PRODUCTION OF BIOPLASTIC FROM AGRICULTURAL WASTE

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PRODUCTION OF BIOPLASTIC FROM AGRICULTURAL WASTE

NUR NAZIRAH BINTI ZULKAFLI

Thesis submitted in partial fulfilment of the requirements
for the award of the degree of
Bachelor of Chemical Engineering (Biotechnology)

Faculty of Chemical & Natural Resources Engineering
UNIVERSITI MALAYSIA PAHANG

JANUARY 2014

SUPERVISOR'S DECLARATION

We hereby declare that we have checked this thesis and in our opinion, this thesis is adequate in terms of scope and quality for the award of the degree of Bachelor of Chemical Engineering (Biotechnology).

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STUDENT'S DECLARATION

I hereby declare that the work in this thesis is my own except for quotations and summaries which have been duly acknowledged. The thesis has not been accepted for any degree and is not concurrently submitted for award of other degree.

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Dedication

To me myself

ACKNOWLEDGEMENT

I would like to thanks the following people and organisations;

- My supervisors Dr. Azilah bt Ajit @ Abd Aziz for her guidance throughout completing my final year project and thesis.
- Technical Unit staffs for their kind and cooperation during experiment handling.

ABSTRACT

Plastic has been a vital part of our life. However, disposal of these non-degradable petroleum-derived plastic has threaten our ecosystem. Hence, extensive research has been conducted to find the best substitute to solve this problem. Much interest has been gained in developing biodegradable plastic. Among other potential biodegradable plastic, polyhydroxybutyrate (PHB) has gained much attention and developed for industrial scale production. PHB are accumulated during fermentation process and act as energy source in microbial cells. However, the major problem in commercializing PHB is its high production cost due to its expensive carbon source and tedious procedures of using pure cultures. Thus, utilization of other cheap and renewable culture has been explored. In this study, agricultural waste has been chosen as the potential carbon source for fermentation using *Bacillus subtilis* to produce PHB. The high glucose content in the sugarcane and pineapple waste juice has making it as the potential substrates. A laboratory study was conducted to screen the effect of five potential factors; temperature, pH, agitation speed, substrate to nutrient ratio and types of waste, towards the production. A total of 16 experiments have been conducted in 48 hours of cultivation time using aerobic condition in shake flask. This study had shown that temperature and agitation speed had given the most significant effect toward PHB synthesis. Temperature is known to give a significant on fermentation since different bacteria requires different temperature for optimum production. Agitation speed should be controlled since too much speed could affect the shear force hence break the bacterial cell. Interaction between factors also has been analysed and interaction between factor of temperature and agitation speed and interaction between temperature and types of waste has shown the highest contribution towards production PHB.

ABSTRAK

Plastik telah menjadi sebahagian penting dalam kehidupan seharian kita. Walau bagaimanapun, yg dihasilkan daripada petroleum ini tidak dapat dilupuskan dan telah menjejaskan ekosistem kita. Oleh itu, kajian yang menyeluruh telah dijalankan untuk mencari pengganti yang terbaik bagi menyelesaikan masalah ini dan kini, penghasilan bioplastik telah mula mendapat perhatian. Di antara bioplastik yang lain, polyhydroxybutyrate (PHB) telah mendapat perhatian dan dibangunkan untuk pengeluaran pada skala industri. PHB dikumpulkan semasa proses penapaian dan bertindak sebagai sumber tenaga dalam sel-sel mikrob. Walau bagaimanapun, masalah utama dalam mengkomersialkan PHB adalah kos pengeluaran yang tinggi disebabkan oleh sumber karbon yang mahal dan prosedur yang ketat dalam penggunaan bakteria. Oleh itu, penggunaan sumber yang murah dan boleh diperbaharui telah diterokai sehingga kini. Dalam kajian ini, sisa pertanian telah dipilih sebagai sumber karbon yang berpotensi untuk penapaian menggunakan *Bacillus subtilis* untuk menghasilkan PHB . Kandungan glukosa yang tinggi di dalam sisa tebu dan nanas telah menjadikannya sebagai substrat yang berpotensi. Kajian makmal telah dijalankan untuk melihat kesan lima faktor yang berpotensi; suhu, pH, kelajuan kacauan, nisbah kandungan substrat kepada nutrien dan jenis buah-buahan terhadap jumlah pengeluaran. Sebanyak 16 eksperimen telah dijalankan dengan tempoh 48 jam penapaian dengan bantuan oksigen di dalam kelalang kon. Kajian ini telah menunjukkan bahawa suhu dan kelajuan kacauan telah memberi kesan yang ketara terhadap penghasilan PHB. Telah diketahui umum bahawa ia memberikan kesan yang ketara terhadap penapaian kerana bakteria yang berbeza memerlukan suhu yang berbeza untuk pengeluaran yang optimum. Kelajuan kacauan perlu dikawal kerana kelajuan yang tinggi boleh menjejaskan daya ricih dan mampu memecahkan sel bakteria. Interaksi antara faktor juga telah dianalisis dan interaksi antara faktor suhu dan kelajuan kacauan menyumbangkan kesan yang paling tinggi terhadap penghasilan PHB.

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LIST OF ABBREVIATIONS

AcC	Acetyl cellulose
HPLC	High Performance Liquid Chromatography
IPP	Isotactic Polypropylene
NY11	Nylon 11
PBS	Poly(butylene succinate)
PCL	Polycaprolactone
PE	Polyethylene
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
PLA	Polylactide
PP	Polypropylene

1 INTRODUCTION

1.1 Overview

This chapter discuss on the outline of research conducted. There are five main parts covered in this chapter, which are the background of study, problem statement, research objective, scopes of research and the significance of study.

1.2 Background of Study and Motivation

History of plastic begins starting back in 1862 by Alexander Parkers where he has invented a moldable material made from cellulose called Parkesine. History is then followed by the invention of celluloid as a substitute for ivory in billiard ball in 1868 by John Wesley Hyatt. This was the beginning of plastic revolution but still, it is not yet applied for modern industrial use. It is then started after the production of Bakelite by American chemist, L.H. Baekeland in 1909. Made by polymerization of phenol and formaldehyde, it is a type of plastic called thermoplastic. The new uses of plastics are continually being discovered to multiple its application in regular basis. After World War II, optic wear such as optical lenses, artificial eyes and dentures of acrylic plastics have been developed. Since then, plastic has become very significant towards people due to its durability, strengths, moldability and multipurpose characteristics making people depend on it in daily life basis.

Plastics used today are originally made of petroleum based which is harmful to the environment. It takes about 100 years to completely decompose a single plastic waste. This is because they are having characteristics of high molecular weight with tightly bonded molecules. These are making them non-degradable and difficult to be disposed thus proportionally leads towards the negative impacts to the environment; includes marine life and mankind (Nisha et al., 2009).

Due to awareness on the large influence of plastic used towards the green of nature, studies have been actively conducted to look investigate the possibility of replacing the non-degradable plastic towards one that can be decomposed in a shorter time and eco-friendly as well. In addition, the use of petroleum as main source of production could be decreased and consequently able to be saved for a little bit longer even though it is depleting now. According to Nisha et al. (2009), through those researches, the concept of biodegradable plastic came as a solution for this problem. These bioplastic wastes will be decomposed by microbial degradation in the environment at proper condition such as sunlight, moisture and oxygen. More or less, through these alternatives, our environment and at the same time natural source can be preserved. Thus, this research aims to overcome the abundant plastic waste leftover by developing an eco-friendly and decomposable plastic from agricultural waste.

Even though bioplastic invention has been discovered since centuries ago, it still facing problem for an industrial production scale. One of the major limiting factors to manufacture this finding is it's highly cost of substrate which is used as carbon supply for bacteria in the fermentation process. After extensive researches conducted, a new way has been discovered to lower the cost of production in which by utilizing waste as the raw material instead of pure sugar.

Therefore, this research aims to investigate the production of PHB utilized from two different agricultural wastes which are from sugarcane and pineapple. Both peels and pulp of these two leftover were used as the carbon source in the fermentation process producing the biopolymer. Five different parameters will be screened using Two-Level Factorial Analysis by Design Expert. The factors include temperature, agitation speed, pH, types of waste and substrate to nutrient ratio.

1.3 Problem Statement

The extensive use of plastic based petroleum for over than a century has resulted a major cause towards the environment. The limited future availability of petroleum, with environment and waste managements has thus brought people's concern into more sustainable alternatives to replace petroleum-derived plastic. Synthesizing biopolymer using microbial fermentation is usually expensive with the usage of microbes, nutrient medium and substrates for carbon source. Hence, a new alternative of utilizing agro-waste to replace the original substrate has been discovered. Considering Malaysia with variety agriculture production such as pineapple, sugarcane and oil palm, wastes from these crops could be recycled to synthesize PHB. Besides that, producing a biodegradable plastic is prior to control the abundant of plastics leftover that damaging the ecosystem and nature.

1.4 Research Objective

This research is conducted to screen the production of biopolymer (PHB) in fermentation using agricultural waste by manipulating five different parameters. The measurable objectives are to determine:

1. The effect of temperature, pH, agitation speed, substrate to nutrient ratio and types of waste that enhanced the production of PHB.
2. Interaction between the parameters that contribute to the production of PHB.

1.5 Research Scope

In this research, fermentation was conducted in 500 mL conical flask. Two-Level Factorial Analysis was utilized using Design Expert version 6 software to obtain an experimental design covers the five identified parameters; temperature, pH agitation speed, substrate to nutrient ratio and types of waste. The fermentation by *Bacillus subtilis* is using sugarcane and pineapple wastes as carbon sources. PHB concentration was assessed to determine the effect of parameters studied.

