SCREENING OF VARIABLES RELEVANT TO BIOMASS YIELD AND BIOPOLYMER YIELD IN SHAKE-FLASK FERMENTATIONS FOR BIOPOLYMER

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SCREENING OF VARIABLES RELEVANT TO BIOMASS YIELD AND BIOPOLYMER YIELD IN SHAKE-FLASK FERMENTATION FOR BIOPOLYMER

NURUL NADIAH BINTI MANAP

A thesis submitted in fulfillment
of the requirements for the award of the degree of
Bachelor of Chemical Engineering (Biotechnology)

Faculty of Chemical & Natural Resources Engineering
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May 2009

I declare that this thesis entitled “Screening of Variables Relevant to Biomass Yield and Biopolymer Yield in Shake-Flask Fermentation for Biopolymer” is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.”

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Date : 2 May 2009

Special Dedication to my family members,
my friends, my fellow colleague
and all faculty members

For all your care, love and support.

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ABSTRACT

The production of biodegradable polymer poly (3-hydroxybutyrate) can be done by the bacteria *Cupriavidus necator* under depletion of nitrogen source and excess source of carbon. In this research, screening of variables which are involved in the production of PHB was done. The experiment was focusing on determining which variables do not give significant effect toward the production of PHB. The significant effect for each variable which are the agitation rate, temperature, glucose, peptone, di-sodium hydrogen phosphate, ammonium sulphate, magnesium sulphate and potassium di-hydrogen phosphate were analyzed by using the factor analysis method. Linear models were used to describe the yield as the dependent variable in terms of factors as the independent variables. Starting with the factor corresponding to the smallest eigen values and proceeding with factors corresponding to successively bigger eigen values, factors were dropped and the mean square error of the resulting linear model is compared with the mean square error of the full model using the F test. For the linear regression involving uncorrelated variables for biomass yield, concentration of di-sodium phosphate and potassium dihydrogen phosphate respectively gave significant effect toward the yield. In addition, for biopolymer yield, the concentrations of peptone, ammonium sulphate, di-sodium hydrogen phosphate and potassium dihydrogen phosphate respectively give a significant effect toward the yield.

ABSTRAK

Penghasilan biodegradasi polimer poly (β-hidroksibutric asid) boleh dilakukan oleh bacteria *Cupriavidus necator* dalam keadaan kurangnya sumber nitrogen dan berlebihan sumber karbon. Dalam kajian ini, penyaringan terhadap pembolehubah-pembolehubah yang terlibat dalam penghasilan PHB telah dijalankan. Eksperimen yang telah dijalankan menfokus kepada penentuan pembolehubah yang tidak memberikan kesan terhadap penghasilan PHB. Kesan kepentingan bagi setiap pembolehubah iaitu kadar goncangan, suhu, gula, pepton, di-sodium hydrogen fosfat, ammonium sulfat, magnesium sulfat dan potassium dihidrogen fosfat telah dianalisa dengan menggunakan analisis faktor. Model lurus telah digunakan untuk mengambarkan hasil sebagai pemboleh ubah yang bergantung dalam erti kata faktor sebagai pemboleh ubah yang tidak bergantung. Bermula dengan faktor yang berkaitan dengan nilai eigen yang paling kecil dan berterusan dengan faktor yang berkaitan dengan nilai eigen yang paling besar secara berturutan, faktor-faktor dikurangkan dan min kesilapan kuasa dua hasil dari model lurus dibandingkan dengan min kesilapan kuasa dua model lengkap dengan menggunakan ujian F. Untuk regresi lurus melibatkan pembolehubah-pembolehubah tidak berkolerasi untuk hasil biojisim, kepekatan di-sodium hydrogen fosfat dan potassium di-hidrogen fosfat memberikan kesan penting. Tambahan lagi, untuk hasil biopolymer, kepekatan di-sodium hydrogen fosfat, potassium di-hidrogen fosfat, peptone dan ammonium sulfat masing-masing memberikan kesan penting terhadap hasil.

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LIST OF SYMBOLS/ABBREVIATIONS

PHB	-	poly (3-hydroxybutyrate)
RPM	-	agitation rate
Temp	-	temperature
G	-	glucose
P	-	peptone
N	-	ammonium sulphate
Na	-	di-sodium hydrogen phosphate
K	-	potassium dihydrogen phosphate
M	-	magnesium sulphate
F	-	factor
MSE	-	mean square error
g/L	-	gram per liter
$F_{0.75}$	-	F test distribution

CHAPTER 1

INTRODUCTION

1.1 Introduction

In this millennium era, with the continuous development of technology, more than 60 percent of equipments were made from plastics or in scientific word polymer. The wide used of plastic is due to its physical and chemical properties which are the quality of strength, lightness, durability and resistance to corrosion [1]. Most of polymer or plastic that being produced are from petrochemical based substance. In other words, polymer or plastic than being produce nowadays has low degradation rate. Therefore, this condition leads to waste pollution and increase the number of land filling area for disposing all the plastic waste. This condition also causes an increase the pollution of soil and environment.

In order to decrease the number of pollution cause by the non degradable plastic, there is a need to produce plastic or polymer from the biodegradable substance. One of the solutions is using the biopolymer that being produce by certain microorganism from fermentation process that can be degrades by the soil itself. There are many types of biopolymer that being synthesis from the microorganisms (e.g. *Azotobacter, Bacillus, Pseudomonas, Rhizobium, Methylotroph, Cupriavidus Necator* etc) and in this research, it will be focusing in producing the poly (3-hydroxybutyrate) or PHB by *Cupriavidus Necator* . The PHB is one of the family members of polyhydroxyalkanoates. Although it has good physical and chemical properties such as biodegradable, biocompatible and

has similar properties toward the propylene but it's production cost is high compare to the production of petrochemical based polymer. In PHB production, about 40% of the total production cost is for raw material [3]. In order to decrease the production cost of producing PHB, the substance that being used in the production need to be minimize. In order to minimize the substances that being used but at the same time maximize the PHB production, the screening of the variables that involve in the process of PHB production need to be done. In this research, 8 variables are screened in order to determine their significant effect toward biomass and biopolymer yield.

