextrose as a sole substrate. Therefore, this study optimizes the immobilized cell density of *Rhizopus sp.* on loofah sponge using only potato dextrose.

Oxygen transport in the fermentation medium was developed using pellet morphology (Wang et al., 2010). In prolonged cultivation, high-density large pellets often have problems associated with oxygen starvation in the pellet core caused by a restriction of oxygen diffusion (Moreira et al., 2003; Pino et al., 2018; Wang et al., 2010). For example, in a study by Shahri et al., (2020), the excessive growth of *Rhizopus* hypha in the aeration part of ALR made it challenging to manage and control the system and eventually decreased the LA production yield. Therefore, it is critical to improve aeration efficiency in the submerged fermentation process.

1.3 RESEARCH OBJECTIVES

This study aims to develop an effective fermentation process to produce LA using lignocellulosic substrates such as BaP and BeP by *Rhizopus sp*. that immobilized onto loofah sponge. To achieve the main objective of this study, three sub-objectives are examined:

1. To compare the yield of lactic acid (LA) produced by *Rhizopus sp.* using BaP and BeP.
2. To optimize the immobilization process for a maximum weight of fungal attachment on loofah sponge using potato dextrose.
3. To examine the effects of different air pulsation frequencies on the produced lactic acid yield from the simultaneous saccharification and fermentation process in the ALR system using the selected substrate.

1.4 RESEARCH SCOPE

In this study, BaP and BeP were tested in determining their suitability as a substrate for LA production by *Rhizopus sp.* to produce a higher LA yield. This test was carried out with different substrate concentrations (40 to 100 g/L) in a shake flask for 72 h. At the same time, cell immobilization onto loofah sponge was optimized using a face-centered central composite design (FCCCD) under the following parameters and ranges: dextrose concentration (0.02 to 0.2 M), agitation speed (0 to 150 rpm), and incubation time (24 to 72 h) to maximize the attached fungal weight. Based on the optimization results, further experiments will be conducted. By keeping the agitation speed range from 0 to 150 rpm, the dextrose concentration was increased to 1.5 M at the fixed incubation time of 24 h. Then the microstructure of the loofah sponge was analyzed by scanning electron microscopy (SEM) before and after immobilization to evaluate the fungal attachment and loofah degradation. Finally, three SSF processes in an immobilized air pulsed ALR were conducted for 8 days by applying different air pulsation frequencies (0.0384 to 0.1667 s^{-1}) to improve the LA yield. The microstructure of the loofah sponge was analyzed by SEM after each SSF run to investigate the effect of different air pulsation frequencies on the fungal morphology.

1.5 RESEARCH METHODOLOGY

In this research, multiple techniques and methodologies were used to improve LA production from immobilized *Rhizopus sp.* using BaP and BeP through the SSF process. An overview of the major procedures of the research methodology of this study is described in Figure 1.1.