1.6 Significant of Research

Microbial production of bioplastic is one of the methods to control and minimize conventional plastic usage. The only limiting factor towards an industrial scale production is its high cost of manufacturing. This research helps in promoting a way in synthesizing biopolymer by exploiting waste obtained from agricultural activities. It could assist in lowering the production cost that restrains a wide synthesis of bioplastic.

2 LITERATURE REVIEW

2.1 Overview

This chapter reviews on the experimental studies on the production of PHB via microbial fermentation. The scopes cover on the development of bioplastic, the selection of PHB for an extensive biopolymer study, potential of agricultural waste as carbon source in PHB production and the potential producer of PHB synthesis. In this chapter, the topics are reviewed to provide a basic insight to the early exposure of studies on production of PHB bioplastic from agricultural waste.

2.2 Bioplastic

Bioplastic is a form of plastic made from renewable biomass, instead of the conventional plastic that derived from petroleum. It can be divided into two groups which are biodegradable plastic or bio-based plastic (Tokiwa et al., 2009). Biodegradable plastic is made up of fossil materials while bio-based plastic is synthesized from biomass or renewable resources. It is mentioned in this study that, biodegradable plastics offer a lot of benefits and these include low accumulation of bulky plastic materials in the environment, increased soil fertility and of course reduced the cost of waste management. According to Reddy et al. (2003), there are three major types of biodegradable plastic that are currently identified; photodegradable, semi-biodegradable and completely biodegradable**.**

Photodegradable plastic has light sensitive groups that connected directly into the backbone of the polymer and act as additive. Exposure towards ultraviolet radiation from several weeks to months cause disintegration of the polymeric structure hence open it for further bacterial degradation (Kalia et al., 2000). Unfortunately, landfills lack of sunlight and thus the plastic remains non-degrade. As for semi-biodegradable plastic, it is a starch-linked plastics that connecting short fragments of polyethylene. It is still remains non-degraded since bacteria do not decompose the plastic due to the fragment of polyethylene that hindered them from attacking the starch (Johnstone, 1990). The last type of plastic is the completely degradable plastic is somehow compromising since it is produced by bacteria to form biopolymer. Some of the existing bioplastic include polyhydroxyalkanoate (PHA), polylactides (PLA), aliphatic polyesters and polysaccharides (Reddy et al., 2003).

In a research done by Tokiwa et al. (2009) on biodegradability of plastics, it is mentioned that there are inter-relationship between biodegradable plastic and bio-based plastic as shown in Figure 2.1 below.

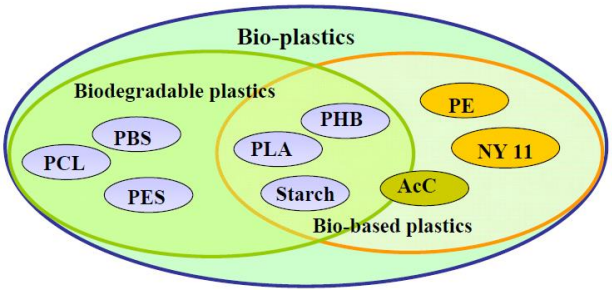

Figure 2-1: Types of Bioplastic belongs into Different Group

Based on the figure shown, even polycaprolactone (PCL), and poly (butylene succinate) (PBS) are petroleum based, however they can be degraded by microorganisms. On the other hand, polyhydroxybutyrate (PHB), polylactide (PLA) and starch blends are produced from biomass or renewable sources, hence making them biodegradable. As for polyethylene (PE) and Nylon 11 (NY11), even though they can be produced from biomass or renewable sources, they are unfortunately non-biodegradable. Acetyl cellulose (AcC) that falls in the bio-based group, it can be either biodegradable or non-biodegradable, depending on the degree of acetylation. If the AcC is having low acetylation, it can be degraded compared to those with high substitution ratios.

In order for a polymer to be decomposed, there are several factors that need to be considered, in which it includes the properties of plastic itself. Both physical and chemical properties of plastics have influenced the mechanism of biodegradation (Tokiwa, 2009). Some other components that play its important roles are surface condition (surface are, hydrophilic and hydrophobic properties), the first order and high order structures which involves chemical structure with molecular weight and glass transition temperature with melting temperature respectively.

Biodegradability of plastic has been giving much attention lately despite of other types of plastic. Limitation of landfills area for plastic disposal urges people to find various ways to overcome the problem. Hence, biodegradable plastic are seen by many as a promising solution to this problem due to their environmental-friendly characteristic. They can be derived from renewable feed stocks, thereby reducing greenhouse gas emissions.

2.3 Polyhydroxybutyrate (PHB)

Polyhydroxybutyrate (PHB) is a biodegradable polymer belongs to the polyhydroxyalkanoate (PHA) family of polyesters. It is a linear polyester of D (-)-3-hydroxybutyric acid, observed as granules in bacterial cells, mainly, Gram positive and Gram negative organisms, under microscope since 1883 by Beijerinck. According to Bregg (2006), PHB can be synthesis via three different biotechnological processes and these are fermentative production employing microorganisms, production in transgenic plants and in vitro synthesis employing isolated enzymes. However, it is most likely produced from microbial fermentation using variety of bacterial producers such as recombinant *E. coli*, *Bacillus* spp. and *Cupriavidus necator*. It is the most widely studied and also known as best characterized derivative (Sathiyanarayanan et al., 2013).

PHB is a partially crystalline polymer that has been chosen to be one of the suitable candidates to replace the conventional plastic. This is due to its mechanical properties that are comparable to those of propylene (PP). Apart from that, its versatile properties with the biodegradability characteristics have making it as an eco-friendly substitute for the synthetic polymer.

According to Wang et al (2012), PHB is a water insoluble biopolymer and relatively resistance towards hydrolytic degradation. This differentiates it from other currently available bioplastics, which are either water soluble or moisture sensitive. Table 2.1 below shows the comparison in physical properties of PHB with those of PP.

Table 2-1: Comparison of Physical Properties of PHB and PP

Properties	***PHB***	***PP***
Melting temperature, °C	175	176
Density, kg/m^3	1.250	0.905
Tensile strength, MPa	40	38
Solvent resistance	Bad	Good
UV resistance	Good	Bad

Besides that, according to Wang et al. (2012), they did mention on the other potential replacement of the existing synthetic polymer. These include polylactides (PLA), aliphatic polyesters, polysaccharides, polypropylene and other copolymers. Among the others, PHB has been given much attention than the rest due to its properties as mention above. PHB is well known for its eco-friendly properties and its complete decomposition to water and carbon dioxide by aerobic microorganism (Wang et al., 2012).

According to Purwadi (n.d.), the composition of PHA was first discovered by Lemoigne who identified the excretion of 3-hydroxybutyric acid by *Bacillus megaterium*. The role of PHB was then proposed by Macrae and Wilkinson in 1958 where they observed that *Bacillus megatarium* stored biopolymer when glucose-to-nitrogen ratio of medium was height. Hence, from that founding, they have concluded that PHB was a carbon and energy-reserve material of the bacteria itself. Figure below shows on the general structural formula of PHB:

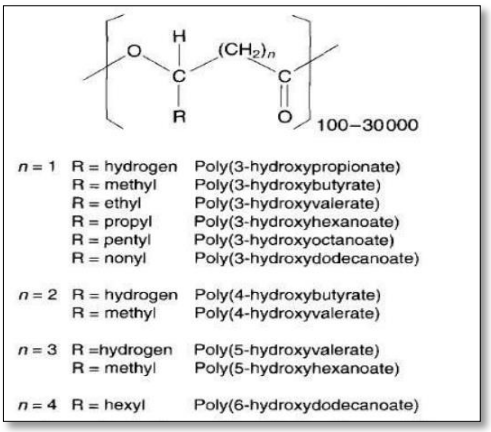

Figure 2-2: General Molecular Structure of PHB

PHB is a type of bioplastic that is having physical and mechanical properties comparable to isotactic polypropylene or known as iPP. IPP is one type of polypropylene, a thermoplastic polymer and it is different from the others in the arrangement of methyl groups that are on the same side of the chain as compared to atactic. The arrangement of methyl groups in atactic are placed randomly on both side of chain and branched. PHB is perfectly isotactic and does not having any branch and hence it flows easily during the process. Even though PHB is not water soluble, but it is 100% biodegradable in the environment when proper condition such as sunlight, moisture, and oxygen those are available.

According to Nisha et al. (2009), the degradation rate of PHB ranges from few months for anaerobic sewage up to several years in seawater besides than having low permeability for O_2, H_2O and CO_2. Therefore, PHB bioplastic have the potential to be commercially produced replacing the current plastic. This is due to its properties that are environmental friendly and the main point is that, it is capable to degrade within much less period as compared to petroleum-based plastic.

Production of PHB and other bioplastics however are known to be very expensive since they are involving expensive carbon source. Apart from that, both the upstream processing and downstream processing were also contribute to the high production cost Therefore, studies have been actively conducted to find the suitable alternative to replace the original glucose. This is where they have come out with the utilization of waste included agricultural waste, sugar industrial wastewater and cafeteria waste.

2.4 Waste as Carbon Source

It is formerly known that the major restriction in commercializing bioplastic is their high production cost. According to Reddy et al. (2003), the cost of PHA using natural producer *A. eutrophus* is US$16 per kg, which is 18 times more expensive than the conventional plastic. This is enough to hamper the commercial application and wide use of biopolymer. In fermentation practise, it is a must for bacteria to get an adequate sugar sources as the main carbon supply for them to produce product. In this case, to get the fine raw sugar for industrial scale PHB production is very costly, in addition with operational cost itself, it is somehow difficult to produce biopolymer industrially. Lee (1996) reported that the price of the product ultimately depends on the cost of substrate, PHA yield on the substrate and efficiency of product formulation in downstream processing.