The screening process will be done by using factor analysis. The method of Factor Analysis has been used to screen the experimental variables which are most relevant to the fermentation [4, 5, 6]. Firstly, the experiment will be conduct based on the random value of variables. The variables that involve are agitation, temperature and concentration of glucose, peptone, magnesium sulphate, di-sodium hydrogen phosphate, potassium dihydrogen phosphate, and ammonium sulphate. The variables are distributed randomly by using 8 dices that being thrown simultaneously. Based on the PHB and cell biomass yield, the variables will be analyzed by using F test method. The F test method is one of the methods in factor analysis. The analyzing process by using the F test will be involving 2 methods. For the first method which is the linear regression involving correlated variables, the variables are assumed to be dependent with each other. The variance and mean square error for each variables will be compare with the variance that being obtained from the F test distribution table with 75 percent confident level. Then, each variable will be drop in order to observe the significant effect of each variable toward the yield. For the second method which is the linear regression involving uncorrelated variables, each variable is assumed to be independent toward each other. Even though each variable are uncorrelated toward each other but their mean and variance value will be the same. The significant effect for each variable will be analyze and being compare its mean square error and also to the first calculation. For both analyzing process, the least significant variables can be drop from the material that will be needed in the experiment. This will decrease the cost of production of PHB.

1.2 Identification of the problem

The development of this research is due to the handling problem of non-biodegradable plastic waste in the world and the high production cost of the biodegradable plastics. The research involves the screening process which can be defined as a process to remove things (variables) that are not acceptable or significant.

1.3 Statement of objective

The purpose of this research is to identify the significant effect of variables and the least significant variables toward the yield of the experiment by using one of Factor Analysis method.

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1.4 Scope of study

To study the significant effect for each variables which are the agitation rate, temperature and concentration of glucose, peptone, magnesium sulphate, di-sodium hydrogen phosphate, potassium dihydrogen phosphate, and ammonium sulphate toward the yield of the biomass and PHB in shake flask fermentation by using one of the method of Factor Analysis [4, 5, 6] which is the method of F test.

1.5 Rational and significant

To identify the significant variables toward the biomass and PHB yield by using F test method.

CHAPTER 2

LITERATURE REVIEW

2.1 Conventional plastic versus biopolymer

Conventional plastic has been used widely around the world in many forms. The conventional plastic mainly being made from many types of polymer as their backbone structure such as polyvinyl chloride, polyethylene, polymethyl methacrylate, acrylics, silicones, and polyurethanes. Plastics have been used to make stationary, utensils, packaging bags and others. In large scale of usage, 50 % of usage of these conventional plastics is for packaging industries solely. The reason for such a wide usage is their versatile qualities of strength, lightness, durability and resistance to corrosion [1]. Nevertheless, the conventional plastics that have been used nowadays are not easy to degrade and it has become an indispensable materials. This property of plastics makes them an environmental hazard [8]. On the other hand, biopolymer is one of the polymer that being produce from the living organism. Examples of biopolymer are starch, proteins, peptides, DNA, and RNA. They are made from combination of monomers which are sugars, amino acids, and nucleic acids respectively. Another type of biopolymer is polyhydroxybutyrates, a class of polyesters produced by certain bacteria. In this research the biopolymer that will be focusing is polyhydroxybutyrates.The

biopolymer can be produce from renewable resources which will decrease the production cost compare with the conventional polymers or plastics. This can lead to the sustainable development of biodegradable plastic industry. In addition, the biopolymer based product is biodegradable compare to conventional polymer or plastics which is petrochemical based product. Consequently, biodegradable biopolymer produced from renewable resources may represent a viable and environmentally friendly alternative to traditional plastics [9].

2.2 Poly-β-hydroxybutyric (PHB)

2.2.1 Background

Poly-β-hydroxybutyric (PHB) belongs to a family of microbial energy or carbon storage compounds collectively known as poly (hydroxyalkanoates) (PHA). PHB can be produce by many microorganisms such as *Alcaligenes eutrophus*, *Bacillus megaterium* or *cynobacterium* (*Nostoc muscorum*). Most of the organisms (e.g. *Azotobacter*, *Bacillus*, *Pseudomonas*, *Rhizobium*, *Methylotroph*, etc) are capable of accumulating PHB up to 30 to 80 percent of their cellular dry weight [2]. In this research, the *Cupriavidus Necator* is being used as the microorganism that will be responsible in producing PHB. The general structure of PHB is as below:

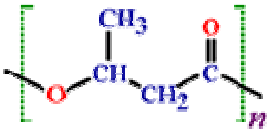

Figure 2.1: General structure of PHB

PHB is a highly crystalline polymer and its melting point is 175°C. It decomposes at 200°C. Its mechanical properties like flexural modulus and tensile strength are similar to polypropylene. It is 100% biodegradable [10]. PHB is resistant towater and ultraviolet radiation, and impermeable to oxygen. It is readily

biodegraded in soil [11]. PHB is a thermoplastic and one of the most widely investigated members of the family of polyhydroxyalkanoates (PHAs) [12].

