OPTIMIZATION OF FERMENTATION FOR PHB BIOPOLYMER USING OIL PALM TRUNK SAP AS MEDIUM COMPONENT

KAMALUDDIN SARIF SUKARDI

UNIVERSITI MALAYSIA PAHANG

OPTIMIZATION OF FERMENTATION FOR PHB BIOPOLYMER USING OIL PALM TRUNK SAP AS MEDIUM COMPONENT

KAMALUDDIN SARIF SUKARDI

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

Faculty of Chemical and Natural Resources Engineering
UNIVERSITI MALAYSIA PAHANG

JANUARY 2012

SUPERVISOR'S DECLARATION

I hereby declared that I have checked this project and in my opinion, this thesis is adequate in terms of scope and quality for the award of the degree of Bachelor of Chemical Engineering (Biotechnology).

Signature:
Name of Supervisor: Prof. Ir. Dr. Jailani Bin Salihon
Position: Dean of Faculty of Chemical and Natural Resources Engineering
Date: 30 January 2012

STUDENT'S DECLARATION

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

Signature:
Name: Kamaluddin Sarif Bin Sukardi
ID Number: KE08060
Date: 30 January 2012

Dedicated to my beloved family

ACKNOWLEDGEMENT

I am grateful and would like to express my sincere gratitude to my supervisor Professor Ir. Dr. Jailani Bin Salihon for his brilliant ideas, invaluable guidance, continuous encouragement and support in making this research possible. He has always impressed me with his outstanding professional conduct, his strong conviction for science, and his belief that this study can be a start of a long-life learning experience. I appreciate his consistent support from my first meeting to these concluding moments. I am truly grateful for his progressive vision about my training in science, his tolerance of my mistakes, and his commitment to my future career.

I also would like to express very special thanks to Mr. Mior Ahmad Khusairi Bin Mohd. Zahari for his guidance, suggestions and co-operation during the study. I also sincerely thanks for the time spent for proofreading and correcting my mistakes during the lab sessions.

My sincere thanks go to all my classmates for their excellent co-operation, inspirations and supports during this research. Special thanks is also dedicated to the staff of the Chemical Engineering and Natural Resources Laboratory, UMP, who helped me in many ways and made my research at Faculty of Chemical and Natural Resources (FKKSA) laboratory smooth and easy

I hereby acknowledge my sincere gratitude to my parents for their helps throughout my study at Universiti Malaysia Pahang (UMP). I would like to acknowledge their loves, supports and sacrifices which were inevitable for the successful completion of this study.

ABSTRACT

The uses of petroleum-based plastics are harmful because it cannot be degraded and is not environmental friendly. To overcome these problems, researchers have introduced a naturally occurring biopolymer which is polyhydroxybutyrate (PHB) as a substitute to the existing synthetic plastics. Polyhydroxybutyrate is an intracellular product of bacteria fermentation at physiological stress condition. PHB is being studied because of its unique properties and because it is biodegradable. Production of bioplastic using PHB in large scale involved higher production costs compared to the production cost of existing petroleum-based plastics. Optimization of fermentation for PHB biopolymer was studied in shake flask by using *Cupriavidus necator* in oil palm trunk sap as the medium component. Three parameters were studied which were the fermentation temperature, agitation speed and percent volume of oil palm trunk sap. The optimum levels of parameters were determined by using mathematical model. Inocula were done in shake flask to cultivate the bacteria in growth medium solution. Fermentations were done in 500 mL shake flasks containing mineral salt, ultrapure water, inoculum and oil palm trunk sap. The flasks were incubated in incubator shaker for 24 hours at different levels of parameter values. The products were analysed for cell dry weight and PHB contents. Based on the experimental results obtained by using the Method of the Path of Steepest Ascent for the determination of the parameter values, the optimum condition of fermentation for PHB biopolymer was at temperature of 31.3°C, 123 rpm for agitation speed and 38.5 % for percent volume of oil palm trunk sap. The concentration of PHB obtained at the optimum condition was 0.825 g/L and the biomass content was 3.55 g/L.

ABSTRAK

Penggunaan plastik berasaskan petroleum adalah berbahaya kerana tidak boleh diuraikan secara semulajadi dan tidak mesra alam. Untuk mengatasai masalah ini, pengakaji telah memperkenalkan sejenis biopolimer semulajadi iaitu Polyhydroxybutyrate (PHB) sebagai bahan untuk menghasilkan plastik bagi menggantikan plastik sintetik yang sedia ada. Polyhydroxybutyrate adalah produk penapaian bacteria yang dihasilkan di dalam sel pada keadaaan persekitaran yang terhad. PHB menjadi tumpuan kajian kerana sifat uniknya iaitu boleh diuraikan secara semulajadi. Penghasilan bioplastik menggunakan PHB berskala besar melibatkan kos pembuatan yang tinggi berbanding kos pembuatan plastik berasakan petroleum yang ada pada masa kini. Kajian ini dilaksanakan bagi mengoptimumkan dan meningkatkan skala penghasilan PHB biopolimer di dalam kelalang menggunakan *Cupriavidus necator* dan perahan batang pokok kelapa sawit sebagai komponen medium penapaian. Tiga parameter yang dikaji adalah suhu penapaian, kelajuan pengadukan dan peratus kandungan perahan batang pokok kelapa sawit. Inokulum dilakukan di dalam kelalang yang mengandungi medium pertumbuhan untuk membiak bakteria. Proses penapaian dilakukan di dalam kelalang berisipadu 500 mL mengandungi garam mineral, air ultrapure dan perahan batang pokok kelapa sawit. Kelalang diletakkan di dalam *incubator shaker* selama 24 jam pada tetapan parameter yang berlainan. Produk hasil dari proses penapaian dianalisis untuk mendapatkan jisim kering sel dan kandungan PHB produk. Berdasarkan keputusan yang diperolehi daripada eksperimen menggunakan teknik Path of Steepest Ascent, parameter optimum proses penapaian adalah pada suhu 31.3°C, 123 rpm kelajuan pengadukan dan 38.5 % untuk peratus kandungan jus perahan dahan pokok kelapa sawit. Kepekatan PHB yang diperolehi pada keadaan optimum adalah 0.825 g/L dan jisim kering sel adalah 3.55 g/L.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

g	Gram
L	Liter
mL	Milliliter
M	Molar
nm	namometer
rpm	Rotation per minute
°C	Degree Celsius
%	Percent
% v/v	Percent volume over volume
µ	Micro