Extensive researches have been done to discover new finding where the use of available cheap residues can be considered. Malaysia has been reported to be the fifth largest country in Asia that producing agricultural waste annually and most of them were dump without being utilized. Some of the available waste that has the potential for substrates replacement includes sugarcane bagasse, oil palm front juice, fruit peels and pulp. Hence, it is a benefit to exploit this advantage to at least minimize the production cost of PHB.

In a research done by Shivakumar (2012), he has studied on several agro-industrial residues as carbon substrate for PHB production. Agricultural wastes involved are soya flour, bagasse, molasses, rice bran and ragi bran using *Bacillus thuringiensis*. He found that these wastes capable to synthesis PHB biopolymer in different quantities. Soya flour capable to produce PHB up to 0.89 g/L, while using molasses is 0.47 g/L. As for ragi bran is 0.21 g/L PHB produced with bagasse and wheat bran are the least with 0.09 g/L and 0.07 g/L PHB produced respectively.

According to Fukui & Doi (1998), plant oils such as olive, corn and palm oils were good substrates for PHB production using *A. eutrophus*. Olive oil shows a significant effect towards the production of PHB where it produce up to 1g over 50 mL of fermentation medium. Apart from that, oleic acid was also good to be used as carbon source with *Pseudomonas putida* as the producer and has been used instead of alkanes since it exhibits less toxicity (Lee et al., 2000). In a research done by Preethi et al. (2012) using Jambul seed as the substrate for PHB production, it is found that the maximum level of PHA accumulation was observed using *Ralstonia eutropha* with 41.7% of PHA produced.

In order to obtain an optimum product secretion, the raw substrate used need to undergo pre-treatment first. Van-Thuoc et al. (2007) reported in order to employ agro-industrial residues as fermentation substrates; it should be subjected to hydrolysis step first in order to release the easily metabolized sugars. Pandey et al. (2009) also have reported the same in his study using *Bacillus sphaericus* NCIM 5149 where it shows an increment in synthesis of PHB since pre-treatment helps to utilize those wastes into simpler sugar, directly for bioconversion into PHB.

2.5 Microorganism

Microbial fermentation of PHB production requires producer for product secretion. There are two major groups of bacteria that have the potential to utilize raw substrates for the production of PHB. These bacteria grow based on the culture conditions required for PHB synthesis. As for the first group, microbes belong to this group requires the limitation of essential nutrients such as nitrogen, magnesium, sulphur and phosphorus for the synthesizing of PHB from an excess carbon sources (Purwadi, n.d.). Bacteria that fall in this group include *R. eutropha*, *Protomonas oleovorans* and *Protomonas extorquens*. The second group of bacteria belongs to those that do not require limitation of nutrient for PHB synthesis. For these particular types of bacteria, biopolymer is accumulated during the growth phase of bacteria and microbes included *Alcaligenes latus* and recombinant *E. coli*. It is nearly 300 bacteria have been identified to have the ability of producing PHB bioplastic and below are three selected bacteria that proved to have the ability to synthesize PHB.

2.5.1 Recombinant *E. coli*

Recombinant *E. coli* bacteria are one of the few bacteria that has used for industrial production of PHB. Apart from that, among the others, it has been chosen to be the best and better commercial producer of PHB since it can use a wider range of cheap carbon sources. PHB polymer produced also is much easier to be extracted and purified from these particular bacteria than the others. According to Nikel et al. (2006), PHB is efficiently produced by a recombinant strain that grown aerobically in a fed batch cultures, using medium supplied with agro-waste. Based on the research done by Nikel et al. (2006), cells have accumulated PHB to 72.9% of their cell dry weight, reaching a productivity of 2.13g PHB per litre per hour. Physical analysis on the recovered PHB shows that its molecular weight is similar to the PHB produced by *Azotobacter* spp. and higher than bioplastic produced from *Cupriavidus necator***.**

Apart from that, in another studies made by Zhang et al. (1994), on the production of polyhydroxyalkanoates (PHA) in sucrose-utilizing recombinant *Escherichia coli* and *Klebsiella* strains, they found that the usage of recombinant *E.coli* as the producer capable to obtain intracellular polymer accumulations to a level as high as 95% biopolymer per cell dry weight with glucose, lactose or whey as the substrate. Together with the finding, they also discover that *E. coli* genetics capable to develop strains which synthesize the copolymer PHB-co-V that can be lysed by only the method of osmotic shock. It contains plasmid that does not need to be initially stabilised by antibiotics in the medium.

Several advantages have been identified for PHB production using recombinant *E. coli* bacteria, and these include its capability to produce PHB bioplastic within only 24 hours. This is compared to the other non-engineered producers, it take up to three days for the production to occur. Apart from that, a wide range of substrates also have been identified to produce PHB using recombinant *E. coli* and these include whey, agricultural wastes and molasses. This was discussed in Chee et al. (2013), where a recombinant *E. coli* strains has been reported to produce PHB on molasses as carbon source. The final dry cell weight, product content and productivity were determined to be 39.5%, 80% and 1 g/L/h respectively.

2.5.2 Cupriavidus necator

Cupriavidus necator also formerly known as *Ralstonia eutropha*, is one of the commonly known bacteria that accumulates PHA in a nutrient-limited condition excluded carbon source. It is a stable organism that formerly known to accumulate PHB with high productivity. Apart from recombinant *E. coli*, *Cupriavidus necator* is also commonly used and studied extensively to accumulate a large amount of PHB, around 80% (w/w) of cell dry weight from simple carbon source (Purwadi, n.d.).

The capability of *Cupriavidus necator* to produce a large amount of bioplastic has been proved by Verlinden (2011). In his study on production of PHA from waste frying oil, he found that the bacteria produced PHB from waste frying oil and the concentration, 1.2 g/L, obtained using the substrate was as high as the concentration that can be obtained from glucose. This has shown its capability on utilizing waste and still producing a significant amount of PHB. Higher PHB yield has been achieved in Fiorese et al. (2009) study's in which the yield is about 95% with 84% of purity when extracted from *Cupriavidus necator* cells at 130°C from 30 minutes without involving any pre-treatment. In another study made by Taniguchi et al. (2003), he has reported that waste plant oils and waste tallow has been discovered to successfully produced PHB with high yield by *Cupriavidus necator*. Currently, among other potential producers, PHB bacterial fermentation using *Cupriavidus necator* is the most cost-effective fermentation process, high productivity and is used widely in industrial processes.

2.5.3 Bacillus subtilis

Bacillus subtilis, is started to be paid attention as the potential producer of PHB after its performance in production of metabolites, bioremediation and generation of bioenergy. It is formerly recognised in the industrial scale production of amino acids, recombinant proteins and fine chemicals but never been tried for the biopolymers production (Singh et al., 2009).

Bacillus subtilis also known as grass bacilli are Gram-positive bacteria and well known bacteria species that are capable to grow within many environments (Earl, Losick & Kolter, 2008). Their capabilities that can be isolated from many environments, making them seems like they are broadly adapted to grow in various environmental condition. *Bacillus subtilis*, like other members of *bacillus* species, may form a highly resistant dormant endospores when undergo nutrient deprivation and other environmental stresses. It has been reported that among potential *Bacillus* spp., the PHB yield vary from 11% to 69% w/w of dry cell weight. Singh et al. (2009) has reported that *Bacillus subtilis* capable to synthesis PHB biopolymer but it is not suitable for an industrial production scale.

3 MATERIAL AND METHODS

3.1 Overview

This chapter explains in detail the research procedures of production of PHB bioplastic from sugarcane and pineapple peels and pulps. This research consists of five main parts which are factor identification, experimental designation, sample preparation, incubation and analysis. Waste juices obtained from both sugarcane and pineapple wastes were incubated with *Bacillus subtilis* bacteria in a flask. The samples were incubated according to the data that have been manipulated from the five factors affecting PHB production. As for the sample analysis, HPLC equipment has been used to determine the PHB concentration by comparing the peak area of samples with PHB standard's.

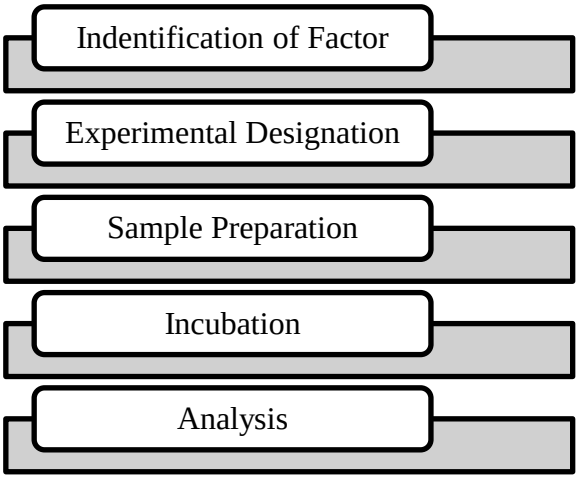

Figure 3-1: Work Flow of Research

3.2 Identification of Factor

There are five factors that have been identified to be studied as the parameters that could affect the production of PHB. All five factors; temperature, pH, agitation speed, types of waste and substrate to nutrient ratio, will used in the experimental designation using Two-Level Factorial Analysis utilized by Design Expert software. This software will arrange the experiments manipulating all five components so that a range of suitable parameter for the experiment could be obtained. Figure below shows on the five factors affecting the production of PHB.