2.2.2 Production of PHB

Mainly, PHB was produced from the microorganisms. PHB will be synthesis by the microorganisms as the intracellular carbon source when condition where source of nitrogen is limit and the source of carbon is excess. PHB is accumulated as a storage material, whose function is to provide a reserve of carbon and energy [12]. There are many ways to produce the PHB. One of the ways is through fermentation process, which will be use in this research. Generally, fermentation process is a process where carbohydrate sources being convert to acid or alcohol. In more specific process, PHB is synthesized as figure 2.2 from acetyl-CoA produce by the bacteria in sequential action of three enzymes. 3-ketothiolase (phbA gene) catalyses theformation of a carbon-carbon bond by condensation of two acetyl-CoA (Masamune et al. 1989a, b). NADP dependent acetoacetyl-CoA reductase(phbB gene) catalyses the stereoselective reduction of acetoacetyl-CoA formed in the first reaction to R-3-hydroxybutyryl CoA. The third reaction of this pathway is catalyzed by the enzyme PHB synthase (phbC gene) that catalyzes the polymerization of R-3-hydroxybutyryl-CoA to form PHB. The EC number is yet to be assigned to PHA synthase (Steinbüchel and Schlegel 1991, Belova et al. 1997).

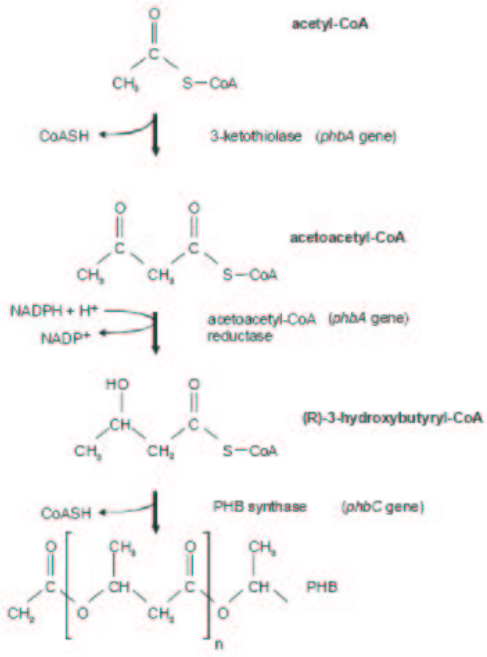

Figure 2.2: Biosynthetic pathway of PHB from acetyl-CoA.

2.2.3 The benefits by producing PHB

There are several benefits from the production of PHB. The biopolymer can be produce from renewable sources such as from plant materials which being planted each year. This will guarantee of the sustainable of development of biodegradable plastic industry. In contrast with the conventional plastics that was being made from petrochemical substances. The petrochemical substance will eventually run out in several decades or years onwards. Therefore, biopolymer can be considered as a good petroleum-derived synthetic plastic subtitutes. PHAs exhibit material properties similar to various synthetic thermoplastics and elastomers currently in use, from polypropylene to synthetic rubber. Besides, upon disposal, they are completely degraded to water and carbon dioxide (and methane under anaerobic condition) by microorganisms in various environments such as soil, sea lakes and sewage [13]. In addition, the technology for the PHB production is already existed and there is no

need production equipment investment because the existing equipment for the production of propylene and polyethylene can be use.

2.2.4 Application of PHB

Due to its properties which are biocompatible and biodegradable thermoplastics, PHB has a big potential in medical fields, agriculture and marine. Other than that, because PHB is resistant to water and ultraviolet radiation and it is impermeable to oxygen, it is especially suitable to use in food packaging [14]. In medical field, there has been a proof application of PHB. Previous report from the laboratory demonstrate that poly-β-hydroxybutyrate (PHB) sheet with unidirectional fiber orientation could be used as a wrap-around implant to guide axonal growth after peripheral nerve injury [15].In addition, we have developed biodegradable conduit for spinal cord repair which is based on strands of PHB fibers coated with alginate hydrogel and supplemented with cultured Schwann cell [16].

2.3 The method of Factor Analysis [4, 5,6]

There are many methods in Factor Analysis [4, 5,6] and one of it is F test method. In this research, in order to screen all the 8 variables toward the biomass and biopolymer yield, the F test method will be conduct. In an unoptimised medium, there might exist components which do not have any effect on biomass and product formation which is the formation of biopolymer. It is also possible that relevant nutrient is present in more than one supplement, or even in the base medium itself.

A linear model will be used to describe the dependent variables which are the yield of biomass and biopolymer in terms of the independent variables which are the 8 variables that will be screen in the experiment. The below formula from linear regression can be used to relate between the yield and variable:

$$Y = a_0+a_1X_1+a_2X_2+a_3X_3+a_4X_4+a_5X_5+a_6X_6+a_7X_7+a_8X_8 \qquad (2.3.1)$$

Where Y is the fermentation yields which are biomass and biopolymer, X_i is an independent variables and a_i is a regression coefficient. If an independent variable X_i has no effect on the fermentation yield, the mean square error of the regression of the resulting linear equation when that variables are dropped will be not be significantly different from the mean square error of the full linear equation. This screening process need to cover as wide a response surface as possible and the combination of levels of experimental variables for each variable for each experiment has to be random distribute. This can be done by using 8 different dices which being thrown simultaneously. Each surface of the dice represents one level value for each variable.

Depending on the choices that being made for all the variables in each experiment, the correlation between two variables might be big or small. Therefore, the correlated standardize variables will be transform into uncorrelated or orthogonal variables. Therefore, each variable will be distributed into different categories of contribution. These orthogonal factors can be used in modeling where the independent variables are truly independent toward each other.