LIST OF ABBREVIATIONS

$(NH_4)_2SO_4$	Sodium hydrogen sulphate
amu	Atomic Mass Unit
EFB	Empty Fruit Bunch
HPLC	High-performance Liquid Chromatography
H_2SO_4	Sulphuric acid
KH_2PO_4	Potassium dihydrogen phosphate
K_2HPO_4	Potassium hydrogen phosphate
$MgSO_4$	Magnesium sulphate
NaOH	Sodium hydroxide
OFP	Oil Palm Fronds
OPT	Oil Palm Trunk
OPTS	Oil Palm Trunk Sap
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
POME	Palm Oil Mill Effluent
PPF	Palm Press Fiber
rpm	Rotation Per Minute
UV-Vis	Ultraviolet-Visible

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

This chapter discusses the overview of this research. It gives a brief introduction to the research conducted. There are five main topics covered in this chapter which are the background of study, problem statement, research objective, scopes of research and the significance of the study.

1.2 BACKGROUND OF STUDY

Plastic plays very important roles in our lives. It makes the tables, chairs, furniture, and packaging for food, furniture, machines and other equipments that used in our daily life. Plastic manufacturing industry was gaining large amount of profit as the demand for plastic was increased due to the growth of population worldwide. However, these petroleum-based plastics were harmful to human and the environment chemically, biologically and physically.

In order to replace the use of synthetic plastics, researchers have been working on new solutions by producing bioplastic using microorganism's activity or fermentation process. Fermentation of specific bacteria were producing intracellular product of polyhydroxybutyrate (PHB) biopolymer by accumulation of excessive amount of carbon from glucose. Bioplastic from PHB biopolymer is suitable as substitute compound for plastic manufacturing because the characteristics of bioplastic are very similar to the synthetic plastics (Doi and Steinbüchel, 2001; Sims, 2003; Postolis et al., 2006). Besides of its biodegradability properties, bioplastics were safer and environmental friendly.

Production of biopolymer from fermentation process to produce bioplastic helps reduces pollutions without uses of toxic chemicals and harmful wastes. The uses of cheap substrates from agricultural waste as the sources of food for the bacteria also help to improve the waste management problems (Gouda et. al., 2001).

1.3 PROBLEM STATEMENT

Usages of petroleum-based plastics had caused severe damages to the environment because it is non-biodegradable materials. It had caused land pollution and also water pollution. The dumping of these plastics to the land may cause diseases and smell to the nearby communities meanwhile the plastics throws to the river and sea may harm or even kills the animals or plants. Petroleum-based plastics were made of various chemicals which are toxic and harmful to living things and the environment. Petroleum resources also have being depleted as the demand for petroleum increasing to generate cars and electricity worldwide. Therefore, a research must be done to study the production of PHB as an alternative for production of bioplastic which is safer for living things and environmental friendly as well as using economically available resources.

1.4 RESEARCH OBJECTIVE

The objective of this study is to optimize the production of PHB biopolymer by fermentation process. Oil palm trunk sap was used as medium component and the fermentation parameters which are the fermentation temperature, agitation speed and percent volume of oil palm trunk sap were manipulated to achieve the objective of the research.

1.5 SCOPES OF RESEARCH

Suitable culture conditions for the fermentations were obtained from literature. Mathematical modelling was performed to determine the fermentation conditions which were the temperature, agitation speed and percent volume of oil palm trunk sap. The mathematical model used in the study was the Method of Path of Steepest Ascent. Fermentations of *Cupriavidus necator* were done in 500 mL shake flask using oil palm

trunk sap as the medium component. *Cupriavidus necator* was cultivated on agar plate using fructose as the carbon source to maintain the regeneration of the bacteria. Two inoculums of *Cupriavidus necator* were prepared to grow the bacteria in culture media contains fructose before the fermentation process. Fermentations in shake flask were done as modelled by the Method of Path of Steepest Ascent. The products after 24 hours fermentation were collected and analysed. Cell dry weight analysis and PHB analysis were to determine the biomass and PHB content of the product. Cell dry weight analyses were done by measuring the dried biomass from the product and PHB contents were analysed by using HPLC. The results of these experiments were collected and analysed. The results of the experiments were discussed and the optimum condition for the fermentation of PHB using *Cupriavidus necator* in medium containing oil palm trunk sap was determined.

1.6 SIGNIFICANCE OF RESEARCH

Biodegradable plastics can be produced by fermentation process of bacteria utilizing oil palm fronds juice as medium component which is environmental friendly and safer for human and living things (Steinbüchel, 1995). Manufacturing of bioplastic also does not involve hazardous and toxic chemicals as compared to the petroleum-based plastics.

The production cost for manufacturing bioplastic can be reduced by using cheap and renewable carbon sources for production of PHB biopolymer in the fermentation process (Choi and Lee, 2001). In the research, oil palm trunk sap was used as the renewable and cheap carbon source as oil palm trunk was an abundant waste in Malaysia.

Besides of using oil palm trunk sap as a renewable carbon source for the fermentation, the oil palm plantation waste is also converted to produce valuable product. Hefty amount of waste is reused to produce value added product which is PHB, helping to solve the waste management problems (Gouda et. al., 2001).

CHAPTER 2

LITERARURE REVIEW

2.1 INTRODUCTION

This chapter discusses the detailed descriptions of the development of the study based on the literature. It gives brief explanations to the study conducted. The topics covered in this chapter include petrochemical plastics, biodegradable plastics, Polyhydroxybutyrate (PHB), limitations for production of polyhydroxybutyrate (PHB**)** and oil palm waste.