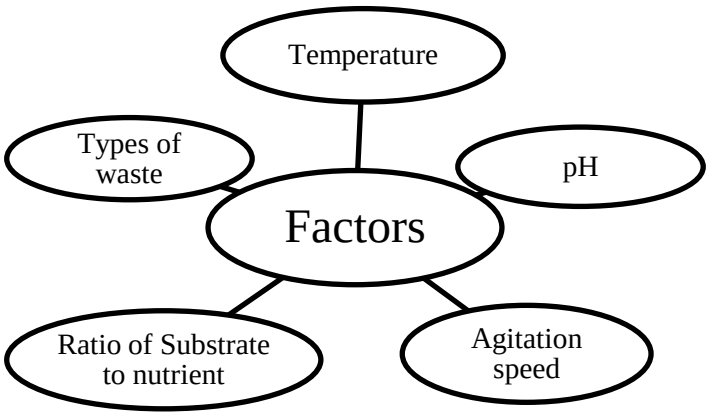

Figure 3-2: Factors Affecting the Production of PHB

Table 3.1: Experimental Designation based on Two-Level Factorial Analysis

Std	Run	Factor 1 A: Temperature	Factor 2 B: pH	Factor 3 C: Substrate:Nutrient	Factor 4 D: Agitation speed	Factor 5 E: Types of Waste
9	1	30.00	4.30	1:1	200.00	P
15	2	30.00	9.30	2:1	200.00	P
5	3	30.00	4.30	2:1	150.00	P
1	4	30.00	4.30	1:1	150.00	S
10	5	37.00	4.30	1:1	200.00	S
16	6	37.00	9.30	2:1	200.00	S
2	7	37.00	4.30	1:1	150.00	P
3	8	30.00	9.30	1:1	150.00	P
4	9	37.00	9.30	1:1	150.00	S
8	10	37.00	9.30	2:1	150.00	P
12	11	37.00	9.30	1:1	200.00	P
7	12	30.00	9.30	2:1	150.00	S
6	13	37.00	4.30	2:1	150.00	S
14	14	37.00	4.30	2:1	200.00	P
11	15	30.00	9.30	1:1	200.00	S
13	16	30.00	4.30	2:1	200.00	S

3.3 Raw Material Preservation

In this study, PHB was synthesized using sugarcane and pineapple peels and pulps as the carbon sources and *Bacillus subtilis* bacteria as the producer. Both carbon sources were obtained from stalls located in Kuantan area while as for the bacteria, it has been provided by the Faculty of Chemical Engineering and Natural Resources (FKKSA) laboratory.

The sugarcane wastes were squeezed into sugarcane squeezer machine to obtain its juice, meanwhile for the pineapple; both the peels and pulps were blended without the addition of water. The juices were then filtered using muslin cloth and centrifuged to remove the remaining solid impurities. These juices were then transferred into sample bottles and stored in freezer at a very low temperature, -8°C, to preserve it. The samples were removed from freezer and left at room temperature when experiment is about to start.

3.4 Experiment Set Up for Screening

3.4.1 Growing of Bacteria

Bacillus subtilis bacteria obtained from FKKSA lab was grown on agar plate. It takes about 24 hours, for the bacteria to grow completely under temperature of 30°C.

3.4.2 Inoculum Preparation

Inoculum was prepared by the preparation nutrient medium for inoculum. Eight grams of nutrient broth powder was diluted in 1 L of ultrapure water and stirred until it is well mixed. 150 mL of prepared nutrient broth was then transferred into a 500 mL conical flask, cotton plugged and autoclaved at 121°C for 20 minutes. The medium was then allowed to cool to room temperature before start bacterial transfer. Under sterilized condition, two loops full of bacteria was inoculated in 150mL sterile nutrient broth. The flask was then transferred into incubator shaker and incubated at 30°C; agitated continuously with 150 rpm agitation speed for 48 hours.

3.4.3 Fermentation Preparation

Fermentation was started with the preparation of fermentation medium. In 1 L of ultrapure water, the nutrient medium prepared consists of 0.2 gram of magnesium sulphate ($\mathbf{MgSO_4}$), 0.1 gram of sodium chloride ($\mathbf{NaCl}$), 0.5 gram of potassium dihydrogen phosphate ($\mathbf{KH_2PO_4}$), 2.5 gram of peptone and 2.5 gram of yeast extract. The fermentation conditions for all 16 experiments were designed using Two-Level Factorial Analysis performed by Design Expert.

Experiment was done in an incubator shaker, with a varied temperature; 30°C and 37°C and agitation speed of 150 rpm and 200 rpm. As for pH manipulation, 2M of sulphuric acid (H_2PO_4) and 2M of sodium hydroxide (NaOH) solutions were used to control the pH of 4.3 and 9.3 of fermentation medium. The next factor to be controlled is types of wastes where pineapple and sugarcane residues were used as substrates in this study. As for the last factor, substrate to nutrient ratio has also been manipulated in this study. Ratios of 1:1 or 2:1 were tested on a total of 150 mL fermentation medium.

For ratio of 1:1, every 67.5 mL of substrate; sugarcane and pineapple juice will be added with 67.5 mL of nutrient medium. Meanwhile, for ratio of 2:1, every 90 mL of substrate used, only 45 mL of nutrient added to make up a total of 135 mL of fermentation medium. Following the preparation, incubation was then done in an incubator shaker with the predetermined parameter. All 16 experiments designed by Design Expert software were incubated for 48 hours. All the processes were repeated until the experimental runs end.

3.4.4 Sample Retrieval

At the end of incubation period, 1 mL of fermentation sample was taken and centrifuged in microcentrifuge at 10 000 rpm for 5 minutes. The supernatant was then discarded and microcentrifuge with left cell pellet was dried in 60°C oven for two days. Drying process will remove the remaining water content in pellet which is prior in the sample preparation for HPLC test.

3.4.5 Analysis of Sample Using HPLC

The results of experiments was analysed by determining PHB concentration in each sample taken. The 48 hours dried samples were then digested in 1ml of 96% purity of sulphuric acid ($\mathbf{H_2SO_4}$) and allowed to homogenize at 90^0C for two hours. Dilution is done where 1 ml of homogenized sample were diluted in 9 ml of ultrapure water. It is then filtered using PES syringe nylon filter (0.22$\boldsymbol{\mu}$m, Milipore Corp., Bedford, MA) into HPLC vial and ready for analysis.

Type of HPLC detector used in this researched was DAD detector (UV-Vis detector). The type of Column used was Aminex Hpx-87 H column 300 x 7.8 um (HPLC column). The Column Pressure was 90 bar. Mobile phase used in the analysis was 0.004 M H_2SO_4 with column flow of 0.7 mol/min. Temperature was set to 35 ^oC and Injection Volume was 40 μL with flow rate 0.7 mol/min. The detector used in this research was 240 nm and the standard used was diluted PHB standard.

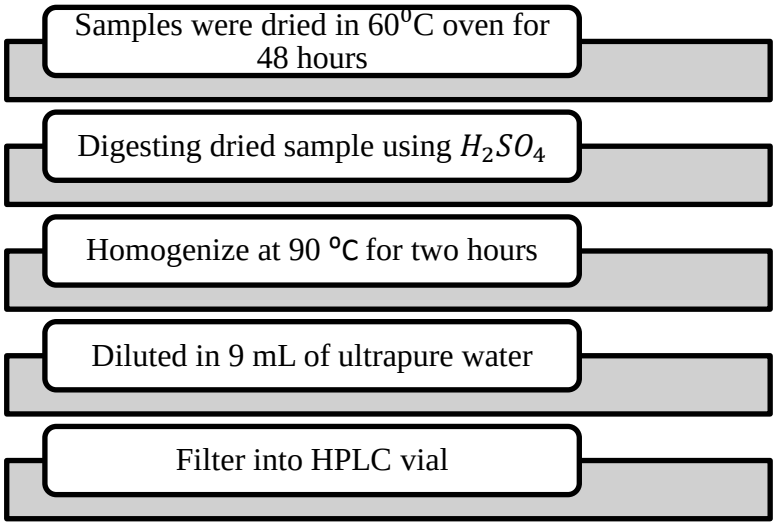

Figure 3-3: Work Flow of Sample Analysis using HPLC

3.4.6 Analysis from HPLC Data

PHB concentration was determined by means of retention time and peaks were quantified by comparing the highest peak area of samples with the results of PHB standard HPLC reading. From the peaks area, concentration of PHB can be determined using equation below:

$$\text{PHB content } \left(\frac{\text{g}}{\text{L}}\right) = \frac{\text{area of sample}}{\text{Standard area of P(3HB)}} \text{ x } 0.1 \frac{\text{g}}{\text{l}} \text{ x dilution factor}$$

Where;

Area of sample refers to the highest peak area in the graph of HPLC, standard area of PHB obtained from the HPLC analysis graph while the dilution factor of PHB content was 10.

4 RESULT AND DISCUSSION

4.1 Screening Result

Result obtained from the experiment was obtained in the form of PHB concentration in both fermentation samples of using pineapple and sugarcane waste juice as the substrates The concentration is as low as 0.4101 g/L up to as high as 0.4226 g/L. Sixteen experiments have been conducted based on the five selected factors determined by Two-Level Factorial Analysis utilized by Design Expert software. Table 4.1 below shows the details experimental set up and results obtained.

Results obtained from this experiment were analysed using Two-Level Factorial Analysis by Design Expert software. It is obtained in the form of percentage contribution towards PHB concentration in the fermentation sample. Result obtained from the screening process was shown below in Figure 4.1. The highest percentage contribution was 29.90% which comes from temperature factor, followed by agitation speed, substrate to nutrient ratio, pH and the least is types of waste with values 28.57%, 0.64%, 0.38% and 0.18% respectively. Apart from that, interaction between the factors also contributes toward the production of PHB. One interaction seems to give a significant contribution towards PHB concentration in the sample and the interaction is between temperature and agitation speed with 32.64% of contribution. Figure 4.1 below shows on the percentage contribution of all five parameters towards the concentration of PHB produced.