CHAPTER 3

METHODOLOGY

3.1 Introduction

For this research, the methodology can be divided into two major steps. The two major steps are the mathematical method which involving the Factor Analysis [4, 5, 6] and the methodology of the experiment. The mathematical method will be focusing in calculation of the data that being obtained from the experiment. On the other hand, the methodology of the experiment will be focusing in the steps to do the experiment in order to obtain the biomass and biopolymer yield.

3.2 Mathematical Method

The mathematical analysis of the experimental data will involve the one of the factor analysis which is the F test method. In this analysis, it can be divided into three major components which are:

3.1.1 Standardization of the variables
3.1.2 Linear regression of the variables and yields
3.1.3 Dropping each variables - F test
3.1.4 Transforming Experimental Variables into Orthogonal Factors

3.2.1. Standardization of the variables

The screening process for this experiment will involve eight different variables.Each variables has its own unit and value. The experiment will be conduct for sixty times for each variable. All the value for each variable will be determine by using the dices which will lead to the random distribution of the data.In order to observe the relationship between the variables and the yield, the variables need to standardize so that they can be compare fairly toward each other.All variables will be standardizing by using the simple formula which is

$$w_{ij} = (x_{ij}-\mu_i)/v \qquad (3.2.1.a)$$

where

a. w_{ij} is the standardize experimental value which has no unit
b. x_{ij} is the experimental value for each variables in its unit
c. μ_i is the mean of a non-standardized experimental variables which define as $\sum x_{ij}/ N$, where N is the total number of experiment
d. v is the root mean square deviation value for each of the variables

The first step for the standardization of the variables is calculating the mean for each variables by using the below formula

$$\mu_i=\sum x_{ij}/ N \qquad (3.2.1.b)$$

where

a. x_{ij} is the value of experimental variables in its unit
b. N is the number of experiment that being conduct

Then, the root mean square for the deviation,v is to be calculate by using the formula

$$v^2 = \sum(x_i-\mu_i)/ N \qquad (3.2.1.c)$$

where

a. v^2 is the value variance of the experimental data for the experiment.
a. x_i is the experiment value for each variable
b. μ_i is the mean value for each variable

Next, all the value that being calculate can be insert into the standardize formula that being mentioned before. The variance and mean of the w_{ij} value should be 1 and 0 respectively. The variance value can be calculate by summation of w_{ij}^2 divide with number of experiment while for the mean of w_{ij}, the summation of w_{ij} divide by the number of experiment.

3.2.2 Linear regression of the variables and yields

After the calculation of standardization of each variable is being done, in order to observe the relationship between the values of standardize experimental variable toward the experimental yield, linear regression is the second major step by calculating the mean square error of the experimental yield. All the calculation can be done by using Microsoft Excel software. Nevertheless, it also can be done by calculate it manually but will require much longer time. The mean square error of the yield can be calculate by using the formula which is

$$\text{Mean square error} = \sum(Y-Y_{new})/N \qquad (3.2.2.a)$$

where

a. Y is the value of the experimental yield
b. Y_{new} is the value of the experimental yield that being calculated by using the linear regression formula which is $Y_{new}= a_0+a_1w_1+….+a_iw_i$. The a_i is the constant value for each variable and w_i is the value of each standardize variable.

As mentioned before, in order to calculate Y_{new}, the linear regression formula ($Y_{new}= a_0+a_1w_1+…. +a_iw_i$) will be use. First, the ai value needs to be determined.

This can be done by using the Matlab software.The mean square error of the experimetal yield will give a clear view whether he experimental result is reliable or not.

3.2.3. Dropping each variables - F test

Repeat the calculation of linear regression between the value of experimental yield and The standardized variable but this time, for each repeating set, one standardize value of variable will be drop. Then, calculate the mean square error of the experimental yield for each set. In order to observe the effect of dropping each value of standardize variable toward the experimental yield, the value o mean square error of complete set (no dropping f variables) divide with the value of mean square error for each set of dropping one variable.

Then, the value for each set will be compare with the value that being obtain from the F distribution test table with the degree of freedom as follow:

a. degree of freedom 1= N-1
b. degree of freedom 2= N-1- number of standardize variable that being drop

If the value of each set larger than the value in the F distribution test table, then the standardize variable is significant toward the experimental yield.

3.2.4 Transforming Experimental Variables into Orthogonal Factor

For this part of calculation each factor will have the same mean as the experimental variables equal to 0.and the same variance as the experimental variables equal to 1.0. Also, zero correlations between them.

The table of levels orthogonal factors for all experiments will be derive from the table of levels of standardized experimental variables of the original experiments by calculating the value of each factor using the equation:

$$F_{rj} = \sum_{1}^{n} {}_{i} \eta_{ri} w_{ij} \qquad (3.2.4\ a)$$

where

a. F_{rj} is the generic j value of each of factor F_r
b. η_r is the r th coefficient of eigen vector associated with the eigen values of the square matrix of correlations between the standardized experimental variables.
c. n is the total number of experimental variables.
d. w_{ij} is the generic value of each standardized variable w_i

Then, the eigen value and eigen vector need to obtain by using the technique of matrices. This can be done by using the Matlab software. Each of standardize variables will be multiply b its own eigen vector. Based from this process, each variable will be independently toward each other. Next, each variable will be drop at a time and the regression analysis will be done. The regression analysis is being done by dropping the value of the variables with smallest eigen value. Then, the mean square error for each dropping will be compare with the mean square error of complete model.