2.2 SYNTHETIC PLASTICS

Early plastic was made of material called Parkesine which was an organic material derived from cellulose. Parkesine can be moulded using heat and it retained its shape when cooled. This material was publicly demonstrated by Alexander Parkes at the 1862 Great International Exhibition in London. Development of plastics continues as John Wesley Hyatt invented celluloid as substitute for the ivory in billiard balls in 1968. However, the material was not strong enough until the addition of camphor, a derivative of the laurel tree.

In 1897, casein plastics were found in the efforts to manufacture chalkboards by mixing milk protein with formaldehyde. 2 years later, Arthur Smith received the first British Patent 16, 275 for processing a formaldehyde resin. Dr. Leo Hendrik Baekland improved the techniques for the production of first fully synthetic resin in early 90's. Bakelite was prepared by reaction of carbolic acid with formaldehyde. In 1909, the first synthetic plastic was introduced to the public at a chemical conference.

There are two types of plastics which are thermoplastics and thermosetting polymers. Thermoplastics do not undergo chemical change in their composition when heated and can be remoulded. Polyethylene, polypropylene, polystyrene and polyvinyl chloride are the examples of thermoplastics. Thermosets can be moulded once and remain in solid form. The chemical reaction occurred during the moulding process was irreversible. It cannot be remoulded such as thermoplastics. Thermoplastics are lighter compared to thermosets because the molecular weights of thermoplastics range from 20,000 to 50,000 amu meanwhile thermosets were assumed to have infinite molecular weight.

Symbol	Acronym	Full name and uses
	PET	Polyethylene terephthalate - Fizzy drink bottles and frozen ready meal packages.
	HDPE	High-density polyethylene - Milk and washing-up liquid bottles
	PVC	Polyvinyl chloride - Food trays, cling film, bottles for squash, mineral water and shampoo.
	LDPE	Low density polyethylene - Carrier bags and bin liners.
	PP	Polypropylene - Margarine tubs, microwave-able meal trays.
	PS	Polystyrene - Yoghurt pots, foam meat or fish trays, hamburger boxes and egg cartons, vending cups, plastic cutlery, protective packaging for electronic goods and toys.
	Other	Any other plastics that do not fall into any of the above categories. For example melamine, often used in plastic plates and cups.

Figure 2.1: Classifications and functions of synthetic plastics

Plastics can be easily moulded with heat and pressure. It has high strength, low friction, light and no electrical conductivity. It is safer to be used in compared to the woods and glass in term of strength and metals in term of electrical conductivity. Besides that, plastic materials became popular among manufacturer and users due to its economical price which is cheaper than glasses, woods and metals.

Malaysia as one of the leading developing countries was also using plastics in several industries such as packaging, furniture and electrical appliances. Based on the statistics, Malaysian solid waste has increased annually, from 17,000 tons per day in 2002 to 19,100 tons per day in 2005. In 2020, it is predicted about 30,000 tons of solid waste produced a day with average of 1kg of waste thrown by each person per day. From the total solid waste discarded by Malaysian, about 24% of the waste cannot be degraded. Around 5600 kg of plastic waste removed every day and if this percentage remains constant annually, about 7200 kg plastics will be discarded daily in 2020.

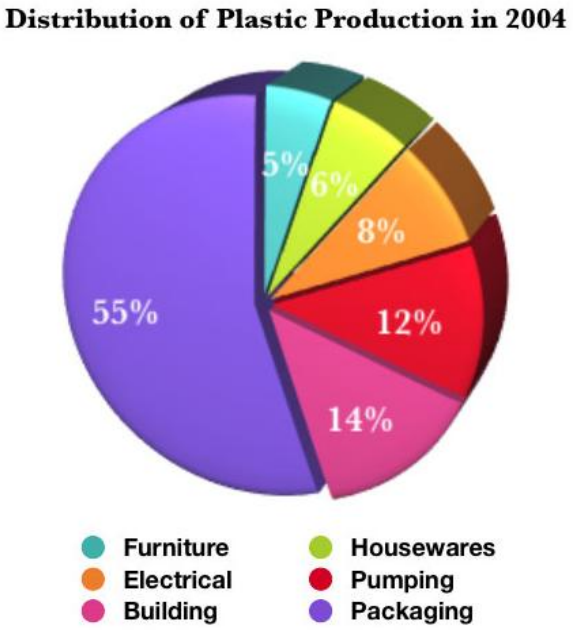


Figure 2.2: Distribution of Plastic Production in 2004

Source: ASEAN Federation of Plastic Industries (AFPI)

Synthetic plastic is non-biodegradable material; it was recalcitrant to microbial degradation (Fletcher, 1993). Plastic cannot be degraded naturally in the soil or water because of its excessive molecular size (Atlas, 1993). Petrochemical plastics were accumulated in environment at a rate of 25 million tonnes per year (Lee et. al., 1991) and to overcome this problem, biodegradable thermoplastics were invented (Edwin, 1988).

2.3 BIODEGRADABLE PLASTICS

Biodegradable plastics or bioplastics can be produced from PHB biopolymer. Bacteria such as *Cupriavidus necator, Alcaligenes eutrophus* (Kim & Cheng, 1995), *Azotobacter chroococcum* (Irina et. al., 2008), and *Bacillus cereus SPV* (Valappil, 2006) produced PHB as an intracellular product as a results of physiological stress conditions (Lee, 1996). The usage of bioplastics as substitute for synthetic plastics was at great advantages as it can be degraded naturally, safer and environmental friendly. The characteristics of bioplastic are very similar to the petroleum-based plastic in term of melting point, stiffness, molecular weight, brittleness and glass transition temperature (Steinbüchel and Fuchtenbusch, 1998; Suzuki et. al., 2001).