Table 4-1: Experimental Designation with Results

Std	Run	Factor 1 A: Temperature	Factor 2 B: pH	Factor 3 C: Substrate:Nutrient	Factor 4 D: Agitation speed	Factor 5 E: Types of Waste	PHB concentration (g/L)
9	1	30.00	4.30	1:1	200.00	P	0.4141
15	2	30.00	9.30	2:1	200.00	P	0.4144
5	3	30.00	4.30	2:1	150.00	P	04125
1	4	30.00	4.30	1:1	150.00	S	0.4112
10	5	37.00	4.30	1:1	200.00	S	0.4121
16	6	37.00	9.30	2:1	200.00	S	0.4134
2	7	37.00	4.30	1:1	150.00	P	0.4192
3	8	30.00	9.30	1:1	150.00	P	0.4126
4	9	37.00	9.30	1:1	150.00	S	0.4226
8	10	37.00	9.30	2:1	150.00	P	0.4212
12	11	37.00	9.30	1:1	200.00	P	0.4115
7	12	30.00	9.30	2:1	150.00	S	0.4122
6	13	37.00	4.30	2:1	150.00	S	0.4219
14	14	37.00	4.30	2:1	200.00	P	0.4119
11	15	30.00	9.30	1:1	200.00	S	0.4101
13	16	30.00	4.30	2:1	200.00	S	0.4111

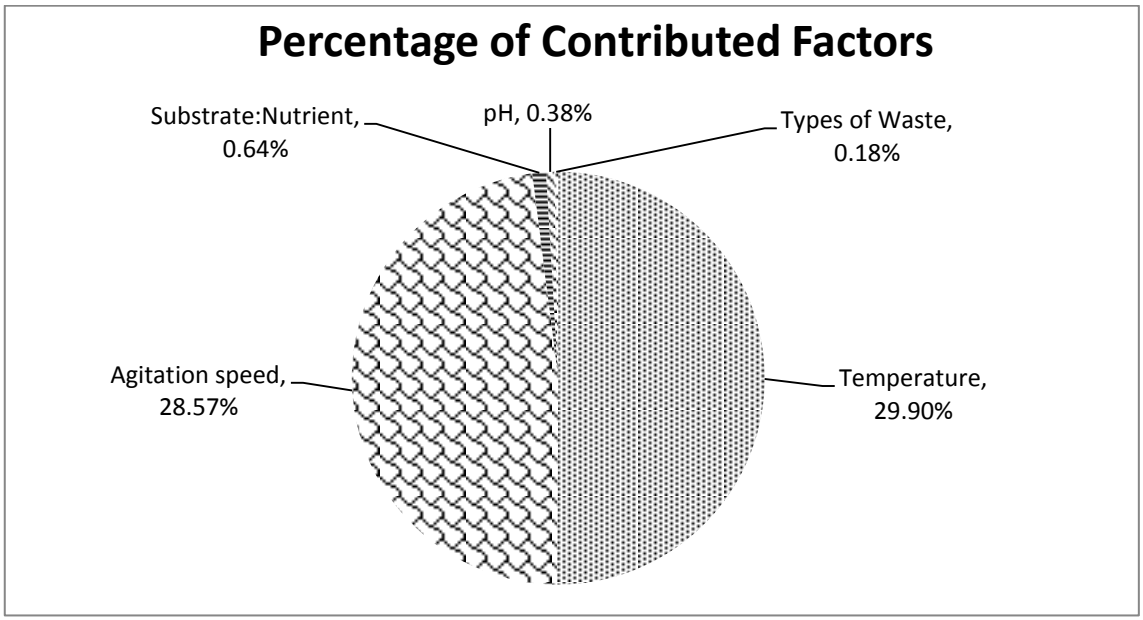

Figure 4-1: Percentage Contribution of Five Factors towards the Concentration of PHB Produced

Based on the results obtained in Figure 4.1 above, it is clearly shown that temperature is contributing the highest percentage toward the concentration of PHB produced in fermentation sample. Temperature plays the most important role in the cultivation of *Bacillus subtilis* since bacteria requires a specific temperature to grow. According to Germany (2001), *Bacillus subtilis,* is a thermophilic type of bacteria which capable to grow from the least allowing temperature, 15^{0}C, up to 55^{0}C the most. However, the selection of optimal temperature for PHB production will be different.

According to Singh et al. (2013), the increase of temperature beyond 40^{0}C has negative impact towards PHB production due to low PHB polymerase enzyme activity that is required for the production. It has been stated in a study using *Bacillus subtilis* strain NG220 as the PHB producer, it shown that PHB is able to be produced as high as 5.201 g/L at temperature 40°C and starts decrease at temperature above that. Apart from that according to Sathiyanarayanan et al. (2013), in a study on optimisation of PHB production using *Bacillus subtilis*, it shown that 30°C is the optimum temperature to synthesis high amount of PHB up to 62% of cell dry weight. It is reported where an extreme temperature results in the decrease of PHB accumulation. This is supported by Tamodogan & Sidal (2011), in their study on PHB production using *Bacillus subtilis* strain ATCC 6633. They have reported that PHB production decreased at extremes temperature due to low enzyme activity at such temperatures.

Agitation speed is the second factor contributed most in the PHB production from both sugarcane and pineapple wastes. In this study, to ensure an efficient and optimum oxygen transfer rate during fermentation, working volume is only 30% of total volume of shake flask. Apart from that, the fermentation medium was agitated at speed of 150 rpm and 200 rpm. In this study, *Bacillus subtilis* bacteria have been used. It is one type of aerobic bacteria that requires oxygen to accumulate PHB during fermentation process. One of the requirement methods for oxygen transfer in aerobic fermentation is by agitating the fermentation medium at a particular agitation speed. Different speed of agitation might results a different quantity of results produced (Grothe et al., 1999).

According to Wei et al. (2011), in a study in the effect of agitation rate on cell growth and PHB production by *Cupriavidus taiwanensis*, he has proved that PHB synthesis has increased with increasing agitation speed from 150 to 200rpm. Based on their study, the increase in agitation speed has enhanced both cell growth and as well as PHB production during the fermentation. However, the speed increase should be controlled since an excessive shear force was produced at agitation speed exceeding 250 rpm. This is because the shear force will damage the cell itself and thus inhibit the synthesis of PHB. Apart from providing a homogeneous fermentation mixture by mixing, agitation rate also affect the dissolved oxygen levels and mass transfer efficiency. These is environmental factor subsequently affect the cellular growth and bioproduct production (Wei et al., 2011). According to this research, it was clearly shown that agitation speed gives a very significant role towards the bacterial growth.

The third factor that contributes the highest PHB production is the factor of substrates to medium ratio with contribution of 0.64% of PHB concentration. In this experiment, two different ratios have been developed in order to identify the effect of different substrate to nutrient ratio supplied in the fermentation process towards the production of PHB. A ratio of 67.5 mL: 67.5 mL and 81 mL: 54 mL has been included as one of the screening factor and based on the results, this factor did gives effect towards the concentration of PHB in the fermentation broth, but it is not too high compared to the other top two factors since the percentage of contribution is only 0.64%. Sandya et al. (2013) reported in a research on biopolymer production using *Pseudomonas* spp. (MTCC) and its application in agriculture, the higher PHB accumulation has been encouraged by the higher content of carbon source, with less nitrogen, phenol and lignin contents since they inhibit the growth and PHB production. This has shown that a higher content of carbon source will be needed in order to ensure an optimum production of the product.

The pH of fermentation broth also contributes 0.38% towards the concentration of PHB. *Bacillus subtilis* able to grow in pH ranging from 4.3 up to 9.3, however, requires only specific pH to ensure an effective cultivation process. According to Aly et al. (2013), in a study on the effect of culture condition on PHB production by *Bacillus cereus*, it has been reported that maximum amount of PHB was produced in cultivation with pH 7. This is agreed by De Vries et al. (2004) and it is mentioned by Valappil et al. (2007) where a low pH conditions inhibit the utilization of PHB polymer.

In another study made by Flora et al. (2010), it is reported that another *Bacillus* species, *Bacillus sphaericus*, requires pH in the range of 6.5 to 7.5 to accumulate a maximum PHB production. Hence, it can be seen that *Bacillus* species requires a pH around 7 to ensure a high production of PHB. Wei et al. (2011) has reported that metabolic processes are highly sensitive to even slight changes in pH. The reduction of polymer accumulation at higher pH values results from the degradative enzymes of polymer breakdown, hence the rate of PHB utilized is equal to the rate of its synthesis.

The factor of types of waste does not contribute much on the production of PHB. This is because of the high sugar content in both pineapple and sugarcane were almost the same. According to Heyne (n.d.), sugar content in sugarcane is mostly sucrose, around 50% glucose and 50% fructose. As for pineapple, it consists of 2.9g glucose, 2.1g fructose and 3.1g sucrose per 100g of pineapple, hence making a total of glucose content after the reducing of sucrose is 48%. This has shown the content of glucose in both pineapple and sugarcane were almost the same. Microbial fermentation process requires sugar supply in the form of glucose, sucrose and fructose as carbon for to ensure a continuous production of product. Therefore, based on the quantity of glucose content in both pineapple and sugarcane juice, it can be conclude that the amount of glucose content in both of the carbon does not affect much in PHB synthesis since the glucose quantity are almost similar and there is not much different.

4.2 Interaction between Factors

Apart from the percentage contribution of factors towards PHB production, screening method also results in the interaction between the factors. There are ten pairs of interaction that contributes to the concentration of PHB produced. However, only two seems to give a significant interaction of the production of PHB. The interaction between temperature and agitation speed factor contributes the highest percentage that is 32.64% followed by interaction between temperature and types of waste, accounts for 5.45% contribution. The other interactions involved are interaction between agitation speed and types of waste, interaction between pH and substrate to nutrient ratio, interaction between pH and agitation speed, interaction between temperature and pH, interaction between temperature and substrate to nutrient ratio, interaction between substrate to nutrient ratio and agitation speed, interaction between pH and types of waste and the last is interaction between substrate to nutrient ratio and types of waste. The percentage of contributions for all of the interactions are 1.36%, 0.31%, 0.31%, 0.24%, 0.015%, 0.015%, 0% and 0% respectively.