3.3 Methodology of the experiment

The procedure for this experiment can be divided into five stages which are

3.2.1 Preparation of the inoculum or culture broth
3.2.2 Preparation of the stock solution
3.2.3 Fermentation process
3.2.4 Analysis of cell dry mass
3.2.5 Analysis of PHB

3.3.1 Preparation of the inoculum or culture broth

The pure culture strain which is *Cupriavidus Necator* need to be breed in the slant agar. The agar is prepared with the specific condition based on the NGY agar for the strain that will be breed. Substance contain in the NGY agar are glucose (10 g/L), peptone (5 g/L), yeast extract (3 g/L), beef extract (0.3 g/L) and agar (30 g/L). Then, transfer the culture by swaping it with inoculum rod into 10 ml of NGY agar in the 100 mL of conical flask. name this flask as starter 1. The composition of the NGY broth are are glucose (10 g/L), peptone (5 g/L), yeast extract (3 g/L) and beef extract (0.3 g/L).Incubate the flask containing the culture broth for 24 hours at 30°C and agitation at 200 rpm. In order to obtain large amount of inoculum, starter 3 will need to be prepare. The process will be started as mineral salt medium was prepared according to its concentration. The composition and concentration of the mineral salt medium are glucose (20g/L), peptone (5g/L), magnesium sulphate (0.2g/L), di-sodium hydrogen phosphate (6.7g/L), potassium dihydrogen phosphate (1.5g/L), and ammonium sulphate (2 g/L). 180 mL of the prepared mineral salt medium was poured into 500 mL conical flask. Name this flask as starter 2. 20 mL culture broth from starter 1 was poured into the starter 2.The starter 3 was incubate for 48 hours at 30°C and 200 rpm.

3.3.2 Preparation of stock solution

The stock solution for the medium that will be used in the fermentation process need to be more concentrated than the given concentration. This is because it is more convenient to dilute the stock solution than to make new medium with different concentration all over again. The dilution of the stock solution can be done by using the formula as follow:

$$M_1V_1=M_2V_2 \qquad (3.3.2.a)$$

where

a. M_1 is the molarity of substance 1
b. M_2 is the molarity of substance 2

c. V_1 is the volume of substance 1

d. V_2 is the volume of substance 2

3.3.3 Fermentation process for screening experiment

For screening experiment, the concentration of glucose, peptone, magnesium sulphate, di-sodium hydrogen phosphate, potassium dihydrogen phosphate,ammonium sulphate, temperature and agitation rate were varied according to the data that already being set before the experiment get started. The data was being set by throwing 8 different dices which represent for each variable. 6 surfaces on each dice will represent the range of value for each variable. The dice was used in order to create a randomization. The data is as in table 3.2 and 4.1.1

Level	RPM	Temp (°C)	G (g/L)	P (g/L)	N (g/L)	Na (g/L)	K (g/L)	M (g/L)
1	160	26	10	2	1	2	0.5	0.1
2	180	28	20	4	2	4	1.0	0.3
3	200	30	30	6	3	6	1.5	0.5
4	220	32	40	8	4	8	2.0	0.7
5	240	34	50	10	5	10	2.5	0.9
6	260	36	60	12	6	12	3.0	1.1

Table 3.2: The level value for each variable

3.3.4 Analysis of cell dries mass

1 mL of the sample was centrifuge at 15000 rpm, 4°C for 4 minutes.The supernatant was refrigerated for further analysis while the cell pellet was washed with deionized water.The cell pellet was dry to constant weight at 90°C for 24 hours. Then, it was cooled in a desiccator for 30 minutes and weighs it. There is a simple way to determine the cell dry mass. A set of data was selected from one fermentation process. The standard curve of optical density versus cell dry weights of cell was determined. Thus, for the next experiment, only the optical density for the sample will be needed to

determine and the cell dry mass for the samples can be obtained from the standard curve.

3.3.5 Analysis of PHB

10 mL of sodium dodecyl sulphate solution (1% w/v, pH 10) was added into the biomass pellet that being obtained from the centrifuged process as describe in cell dry mass measurement.The mixture was incubate at 200 rpm, 37°C for 60 minutes. Then, the solid that being recovered from the centrifugation process was washed with sodium hypochlorite solution (5.64% w/v) that has been diluted for 20 mL.Centrifuge the mixture at 6000 rpm for 4 minutes and then wash it with 20 mL deionized water and centrifuge the mixture again.The pellet that already obtained from the centrifugation process was dry at 90°C for 24 hours to constant weight in aluminium dishes.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Experimental results

Table 4.1.1 shows the values for each variable according to the dices method. Each variable contain 6 different values which represent 6 surfaces of dices. Then, table 4.1.2 shows the value of biomass and biopolymer yield for each running of the experiment. All the data has been analyze by using two different factor analysis methods. For the first method is linear regression involving correlated variables. In this method, each variable was assumed dependently toward each other. The constant value for linear regression for each model for both biomass and biopolymer yield is shown in table 4.1.3 and 4.1.4.The results can be observed in table 4.1.5 and 4.1.6. Next, second method which is linear regression involving uncorrelated variables where the standardize variables were transform into orthogonal factors. In order to transform, the value of eigen vector and eigen value were determine by using MATLAB software based on the correlation matrix of all the variables. The correlation matrix of all variables is shown in table 4.1.7. The value of eigen value can be observed in table 4.1.8 while for eigen vectors in table 4.1.9. Then, for the orthogonal factor method, the result can be observed in table 4.1.12 and 4.1.14 respectively for biomass and biopolymer. Table 4.1.10 consists of standardize value of variables in a form of orthogonal factor after being multiplied by eigen vector. For table 4.1.11 and 4.1.13, each table consists of coefficient and constant for linear model of biomass and biopolymer respectively.