2.4 POLYHYDROXYBUTYRATE (PHB)

PHB as shown in Figure 2.3 is a chain of monomer or polymer of polyhydroxyalkanoates (PHAs) family that isolated and characterized in 1962 by Maurice Lemoigne, a French microbiologist as a constituent of bacterium *Bacillus megaterium* (Lemoigne, 1926). Continuous research has been done to produce PHB as the results of physiological limitation. PHB can be produced by several bacteria using glucose and other reducing sugar as their carbon source. The carbon sources include agricultural wastes such as cane molasses, beet molasses, whey and starch. Besides that, various nitrogen-rich media such as corn steep liquor, yeast extract and collagen hydrolysate, casein hydrolysate, typtone and casamino acids (Lee and Chang, 1994; Bormann et. al., 1998; Khanna and Srivasta, 2005).

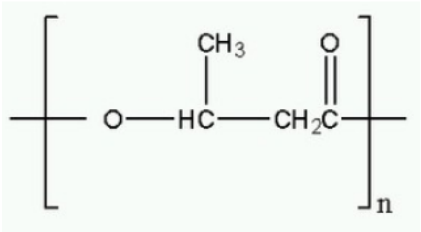
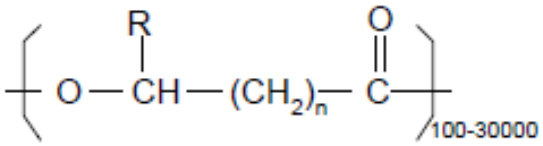


Figure 2.3: Structure of Polyhydroxybutyrate (PHB)

2.4.1 Physical and Chemical Properties of PHB

Polyhydroxybutyrate (PHB) can be produced via bacterial fermentation. PHB belongs to the class of polyhydroxyalkanoates (PHA) as shown in Figure 2.2. PHAs are thermoplastic polymers with physical properties range from hard rigid solids to elastomers (Gunaratne et. al., 2008). It can be biodegraded with water and carbon dioxide.



n =1	R =	hydrogen	poly (-3-hydroxypropionate)
		methyl	poly (-3-hydroxybutyrate)
		ethyl	poly(3-horoxyvalerate)
		propyl	poly (-3-hydroxyhexanoate)
		pentyl	poly (-3-hydroxyoctanoate)
		nonyl	poly (-3-hydroxydodecanoate)
n = 2	R =	hydrogen	poly (-4-hydroxybutyrate)
n = 3	R =	hydrogen	poly (-5-hydroxyvalerate)

Figure 2.4: General structure of Polyhydroxyalkanoates (PHAs)

Source: Ojumu, T.V., 2004

PHB have relatively high melting temperatures (T_m), to 170°C which is close to its thermal decomposition temperature (Madison and Huisman, 1993). The glass transition temperatures (T_g) and crystallinity of PHB is relatively high as shown in Table 2.1.

According to Madison and Huisman (1993), PHB exists in forms of fluid, amorphous state. After extraction from the cell with organic solvent, PHB becomes crystalline, stiff but brittle material and because of its brittleness it is not stress resistant.

Table 2.1: Physical properties of various PHAs and propylene

Property	PHB	P(HB-HN)[a]			Polypropylene
		3 mol %	14 mol %	25 mol %	
Melting point(^{0}c)	175	169	150	137	176
Glass-transition temp (^{0}C)	15	-	-	-1	-10
Crystalline (%)	80	-	-	40	70
Young's modulus	3.5	2.9	1.5	0.7	1.7
Tensile strength (MPa)	40	38	35	30	34.5
Elongation to Break (%)	6	-	-	-	400
Impact strength (v/m)	50	60	120	400	45

Data adapted from Lee (1996) and Poirier et al. (1995).
- data not available
[a] poly (-3-hydroxybutyrate-co-3yhdroxyvalerate)

Source: Ojumu, T.V., 2004

2.4.2 Raw Material for Production of PHB

In the production of PHB biopolymer, there were several types or raw materials that can be used. The considerations have to be made for the raw materials were the availability and price of the raw materials. Besides that, the raw materials must be containing sugars such as glucose, sucrose, fructose and mannose to produce PHB. Agricultural wastes which were highly available with cheaper price compared to the pure chemicals were the main focused to produce bioplastic nowadays because it was renewable resources (Madison and Huisman, 1999).

Examples of raw material used in the production of PHB include methanol (Suzuki et. al., 1986), ethanol (Alderet et. al., 1993), beet molasses (Page, 1992), starch and whey (Kim, 2000; Ghaly et. al., 2003), and cassava hydrolysate medium (Aremu et. al., 2011).

2.4.3 Bacteria for Production of PHB

Bacteria had been widely used in this field to improvise the production of bioplastic. Table 2.2 below represented types of bacteria that had been used by researchers to produce PHB and its substrate.

Table 2.2: Microorganisms and Carbon Source Used for Production of PHB

Microorganism	Carbon Source	Author
Hydrogenophaga. pseudoflava DSMZ 1034	Glucose and Fructose	Mahmoudi et. al., 2010
Rhodopseudomonas palustris KU003	Glucose	Ramchander et. al., 2010
Pseudomonas Putida Strain KT24400	Glucose	Aremu et. al., 2011
Activate sludge performing EBPR	Glucose	Rodgers and Wu
Ralstonia eutropha ATCC 17697 and *Alcaligenes latus* ATCC 29712	Glucose and Sucrose	Azhar et. al., 2009
Bacillus megaterium and *Alcaligenes eutrophus*	Glucose	Kumaravel et. al., 2010
Bacillus megaterium ATCC 6748	Fructose	Chaijamrus and Udpuay, 2008
Rhodobacter spharoides ES16 (wild type and mutants)	Glucose, fructose and acetate	Sangkharak and Prasertsan, 2008
Rhanella aquatilis and *Stenotrophomonas maltophilia*	D-xylose, sorbose, fructose, glucose, lactose and glycerol	Singh and Parmar, 2011