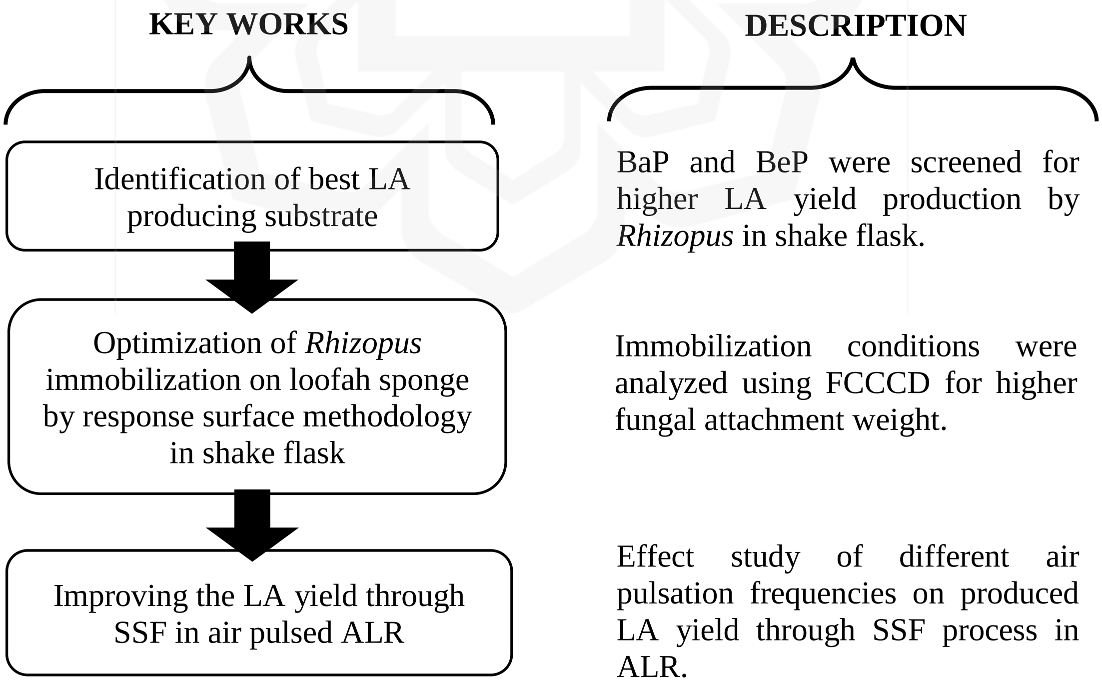

Figure 1.1 Overview of main research methods and their descriptions

1.6 THESIS ORGANIZATION

This thesis consists of five chapters. Chapter One begins with a brief background about the research, including the importance of LA in various applications and issues affecting its production, such as finding suitably low-value substrates, reproducibility, and production yield. This chapter includes a short introduction to *Rhizopus sp*., BaP, BeP, and loofah sponge as the main materials for this research. In addition, the problem statement and its significance, objective, and scope of the study are included in this chapter.

Chapter Two starts with an introduction to the LA uses and production methods. This chapter also includes a literature review on low-value substrates used for LA production, the SSF process, fungal cell immobilization, and factors affecting LA production such as pH, neutralizing agents, temperature, and oxygen supply.

Chapter Three explains the materials used in this study, followed by the experimental procedures from selection substrate for higher LA yield, optimizing cell immobilization on a loofah sponge, SSF process in air pulsed ALR, and analytical tests involved.

Chapter Four presents the results and discussion of this research, starting with the selection of higher LA yield-giving substrate. The optimized cell immobilization conditions (potato dextrose concentration, agitation speed, and incubation time) on the loofah sponge and the best air pulsation frequency in the ALR during the SSF process to improve the LA yield are provided. The morphological properties after immobilization and SSF processes are discussed based on the SEM results obtained.

Finally, Chapter Five concludes the studies carried out in the research and summarizes recommendations for improvement of future work.

CHAPTER TWO

LITERATURE REVIEW

2.1 INTRODUCTION

Lactic acid (2-hydroxy propionic acid) (LA) is among high-quantity microbial and chemical compounds with an annual international manufacturing quantity of 370,000 metric tons (Miller et al., 2019). Historically, the number one use of LA has been in food acidulation and preservation. LA is utilized in leather-based tanning, cosmetics, and pharmaceutical applications, among many different fields (Miller et al., 2017). However, in recent years, polylactic acid (PLA) synthase as a polymer used in biodegradable plastics manufacturing has prevailed (Shahri et al., 2020).

The production strategies of LA can be divided into two: chemical synthesis and fermentation process. The biotechnological pathway is the favored approach in chemical manufacturing. A given microorganism can synthesize the most effective shape LA isomer from the two optical isomers, i.e: D (-) and L (+). For human consumption, only L-form is allowed (Shahri et al., 2020). Unlike *Lactobacillus, Lactococcus, Streptococcus, Bacillus,* and *Enterococcus* bacteria, the production of L-lactic acid by the fungus *Rhizopus oryzae* exclusively generates the L-isomer, has simple nutritional requirements, and allows for easy product recovery; thus, it is a preferred over the use of bacteria (Efremenko et al., 2006; Fu et al., 2016; Miller et al., 2019; Skory, 2004).

2.2 LOW-VALUE SUBSTRATES

Many studies on LA production by *Rhizopus* species used sugar and starch-based substrates (Ahmadi et al., 2006; Shuizhong et al., 2016; Wang et al., 2014). Recognizing that using these substrates represents 30–40% of total production costs, recent studies have shifted the focus on abundant, low-cost lignocellulosic feedstocks for lactic acid production by *Rhizopus* fungus (Zhang et al., 2015). As summarized in Table 2.1, several lignocellulosic materials have been used as substrates for LA production. However, BaP and BeP have never been used before for this purpose.

Banana is a large herbal plant belonging to the genus *Musa*. It is a tropical fruit rich in many vitamins as it is lignocellulosic and is mainly planted in Asia, Latin America, and Africa. Malaysia produced more than 330.956 tons of bananas in 2018 (Taib et al., 2021). Around 440 kg of banana peel waste is generated from each ton of plucked banana (Taib et al., 2021). These wastes are among the most plentiful agricultural residues in the equatorial region. For example, Makanan Ringan Mas Industry is a Small and Medium Industry (SMI) located in Parit Raja, one of the industries targeted at food production. This industry produces 101.82 kg/month with a total density of 32303.04 kg/m^3 per month of food waste observation. In this industry, banana peel waste presents the highest rate of waste generated (27%), followed by tapioca peel waste rate (25%) and shredded coconut waste (16%) (Abd Kadir et al., 2017; Zain et al., 2020). The problems faced by the SMIs such as Makanan Ringan Mas Industry are inappropriate waste disposals such as dumping wastes into ditches and open burning, causing surface and groundwater contamination, atmospheric and hydrologic pollution, and negative environmental impact (Abd Kadir et al., 2017).

On the other hand, sugar beets are root vegetables adapted to grow in temperate zones with a day temperature of 15 to 20 ^{o}C and a night temperature of 10 to 15 ^{o}C.