Table 4.1.1: Experimental data for variables (dices method)

Run	RPM	Temp (°C)	G (g/L)	P (g/L)	N (g/L)	Na (g/L)	K (g/L)	M (g/L)
1	180	26	50	4	1	2	1.5	0.3
2	260	30	50	10	1	12	0.5	0.7
3	260	30	20	10	2	10	0.5	0.1
4	180	32	10	4	4	10	0.5	0.1
5	200	32	50	2	2	8	1	0.5
6	200	32	20	2	1	8	2	1.1
7	220	32	10	6	4	8	2	1.1
8	220	32	50	8	1	8	1.5	0.3
9	220	32	30	4	3	6	1.5	0.3
10	260	32	20	6	1	12	3	0.1
11	260	32	50	6	1	12	0.5	0.5
12	260	32	50	12	6	12	1	0.3
13	180	34	50	10	5	10	2	0.7
14	200	34	40	2	1	12	3	0.5
15	200	34	40	6	6	10	2	0.5
16	200	34	50	2	3	4	0.5	0.7
17	220	34	60	4	5	10	2	0.5
18	240	34	20	12	4	4	0.5	0.7
19	260	34	40	10	3	10	2	0.3
20	260	34	50	12	2	6	2	0.1
21	160	36	60	4	2	12	2	0.7
22	160	36	30	10	6	10	2.5	0.5
23	180	36	10	2	3	4	0.5	0.9
24	200	36	10	6	2	6	2.5	0.9
25	200	36	30	2	2	2	2.5	0.7
26	240	36	10	2	6	6	2.5	1.1
27	260	36	20	6	2	8	0.5	0.9
28	260	36	50	4	4	6	0.5	0.7
29	160	28	20	6	2	2	2.5	0.3
30	160	28	60	10	3	10	2.5	0.7

Table 4.1.2: Experimental data for biopolymer and biomass yields

Run	Biomass (g/L)	PHB (g/L)
1	3.916	0.63
2	1.036	1
3	1.9838	0.73
4	4.149	0.91
5	2.865	0.765
6	3.111	1.27
7	2.1721	0.19
8	4.6502	2.2
9	3.523	0.63
10	2.096	0.43
11	7.012	0.81
12	6.896	4.145
13	3.755	2.75
14	4.7379	0.86
15	3.318	0.75
16	6.954	1.475
17	2.0332	0.6
18	1.1702	0.39
19	6.605	0.33
20	4.538	0.38
21	1.5458	0.025
22	0.195	0.14
23	1.2776	0.54
24	0.9421	0.135
25	1.1344	0.74
26	1.2516	0
27	1.3536	0.42
28	3.2814	2.975
29	3.3083	2.45
30	5.5538	2.65

Table 4.1.3: The coefficient and constant values for linear model for biomass

Model	a_0	a_1	a_2	a_3	a_4	a_5	a_6	a_7	a_8
12345678	3.2122	0.3193	-0.5189	0.7939	-0.2856	0.2209	0.0603	-0.1047	-0.3829
2345678	3.2122		-0.4268	0.7515	-0.182	0.1437	0.1178	-0.2144	-0.4183
1345678	3.2122	0.1724		0.8178	-0.1533	0.0295	0.0272	-0.156	-0.5401
1245678	3.2122	0.1868	-0.5656		-0.2126	0.1688	0.2898	-0.1906	-0.5032
1235678	3.2122	0.2267	-0.4448	0.7731		0.1275	0.0187	-0.1164	-0.3288
1234678	3.2122	0.2595	-0.426	0.781	-0.2047		0.0716	-0.1191	-0.371
1234578	3.2122	0.3322	-0.5142	0.8104	-0.2751	0.2241		-0.0951	-0.387
1234568	3.2122	0.3644	-0.5321	0.8052	-0.291	0.2285	0.0428		-0.3772
1234567	3.2122	0.3655	-0.6475	0.8442	-0.2066	0.2009	0.0843	-0.0866	

Table 4.1.4: The coefficient and constant values for linear model for biopolymer

Model	a_0	a_1	a_2	a_3	a_4	a_5	a_6	a_7	a_8
12345678	1.044	-0.0044	-0.3426	0.3611	0.1086	0.2829	-0.0696	-0.1092	0.0764
2345678	1.044		-0.3439	0.3617	0.1072	0.2839	-0.0704	-0.1077	0.0769
1345678	1.044	-0.1013		0.3769	0.196	0.1565	-0.0915	-0.1431	-0.0274
1245678	1.044	-0.0646	-0.3639		0.1418	0.2592	0.0348	-0.1483	0.0217
1235678	1.044	0.0309	-0.3708	0.3691		0.3184	-0.0538	-0.1048	0.0559
1234678	1.044	-0.081	-0.2236	0.3446	0.2123		-0.0552	-0.1276	0.0916
1234578	1.044	-0.0193	-0.348	0.3421	0.0966	0.2791		-0.1203	0.0812
1234568	1.044	0.0426	-0.3564	0.3729	0.103	0.2908	-0.0879		0.0824
1234567	1.044	-0.0136	-0.3169	0.3511	0.0929	0.2868	-0.0744	-0.1128	

Table 4.1.5: Evaluation on linear regression involving correlated variables for biomass

No	Model	MSE	MSE_{NEW}/MSE_9	$F_{0.75}$
1	12345678	2.407618592	1	
2	2345678	2.475684082	1.028271	
3	1345678	2.58405671	1.073283	
4	1245678	2.936332297	1.2196	
5	1235678	2.462036283	1.022602	
6	1234678	2.444086612	1.015147	
7	1234578	2.410501935	1.001198	
8	1234568	2.4167955	1.003812	
9	1234567	2.514175844	1.044258	

Note: / indicates significant

Table 4.1.6: Evaluation on linear regression involving correlated variables for biopolymer