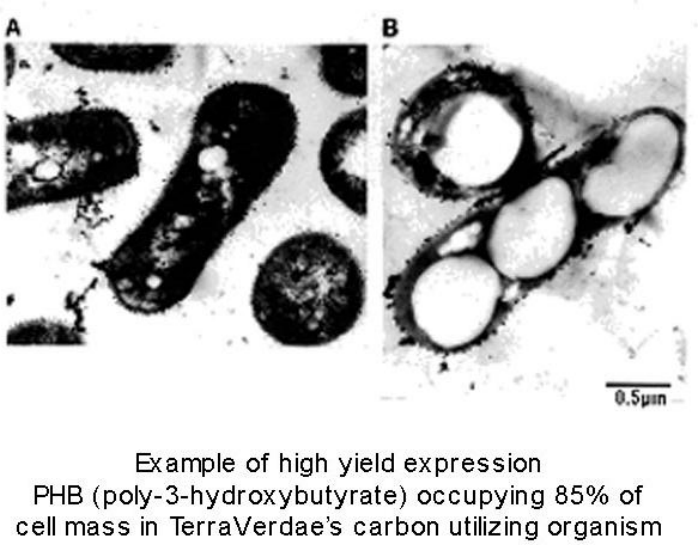


Figure 2.5: PHB in cell mass

Adapted from TerraVerdae

Cupriavidus necator or *Ralstonia eutropha* is *Alcaligenes eutrophus* is the main concern in this research. It was a non-spore forming bacteria. This bacterium is able to survive and flourish in mill molar concentrations of heavy metal that might be toxic for some other microorganisms. From the research done by Kim and Cheng (1995), Polyhydroxybutyrate (PHB) was found to be the intracellular fermentation product of *Cupriavidus necator* by accumulating carbon from glucose.

2.4.4 Transgenic Plant Producing PHB

Besides of using microorganisms, few researchers also had reported their findings on the production of PHB through the genetic engineering technology. Few transgenic plants were created to synthesis of PHAs, preferably PHB. The preferred plants for the transgenic method were preferable do not produce storage as oils and carbohydrates and also having C_4 NAD-malic enzyme photosynthetic pathway. Besides of producing PHB, these plants can produce lignocellulosic biomass for the production of biofuels (Mariya et. al., 2009). Table 2.3 below was shown the types of plants that can be used for the production of PHB.

Table 2.3: Transgenic Plant for the production of PHB

Plant	Yield	Author
Switchgrass, *Panicum Virgatum L*.	Producing at least 1% dry weight of PHA	Mariya et. al., 2009
Sugarcane, *Saccharum spp*. hybrids	PHB accumulated in leaves to a maximum of 1.88% of dry weight	Petrasovits et. al., 2007
Tobacco, *Nicotiana tabacum*	3.2 mg/g dry weight	Bohmert et. al., 2002
Cotton, *Gossypium hirsutum* L. Cv DP50	-	Maliyakal et. al., 1996
Potato, *Solanum tuberlosum*	0.09 mg/g dry weight	Bohmert et. al., 2002

2.5 LIMITATIONS FOR PRODUCTION OF POLYHYDROXYBUTYRATE (PHB)

Expensive raw material cost of fine chemicals such as glucose, sucrose and fructose become one of the obstacles for the mass production of PHB. These fine chemicals also do not promote higher polymer production compared to the unrefined carbon sources (Chaijamrus and Udpuay, 2008).

Other than that, the cost for microbial large scale production of PHB is very expensive. The production cost of petrochemical derived plastics is cheaper compared to the production of bioplastic with similar properties to the petrochemical derived plastics (Steinbüchel and Fuchtenbushed, 1998).

Besides that, fermentation conditions for the production of PHB biopolymer were not yet been optimized. Therefore, the yield of the production is very low. Researches that have been done previously done not reported the optimized condition for the production of this natural polymer. Therefore, it is needed for research on the production of PHB with high productivity and high yield.

Recovery of PHB from the fermentation media also was not economically attractive process. The separation of this polymer from the cells required large amount of solvent (Lee, 1996) and increasing the possibility of the product to be degraded. Solvent extraction also present some safety concerns (Luzier, 1992) eventhough it is effective to recovery the product from the cells. Cheaper cost for the recovery of PHB reduced the molecular weight of product by using aqueous extraction process (Luzier, 1992).

2.6 OIL PALM WASTE

Currently, Malaysia was reported as the second largest producer in palm oil industry after Indonesia. In 2009, Malaysia was reported to produce about 18.5 metric tonnes of oil palm. Malaysia became the main exporters of oil palm industry which contributes to 41% of palm oil production in the world with approximately 15700 metric tons of palm oil.

Became the second largest producer of oil palm in the world, it contributes to the economic growth of the country as the price for oil palm is keep increasing. However, there are disadvantages from the oil palm industries such as generation huge amount of wastes. There are several types of waste generated from the industry which include empty fruit bunches, fronds and trunks and also liquid chemical waste or palm oil mill effluent (POME). This biomass of trunks and fronds were contributing to waste management problems to the country.

According to Singh (1999), combined dry matter of biomass produced for a generation of oil palm per hectare is 377 tonnes; a huge amount of biomass was produced. Annually, the oil palm industries generates about 30 million tonnes of lignocellulosic biomass in the form of oil palm trunks (OPT), empty fruit bunches (EFB), oil palm fronds (OPF), and palm pressed fiber (PPF).