Figure 4.2 below shows on the percentage contribution between factors. The highest interactions that gives a significant amount of contribution is the interaction between temperature and agitation speed, hence it will be discussed further in the next subtopic.

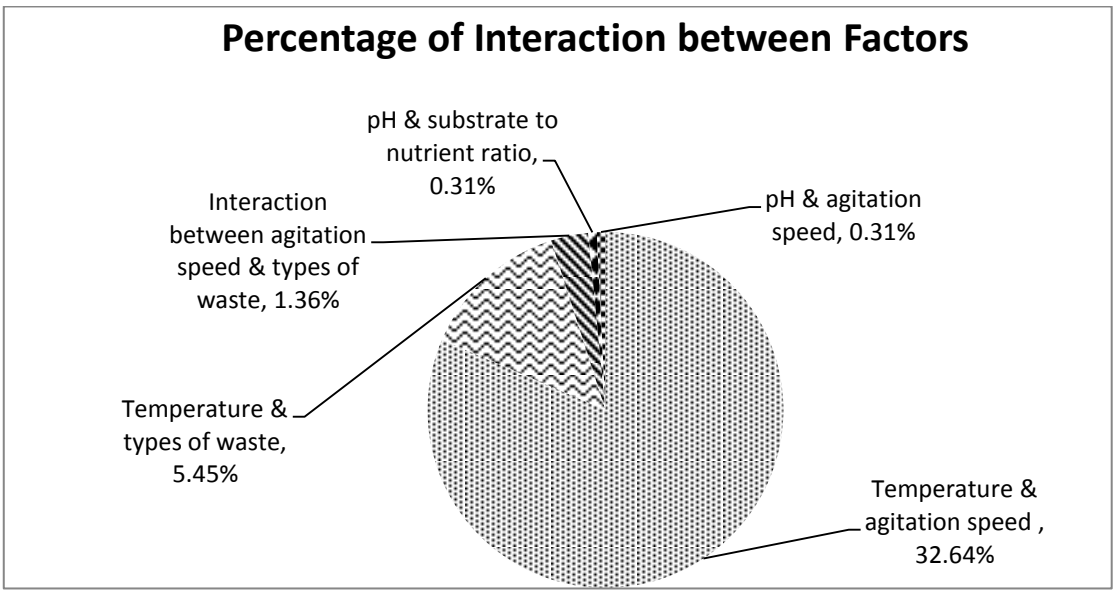

Figure 4-2: Percentage Contribution of the Interaction between Factors for Screening Process on PHB Production

4.2.1 Interaction of Temperature Factor with Agitation Speed

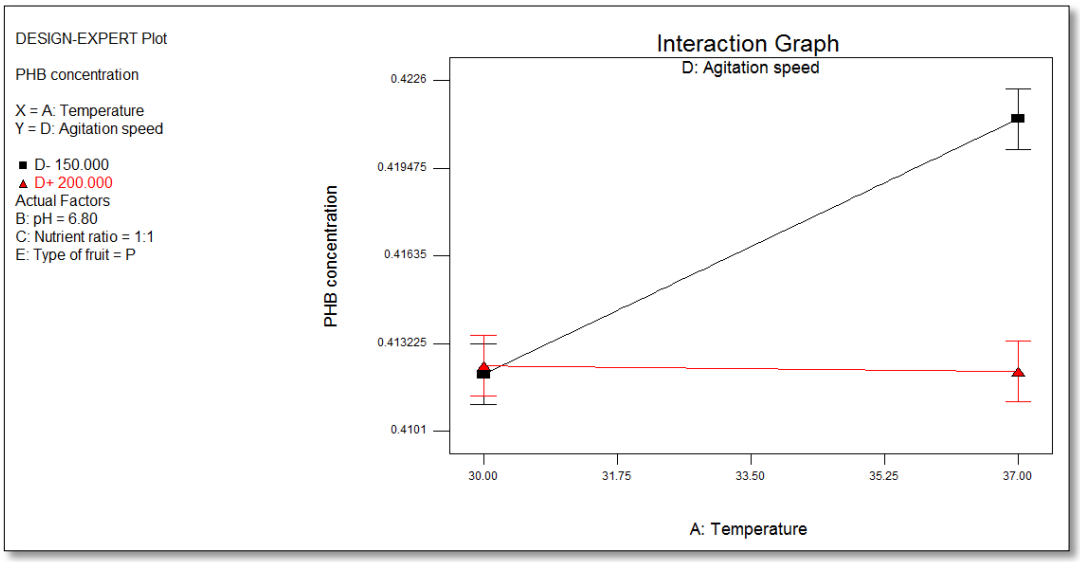

Figure 4-3: Graph of Interaction of Temperature with Agitation Speed factor

The interaction between factors also contributes to the production of PHB. The interaction between factor temperature and agitation speed gives 32.64% contribution towards the synthesis of PHB, which is higher than the other interactions. Based on Figure 4.3, it shows the interaction between factor temperature and agitation speed with responds to the concentration of PHB. When the agitation speed is low at 150 rpm (black line), the concentration of PHB has increased as temperature increase. Besides, the angles steeply upward, hence, representing a strong positive effect towards agitation speed as temperature increase. This is differ when agitation speed is high at 200 rpm (red line), the concentration of PHB decreases as temperature increase. This can be explains since these factors are related with the PHB synthase enzyme activity. Factor agitation speed has a strong effect towards the growth of *Bacillus subtilis* hence towards the secretion of enzyme by the bacteria. It is responsible in PHB production during the limitation of nutrient throughout the fermentation process. However, this enzyme is only active at certain range of temperature only. This has shown the interrelation of agitation speed and temperature towards PHB production.

Zahari et al. (2012) has reported in a study on PHB production using oil palm front juice by *Cupriavidus necator*, regarding the effect of agitation speed towards the growth of bacteria. It is mentioned that other than providing homogeneous cells by mixing and heat dispersion in the fermentation broth, agitation also gives a better aeration by increasing the oxygen transfer rate in fermentation medium. Generally, slower agitation speed may results the aggregation of cells and hence making the culture medium more heterogeneous. As a result, it causes the cell growth to be decreased and hence affecting the production of PHB. In another study made by Feng et al. (2003), it has been reported that a higher agitation speed increased the amount of dissolved oxygen and dispersion of macromolecules in the medium, thus contributes to the greater growth and better enzyme production.

In addition, enzyme is a protein; and protein is very sensitive towards temperature. Therefore, it is a must for temperature to be controlled as higher temperature would results in a denaturation of enzyme responsible for the PHB secretion. According to Wei et al. (2011), in a study on screening and evaluation of polyhydroxybutyrate-producing strains from indigenous isolate *Cupriavidus taiwanensis* strains, it is mentioned that temperature and agitation rate affect the dissolved oxygen levels and mass transfer efficiency and the factors profoundly affect cellular growth and bioproduct production. An effective temperature relation and agitation speed enhance the production of PHB hence results in higher concentration. Soetaert and Vandamme (2010) stated that the critical dissolved oxygen (O_2) level depends on the microorganism, culture temperature and the substrate being oxidized. The higher critical dissolve O_2, the greater the likelihood that O_2 transfer will become limiting. Hence, it is clearly shown the interaction between agitation speed and temperature is very important for an optimum PHB secretion. It is vital to control the temperature and agitation rate so that a sufficient O_2 is transfer with suitable temperature for cultivation.

4.3 Non Interacting Factors

There were a total of eight pairs of factors in this experiment that gives a minor interaction or did not interact at all with each other. The factors are as follows:

- Factor of agitation speed and types of waste
- Factor of pH and substrate to nutrient ratio
- Factor of pH and agitation speed
- Factor of temperature and pH
- Factor of temperature and substrate to nutrient ratio
- Factor of substrate to nutrient ratio and agitation speed
- Factor of pH and types of waste
- Factor of substrate to nutrient ratio and types of waste

As for the first interaction, interaction between agitation speed and types of waste, it contributes 1.36% towards PHB production. It is not possible to correlate both of the factors since agitation speed is only affected by the viscosity of the fermentation broth itself. It does not affect by the residues' types used in cultivating with *Bacillus subtilis* for PHB production. The second factor interaction between pH and substrate to nutrient ratio, contributes 0.31% towards PHB synthesis. This interaction does not affect the fermentation condition. This is the same condition with interaction between factor of pH and types of waste with 0.31% of contribution. It is because the mixture of substrate and nutrient pH will be standardized by adding buffer into desired pH before experiment started. Hence, the varying of substrate to nutrient ratio and types of waste did not affecting the pH. For pH factor with agitation speed factor which contributes 0.31%, these parameters did not interact because pH measurement is not dependent with agitation speed.

The interaction between temperature factor with pH gives 0.24% of contribution towards PHB production. It is supposed to give a significant effect towards the fermentation process hence towards the concentration of PHB. This is because of the PHB polymerase enzyme activity during the cultivation process. The pH factor contributes to the release of the polymerase enzyme by *Bacillus subtilis* where it enhances the secretion of PHB. However, this enzyme only secreted and active at certain ranges of temperature only. Lower or high temperature will affect the synthesis of PHB. In this study, the range of pH has been taken either pH 4.3 or pH 9.3 in which according to literature, *Bacillus subtilis* capable to live in that range of pH. The selection of pH is done based on the capability of *Bacillus subtilis* to grow within that condition. This is because there are no literatures that specifically discuss on the effect of pH towards PHB production when utilizing waste of pineapple and sugarcane as fermentation substrate. Based on this result, it can be conclude that even though *Bacillus subtilis* have the ability to grow within that range of pH, but for the purpose of PHB production, that pH is not suitable. Hence, based on the finding from this study, interaction between temperature and pH factor did not affect much toward concentration of PHB due to the wide range of pH value that is not suitable for PHB secretion.