No	Model	MSE	MSE_{NEW}/MSE_9	$F_{0.75}$
1	12345678	0.68798453	1	
2	2345678	0.68799727	1.000019	
3	1345678	0.76490738	1.111809	
4	1245678	0.79736563	1.158988	
5	1235678	0.69585786	1.011444	
6	1234678	0.74779508	1.086936	
7	1234578	0.69182756	1.005586	
8	1234568	0.69796636	1.014509	
9	1234567	0.69223163	1.006173	

Note: / indicates significant

Table 4.1.7: Correlation matrix for the variables

	RPM	T	G	P	N	Na	K	M
RPM	0.9983	0.1069	-0.01386	0.3143	-0.08341	0.223	-0.3470	-0.1495
T	0.1069	1.0000	-0.1788	-0.1955	0.3500	0.01598	0.04487	0.4022
G	-0.01385	-0.1788	1.0000	0.1839	-0.0576	0.2944	-0.0482	-0.2469
P	0.3143	-0.1955	0.1839	1.0000	0.1901	0.2971	-0.07413	-0.3393
N	-0.08341	0.3500	-0.0576	0.1901	1.0000	0.07515	-0.00247	0.1421
Na	0.2230	0.01598	0.2944	0.2971	0.07515	1.0000	0.06156	-0.1831
K	-0.3470	0.04487	-0.0482	-0.0741	-0.002471	0.06156	1.0000	0.0290
M	-0.1495	0.4022	-0.2469	-0.3393	0.1422	-0.1831	0.0290	1.0000

Table 4.1.8: The coefficient of eigen vectors

	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8
F_1	-0.5272	0.2548	0.1277	0.3540	0.1669	0.4715	0.4116	-0.3097
F_2	0.4891	0.1670	0.4736	0.08405	0.2742	-0.0800	0.5464	0.3484
F_3	-0.1302	0.4128	0.1486	-0.5841	0.4640	-0.2993	-0.08951	-0.3696
F_4	0.4268	0.4336	-0.3284	0.09235	-0.4500	-0.1223	0.2663	-0.4745
F_5	-0.4370	-0.2007	0.09407	-0.3523	-0.4588	-0.3643	0.5285	0.1154
F_6	0.07314	-0.5576	-0.2935	0.2414	0.4447	-0.3602	0.2795	-0.3656
F_7	-0.2708	0.3367	0.1034	0.5694	-0.01789	-0.6349	-0.2229	0.1589
F_8	-0.1185	0.2904	-0.7238	-0.1067	0.2658	0.03297	0.2145	0.4987

Table 4.1.9: The eigen values

	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8
F_1	0.3292	0	0	0	0	0	0	0
F_2	0	0.5296	0	0	0	0	0	0
F_3	0	0	0.5631	0	0	0	0	0
F_4	0	0	0	0.8505	0	0	0	0
F_5	0	0	0	0	0.9106	0	0	0
F_6	0	0	0	0	0	1.2584	0	0
F_7	0	0	0	0	0	0	1.5009	0
F_8	0	0	0	0	0	0	0	2.0561

Table 4.1.10: The orthogonal factors for each variable

Run	F1	F2	F3	F4	F5	F6	F7	F8
1	-0.3259	-1.41475	0.483669	-0.53624	-1.05294	-2.37189	-0.17647	-1.79801
2	0.4120	-0.60632	0.483669	0.97194	-1.05294	1.581257	-1.94122	0.968159
3	0.4120	-0.60632	-0.50565	0.97194	-0.50832	0.790629	-1.94122	-3.18109
4	-0.3259	-0.20211	-0.83543	-0.53624	0.580935	0.790629	-1.94122	-3.18109
5	-0.1414	-0.20211	0.483669	-1.03897	-0.50832	0	-1.05885	-0.41493
6	-0.1414	-0.20211	-0.50565	-1.03897	-1.05294	0	0.705899	3.734328
7	0.043047	-0.20211	-0.83543	-0.03352	0.580935	0	0.705899	3.734328
8	0.043047	-0.20211	0.483669	0.469213	-1.05294	0	-0.17647	-1.79801
9	0.043047	-0.20211	-0.17588	-0.53624	0.036308	-0.79063	-0.17647	-1.79801
10	0.412025	-0.20211	-0.50565	-0.03352	-1.05294	1.581257	2.470646	-3.18109
11	0.412025	-0.20211	0.483669	-0.03352	-1.05294	1.581257	-1.94122	-0.41493
12	0.412025	-0.20211	0.483669	1.474668	1.670187	1.581257	-1.05885	-1.79801
13	-0.32593	0.202108	0.483669	0.97194	1.125561	0.790629	0.705899	0.968159
14	-0.14144	0.202108	0.153895	-1.03897	-1.05294	1.581257	2.470646	-0.41493
15	-0.14144	0.202108	0.153895	-0.03352	1.670187	0.790629	0.705899	-0.41493
16	-0.14144	0.202108	0.483669	-1.03897	0.036308	-1.58126	-1.94122	0.968159
17	0.043047	0.202108	0.813444	-0.53624	1.125561	0.790629	0.705899	-0.41493
18	0.227536	0.202108	-0.50565	1.474668	0.580935	-1.58126	-1.94122	0.968159
19	0.412025	0.202108	0.153895	0.97194	0.036308	0.790629	0.705899	-1.79801
20	0.412025	0.202108	0.483669	1.474668	-0.50832	-0.79063	0.705899	-3.18109
21	-0.51042	0.606323	0.813444	-0.53624	-0.50832	1.581257	0.705899	0.968159
22	-0.51042	0.606323	-0.17588	0.97194	1.670187	0.790629	1.588273	-0.41493
23	-0.32593	0.606323	-0.83543	-1.03897	0.036308	-1.58126	-1.94122	2.351243
24	-0.14144	0.606323	-0.83543	-0.03352	-0.50832	-0.79063	1.588273	2.351243
25	-0.14144	0.606323	-0.17588	-1.03897	-0.50832	-2.37189	1.588273	0.968159
26	0.227536	0.606323	-0.83543	-1.03897	1.670187	-0.79063	1.588273	3.734328
27	0.412025	0.606323	-0.50565	-0.03352	-0.50832	0	-1.94122	2.351243
28	0.412025	0.606323	0.483669	-0.53624	0.580935	-0.79063	-1.94122	0.968159
29	-0.51042	-1.01054	-0.50565	-0.03352	-0.50832	-2.37189	1.588273	-1.79801
30	-0.51042	-1.01054	0.813444	0.97194	0.036308	0.790629	1.588273	0.968159