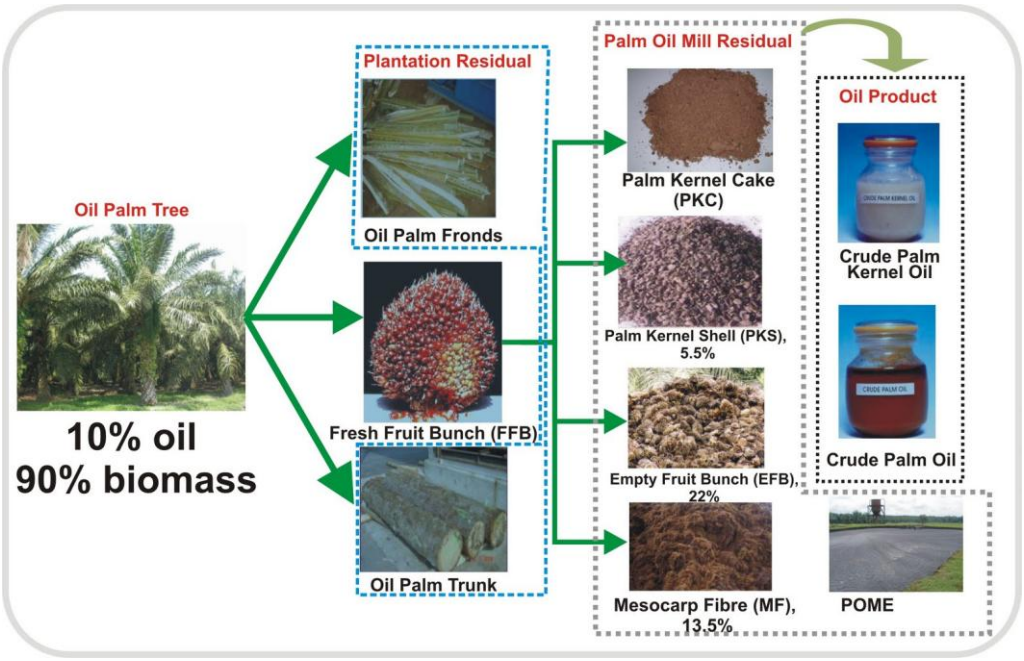


Figure 2.6: Biomass generated from oil palm tree

Recycling the empty fruit bunches, old fronds and palm oil mill effluents as organic fertilizers for the soil to return the nutrients have save the cost for using inorganic fertilizers. Dry fibers from empty fruit bunches were recycled to produce paper and production of panel products. POME was treated to recover the high level of plant nutrients to improve the soil and increases yield.

2.6.1 OIL PALM TRUNK SAP

Oil palm trunks can be recycled to produce energy. Oil palm trunks were processed to produce ethanol. To diversify the usage of oil palm trunk sap, this research was proposed to maximize the usage of the biomass waste from the plantation sector. Other than producing value added product from oil palm plantation waste, this research also offers solution to the waste management problems in discarding this waste.

Oil palm trunk sap was obtained from the oil palm trunk. The trunk was cut and cleaned. The inner part of the trunk was taken and squeezed to obtain the sap. Oil palm trunk sap was collected and processed in the laboratory to obtain the sap without ash and fibers.

CHAPTER 3

METHODOLOGY

3.1 INTRODUCTION

The research is conducted to optimize and scale-up the production of PHB biopolymer by fermentation of *Cupriavidus necator*. Oil palm trunk sap is used as cheap carbon sources to feed the bacteria. Fermentation parameters which are temperature, agitation and oil palm percent volume adjusted to obtain the maximum PHB content from the fermentation process.

The general experimental procedures are shown as Figure 3.1 below:

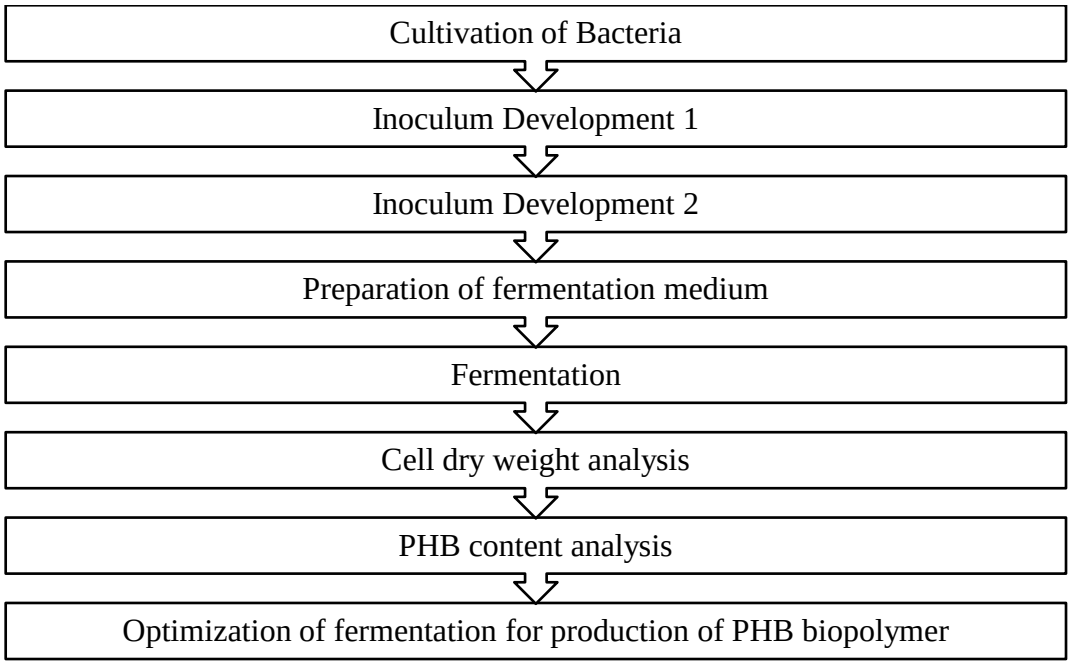


Figure 3.1: General Procedures of fermentation process

3.2 RESEARCH METHODOLOGY

3.2.1 Mathematical Modelling

Mathematical modelling was used to determine the sets of parameters for the experimental process. The mathematical modelling for this experiment is the method of the path of steepest ascent. For the Method of the Path of Steepest Ascent, the calculation for the sets of fermentation parameters were done as followed:

The linear regression equation relating the relation between the yields of product, y_n to the fermentation parameters, $x_{1n,}$ x_{2n}, and x_{3n} were as Equation 3.1 below:

$$y_n = 1.161 + 0.056x_{1n} - 0.129x_{2n} - 0.394x_{3n} \quad (3.1)$$

y_n was differentiated to obtain $\frac{\partial y}{\partial xi}$, and the value for each $\frac{\partial y}{\partial xi}$ is as followed:

$$\frac{\partial y}{\partial x_1} = 0.056, \frac{\partial y}{\partial x_1} = -0.129 \; and \; \frac{\partial y}{\partial x_1} = 0.394$$

For the chosen range, l . Equation 3.2 is used as below:

$$l = \frac{n}{\sqrt{\sum(\frac{\partial y}{\partial x})^2}} \quad (3.2)$$

and the value of $\sqrt{\sum(\frac{\partial y}{\partial x_i})^2} = 0.6468$

The chosen range values, l for the value of *n* from 1 to 10 are shown in Table 3.1.

Table 3.1: Chosen Range Value of *n* from 1 to 10

n	**l**
1	2.3904
2	4.7807
3	7.1711
4	9.5615
5	11.9518
6	14.3422
7	16.7326
8	19.1229
9	21.5133
10	23.9037

x_i was calculated at different *n* value from 1 to 10 using Equation 3.3 as below:

$$x_i = l\frac{\partial y}{\partial x_i} \tag{3.3}$$

The results were presented in Table 3.2 as below for *n*, l , and x_i:

Table 3.2: Value of l and x_i for *n* from 1 to 10

n	***l***	x_1	x_2	x_3
1	2.3904	0.1339	-0.3084	0.9418
2	4.7807	0.2677	-0.6167	1.8836
3	7.1711	0.4016	-0.9251	2.8254
4	9.5615	0.5354	-1.2334	3.7672
5	11.9518	0.6693	-1.5418	4.7090
6	14.3422	0.8032	-1.8501	5.6508
7	16.7326	0.9370	-2.1585	6.5926
8	19.1229	1.0709	-2.4669	7.5344
9	21.5133	1.2047	-2.7752	8.4762
10	23.9037	1.3386	-3.0836	9.4181

The parameters values for the experimental procedures can be determined by using the calculated values of x_i. The real parameters values were as presented in Table 3.3.

Table 3.3: The Real Parameter Values of *n* from 1 to 10

No. of experiment, *n*	**Temperature, (°C)**	**Agitation Speed, (rpm)**	**Percent Volume of OPTS, (% v/v)**
1	30.3	185	19.7
2	30.5	169	24.4
3	30.8	154	29.1
4	31.1	138	33.8
5	31.3	123	38.5
6	31.6	107	43.3
7	31.9	92	48.0
8	32.1	77	52.7
9	32.4	61	57.4
10	32.7	46	62.1

Experiments were conducted based on the real parameter values as stated in Table 4.3 for *n* values of 1, 3, 5, 6, 7, 8 and 9. Cell dry weight analysis and PHB analysis were performed at the end of the experiments to determine the biomass and product concentration for each experiment.

3.2.2 Cultivation of Bacteria

Cultivation of bacteria has to be done to maintain the regeneration of the species. *Cupriavidus necator* was used in the research and cultivated on the agar plate. The agar plate was prepared by mixing 5.0 g of peptone, 10.0 g of fructose, 3.0 g of yeast extract, 0.3 g of beef extract, and 15.0 of agar powder in a 1.0 l Scott bottle. Ultra pure water was added into the Scott bottle until the volume is 1.0 L. The solution was mixed using magnetic stirrer and then autoclaved in autoclave at 121°C for 20 minutes. The Scott bottle cap was wrapped with aluminium foil and loosens to avoid build up of pressure in the bottle during autoclave. The agar medium let to cool to about 50°C after the autoclave has finished.

10 mL of agar solution transferred into a petri plate and let to solidify for about 15 minutes. The steps repeated to get about 20 petri plates of agar medium and the remaining agar solution keep in refrigerator. After the agar solution solidified, a loop of bacteria from the previous culture was transferred onto the agar plate using inoculating loop in the laminar air flow hood with Bunsen burner lighted on for sterilization purpose. The agar plates contain the bacteria then incubated in incubator for 24 hours at 30°C. After 24 hours, the agar plates transferred into refrigerator at 4°C to avoid it from contaminated.

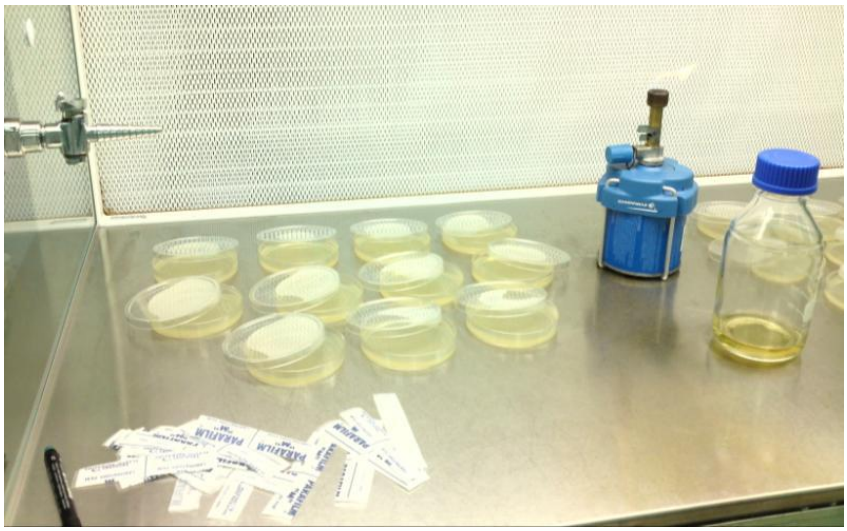


Figure 3.2: Cultivation of Bacteria

3.2.3 Inoculum Development 1

Inoculum development 1 was proceeds after the suitable parameter for fermentation obtained. This step followed with Inoculum development 2 before fermentation process and the purpose of these inoculum developments is to make sure the bacteria is alive and also to amplify the amount of bacteria. Inoculum development 1 was done by transferring the cultured bacteria into a growth medium. The growth medium was prepared by mixing 5.0 g of peptone, 10.0 g of fructose, 3.0 g of yeast extract and added with ultra pure water until the volume is 1.0 L in a 1.0 L Scott bottle. The solution was stirred and sterilised in autoclave. The Scott bottle cap was loosen and covered with aluminium foil. After the sterilisation process complete, the solution was cooled down in room temperature.