Other than that, there is no interaction between substrate to nutrient ratio with temperature factor. The same result is also obtained for the next interaction factor of substrate to nutrient ratio and agitation speed. This is because both substrate to nutrient ratio and agitation speed did not depends on the temperature factor and substrate to nutrient ratio respectively. Therefore, these has resulted zero interaction between them. The same idea also valid for the last interaction factors of substrate to nutrient ratio and types of waste. If there is any change happen towards the types of waste, the ratio will still remain the same.

5 CONCLUSION AND RECOMMENDATION

5.1 Conclusion

Biopolymer such as polyhydroxybutyrate (PHB) has been found to be the potential replacement for existing conventional petroleum-derived plastic. However, the cost of production itself has limiting it for an industrial scale production. Agricultural waste such as pineapple and sugarcane peels and pulp has the potential to be the cheaper carbon source to synthesis PHB. The finding from this research, out of five factors, two factors have given much effect on the production. The factors are temperature factor and agitation speed factor while the other three factors that gives minor effect towards production of PHB are pH, substrate to nutrient ratio and types of waste with percentage of contribution were 29.90%, 28.57%, 0.64%, 0.38% and 0.18% respectively. There are also two interactions of factors that found to have a significant effect towards the process that is interaction of temperature factor with agitation speed and interaction of temperature factor with types of waste.

5.1 Recommendation

There are several improvements that can be made to get a better result. Based on this research, temperature has contributes the highest percentage of contribution towards the production of PHB. Hence, an extensive study can be made on the interaction of temperature with the other four factors to get the connection between them to enhance a better synthesis of PHB. Besides that, apart from screening method, optimization could be conducted in the future research, to obtain the suitable fermentation condition to optimized PHB production.

On the other hand, the screening of factors on the production of PHB by *Bacillus subtilis* utilizing pineapple and sugarcane peels and pulps could be improved. The range of pH can be tapered in the future research since it is one of the biggest contribution factors based on the literature done by other researches. Apart from that, the addition of growth medium with incubation time factor in screening process could enhance growth of *Bacillus subtilis*. A better production of PHB could be obtained with more varying potential contributing factors on PHB production. Since mentioned earlier, *Bacillus subtilis* is not suitable for an industrial scale production; the usage of other highly potential bacteria such as *Cupriavidus necator* and recombinant *E. coli* is prior for a better PHB production. Besides, the stains used in this research also seemed not suitable for PHB production based on the concentration of PHB obtained were almost the same although varied in parameters.

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APPENDICES

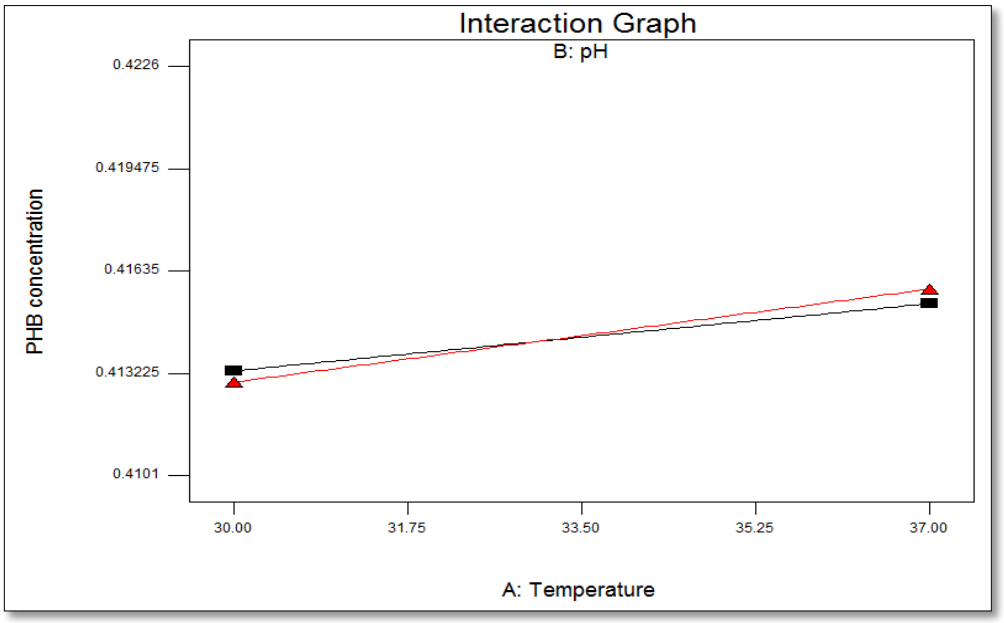

Figure 7-1: Graph of Interaction between pH with Temperature factor.

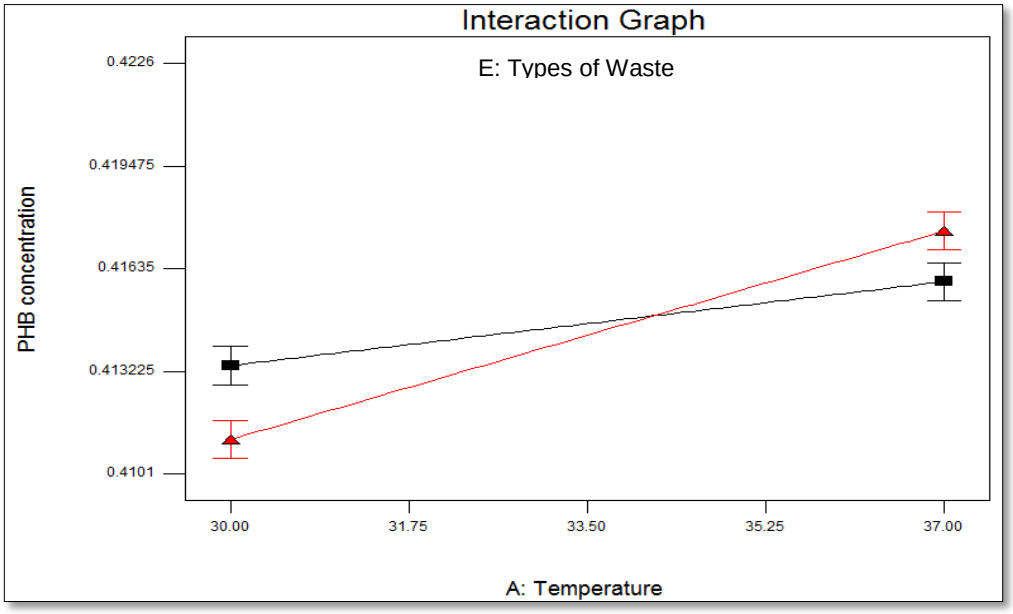

Figure 7-2: Graph of Interaction between Types of Waste with Temperature factor.

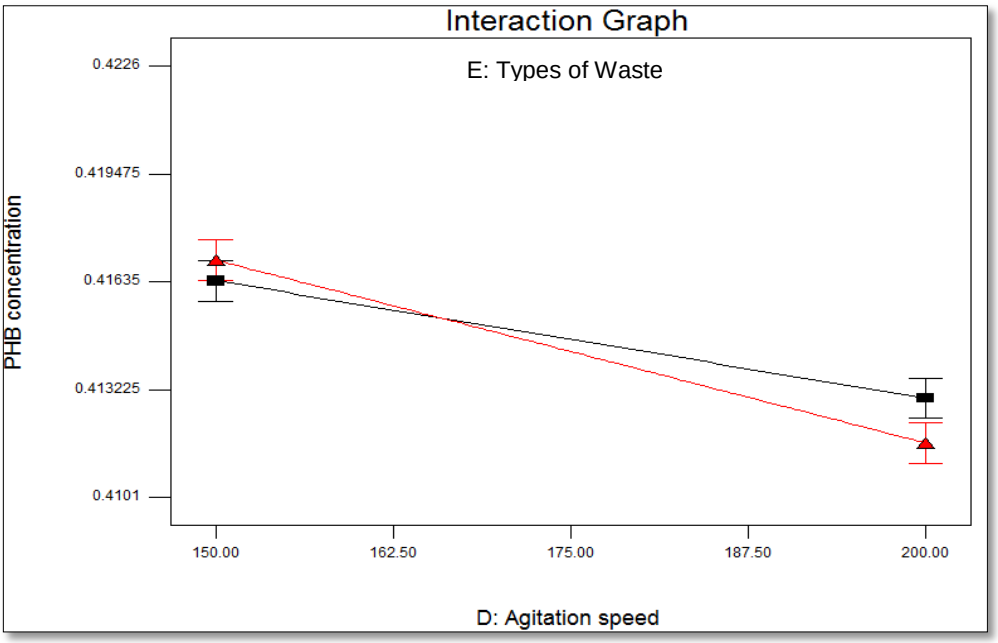

Figure 7-3: Graph of Interaction between Types of Waste with Agitation Speed factor