Table 4.1.11: The coefficient and constant value for linear models for biomass

MODEL	a_0	a_1	a_2	a_3	a_4	a_5	a_6	a_7	a_8
12345678	3.2122	0.9699	-0.9798	1.41	-0.3358	0.2426	0.0479	-0.0698	-0.1862
2345678	3.2122		-0.8059	1.3347	-0.214	0.1578	0.0936	-0.1428	-0.2034
345678	3.2122			1.4036	-0.1248	0.0045	0.0512	-0.1433	-0.2648
45678	3.2122				-0.0703	-0.0494	0.2106	-0.1711	-0.3303
5678	3.2122					-0.3207	-0.1678	0.1998	-0.064
678	3.2122						0.195	-0.1684	-0.3253
78	3.2122							-0.1575	-0.3474
8	3.2122								-0.3507

Table 4.1.12: Evaluation on linear regression involving uncorrelated variables of biomass

No	Model	MSE	MSE_{NEW}/MSE_9	$F_{0.75}$
1	12345678	2.407618595	1	
2	2345678	2.475684091	1.028271	
3	345678	2.605670848	1.082261	
4	45678	3.146900968	1.30706	
5	5678	3.14966934	1.308209	
6	678	3.152962635	1.309577	
7	78	3.210904548	1.333643	/
8	8	3.266729865	1.35683	/

Note: / indicates significant

Table 4.1.13: The coefficient and constant value for linear models for biopolymer

MODEL	a_0	a_1	a_2	a_3	a_4	a_5	a_6	a_7	a_8
12345678	1.044	0.0372	-0.0728	-0.0553	0.3106	0.1277	0.6413	-0.6469	-0.0133
2345678	1.044		0.0374	-0.0718	-0.0559	0.3118	0.1261	0.6423	-0.6493
345678	1.044			-0.0121	-0.0722	-0.0901	0.1883	0.1978	0.6979
45678	1.044				-0.0446	-0.086	-0.0109	0.1615	0.2249
5678	1.044					-0.075	-0.0968	0.0234	0.2081
678	1.044						-0.0602	-0.0947	0.039
78	1.044							-0.0646	-0.0925
8	1.044								-0.0666

Table 4.1.14: Evaluation on linear regression involving uncorrelated variables toward biopolymer

No	Model	MSE	MSE_{NEW}/MSE_9	$F_{0.75}$
1	12345678	0.68798453	1	
2	2345678	0.687997275	1.000019	
3	345678	0.772373621	1.122661	
4	45678	0.906182128	1.317155	
5	5678	0.934494091	1.358307	/
6	678	0.969297689	1.408895	/
7	78	0.971617974	1.412267	/
8	8	0.990864287	1.440242	/

Note: / indicates significant

4.2 Discussions

For the first method which is the linear regression involving correlated variables, the evaluation of the variables can be observed in table 4.1.5 for biomass yield and table 4.1.6 for biopolymer. For both yield, the evaluation shows that all the variables are not significant toward the biomass and biopolymer yield. The second method which is the linear regression involving uncorrelated variables where all the variables being transform into orthogonal factors by using the eigen vectors and eigen value shows different result. For biomass yield, by dropping variables 6 and 7 which are the concentration di-sodium phosphate and potassium dihydrogen phosphate respectively will give significant effect. Nevertheless for biopolymer yield, by dropping variables 4, 5, 6 and 7 which are the concentrations of peptone, ammonium sulphate, di-sodium hydrogen phosphate and potassium dihydrogen phosphate respectively give a significant effect toward the yield. By comparing for both method, the second method is successfully describe the correlation of all the variables toward the biomass and biopolymer yield.

The most crucial step in this experiment is the inoculum development. For each run of the experiment, fresh inoculum needs to be used. Other than that, all equipments that maybe in contact with the bacteria need to be sterilize in order to avoid any contamination. This is also to increase the accuracy of the experimental results. For the process of regeneration of bacteria, if restoring agar slant in 4°C,

there is a need to layer the agar slant with parafilm oil in order to avoid any contamination. The universal bottle that being used must be sterile first by using autoclave. All process transferring the bacteria into medium need to be done in sterile condition. The sterile condition can be achieved by doing all the procedures inside the laminar flow.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

In this research, the linear regression involving correlated variables show that all variables give an insignificant effect toward the biomass and biopolymer yield while for linear regression involving uncorrelated variables; variables 6 and 7 which are the concentration of di-sodium hydrogen phosphate and potassium hydrogen phosphate give significant effect. For biopolymer yield, the concentration of peptone, ammonium sulphate, di-sodium phosphate and potassium dihydrogen phosphate give a significant effect toward the yield.

In order to improve this research in the future, increasing the number of experiments may help in reducing the error and increasing the accuracy of the experimental data. In addition, the development of inoculum needs to be done seriously in order to get good results.

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