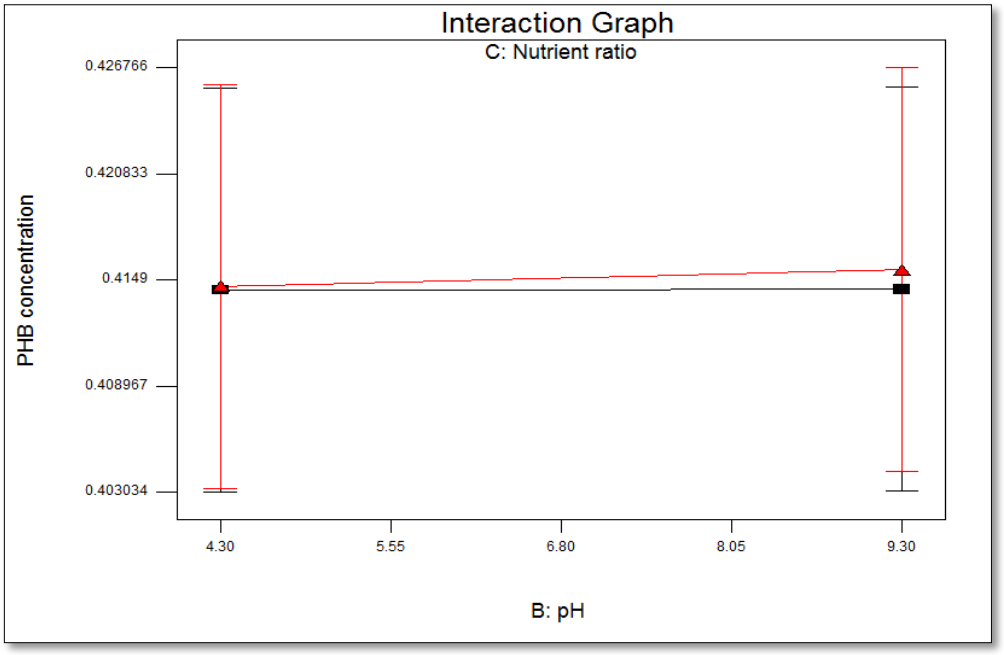

Figure 7-4: Graph of Interaction between Substrate to Nutrient ratio with pH factor

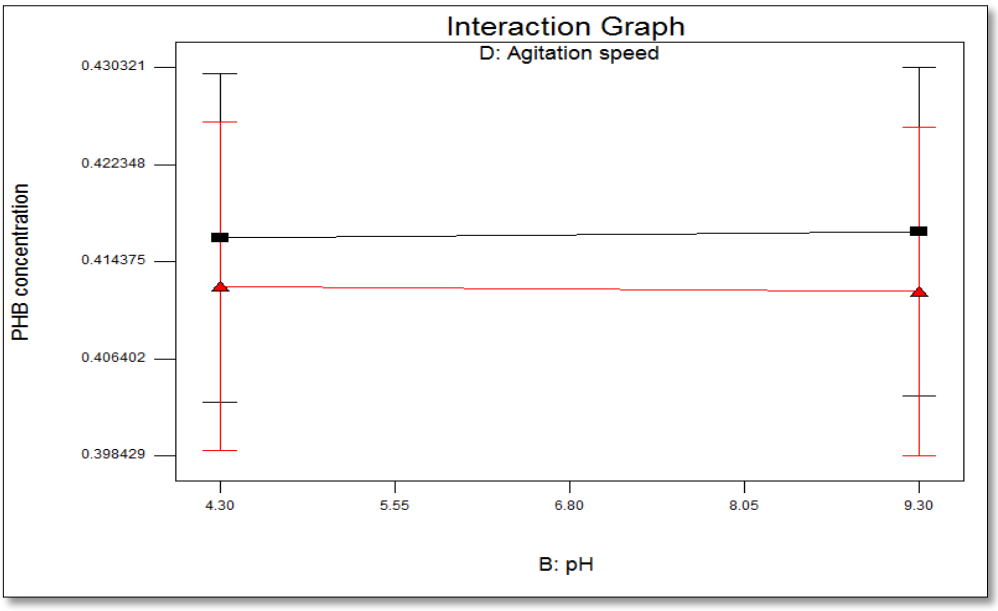

Figure 7-5: Graph of Interaction between Agitation Speed with pH factor

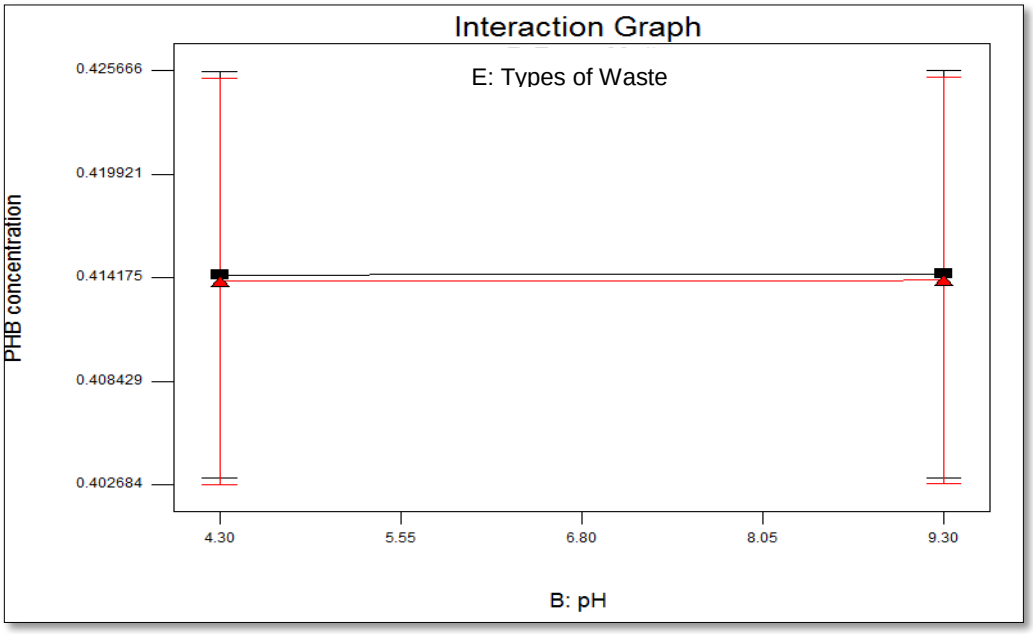

Figure 7-6: Graph of Interaction between Types of Waste with pH factor

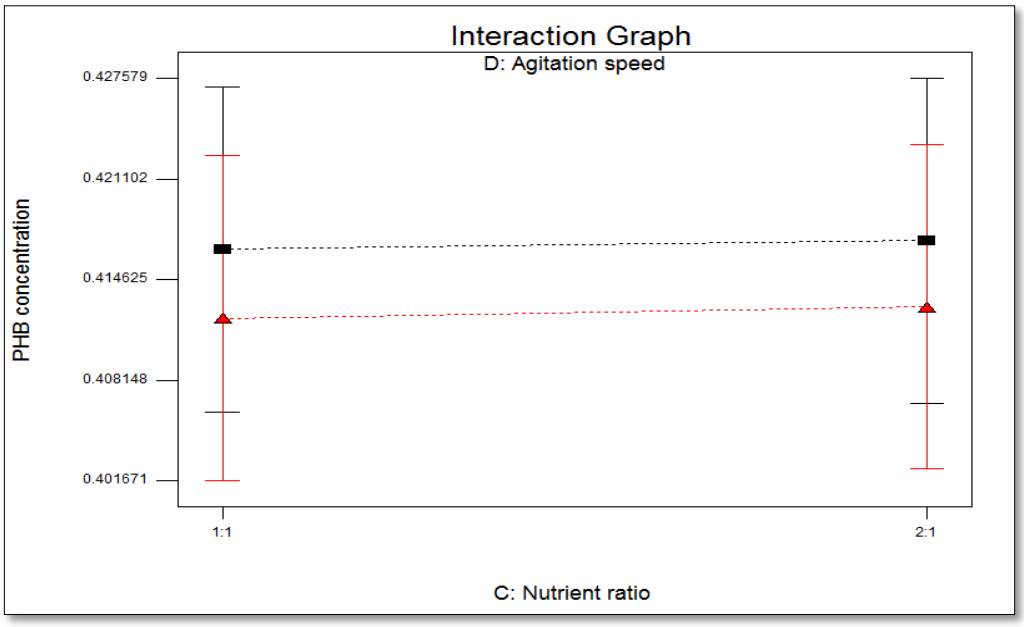

Figure 7-7: Graph of Interaction between Agitation Speed factor with Substrate to Nutrient Ratio factor

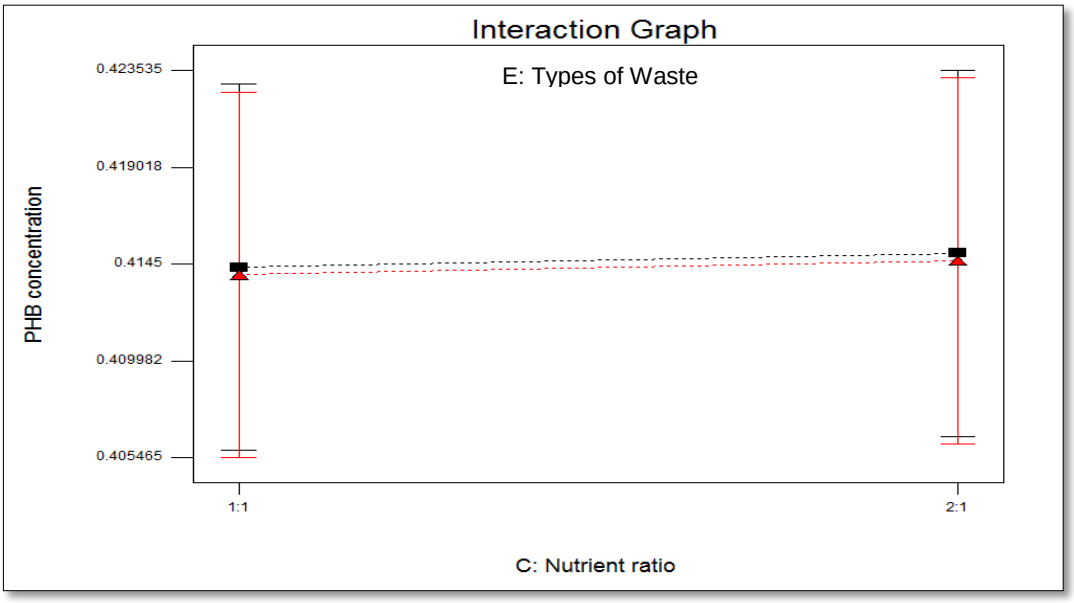

Figure 7-8: Graph of Interaction between Types of Waste factor with Substrate to Nutrient Ratio factor