Two 10 ml sterile test tubes filled with 5 mL of the prepared solution each. The remaining prepared solution was keeps in refrigerator for sterile purpose. A loop of bacteria from the agar plate was transferred into the test tubes each. Finally, the test tubes opening were closed with sterile cotton and the test tubes were incubated at 30°C and 200 rpm for 24 hours in incubator shaker.

3.2.4 Inoculum Cell Washing

Inoculum cell washing was done to make sure that the inoculum prepared earlier does not contaminated. 1 mL of the inoculum prepared was transferred into an Eppendorf tube and centrifuged at 10000 rpm for 5 minutes by using biofuge. The supernatant was removed and the 1 mL of ultra pure water was added and mixed well. The Eppendorf tube was centrifuged again at the same 10000 rpm for 5 minutes. The supernatant from the second centrifugation process was removed. The remaining pellet in the Eppendorf tube was added with 1 mL of ultra pure water and shaken. The solution was transferred into 10 mL test tube and was added with 9 mL of ultrapure water. The solution mixed using vortex mixture and then it was transferred into vials for optical density test. The optical density of the pellet was observed using UV-Visible Spectrophotometer at 600nm wavelength.

3.2.5 Inoculum Development 2

The remaining growth solution from inoculum development 1 taken and 40 mL of the growth media transferred into a 250 mL sterile Erlenmeyer flask. The 10 mL inoculums prepared earlier in the Inoculum Development 1 were transferred into the flask and shaken slowly. The opening of the flask covered with sterile cotton and the flask incubated in incubator shaker for 24 hours at 30°C and 200 rpm. After 24 hours incubated, 1 ml of the sample taken for cell washing and the optical density of the sample was observed by using UV-Visible Spectrophotometer at 600nm wavelength. The cell washing procedures were as done in cell washing of the first inoculum.

3.2.6 Preparation of Mineral Salt Medium

Before the fermentation process can be preceded, solution of salt medium has to be prepared. 6.7 g of KH_2PO_4, 1.5 g of K_2HPO_4, 1.0 g of $(NH_4)_2SO_4$ and 0.2 g $MgSO_4$ were mixed in 1 L Scott bottle. Ultrapure water was added until the volume was 1 L and the solution was stirred using magnetic stirrer to dissolve the mineral salt compounds until a clear solution was obtained.

3.2.7 Preparation of Oil Palm Trunk Sap

Oil palm trunk sap was prepared by centrifugation of raw oil palm trunk sap. The raw oil palm trunk sap was centrifuged using refrigerated centrifuge at 4°C and 8000 rpm for 15 minutes. The supernatant from the centrifugation process was transferred into the Scott bottle and the remaining pellet removed. The centrifugation was repeated until 1.0 L of supernatant oil palm trunk sap was produced. Then, the Scott bottle cap was wrapped with aluminium foil and the loosened for autoclave. The oil palm trunk sap collected was autoclaved at 121°C for 20 minutes. The sterilised oil palm trunk sap was cooled in room temperature for usage. For storage of the oil palm trunk sap, it was kept in refrigerator at 4°C.

3.2.8 Fermentation Process

Fermentation process was proceeds after the inoculum development 2 incubated for 24 hours. 20 mL of mineral salt medium prepared were transferred into sterilized 500 ml Erlenmeyer Shake Flask and it was added with ultra pure water according to the volume as shown in Table 1. The pH was adjusted to pH 6.0 with 2M NaOH or 2M H_2SO_4 as the optimum pH for the fermentation is at pH 6.0. The mineral salt was sterilised in autoclave at 121°C for 20 minutes. After the sterilisation complete, the mineral salt and oil palm trunk sap were let to cool at room temperature. The flask then filled with oil palm trunk sap and inoculum as tabulated in Table 3.4.

Table 3.4: Composition of the Components for Fermentation Process.

n	Volume of ultra pure water, (mL)	Percent volume of oil palm trunk sap, (mL)	Volume of Inoculum 2, (mL)	Volume of salt medium, (mL)	Total volume, (mL)
1	120.6	19.7	20	20	200
3	101.8	29.1	20	20	200
5	83.0	38.5	20	20	200
6	73.4	43.3	20	20	200
7	64.0	48.0	20	20	200
8	54.6	52.7	20	20	200
9	45.2	57.4	20	20	200

The flask opening was covered with sterile cotton and shaken slowly. Finally, the Erlenmeyer flask was incubated in incubator shaker. The fermentation conditions were as tabulated in Table 2 and the flask was incubated for 48 hours.

Table 3.5: Conditions for Fermentation Process.

n	Temperature, (°C)	Agitation speed, (rpm)	Duration, (hour)
1	30.3	185	24
3	30.8	154	24
5	31.3	123	24
6	31.6	107	24
7	31.9	92	24
8	32.1	77	24
9	32.4	61	24

3.2.9 Cell Dry Weight Analysis

Cell dry weight analysis was performed to determine the amount or concentration of cells in the samples. Empty Eppendorf tube was dried for 48 hours in oven and the weight of the tube was measured and recorded. 1 mL of the product was taken from the flask and transferred into the dried Eppendorf tube. The sample was centrifuged at 10,000 rpm for 5 minutes by using biofuge. The supernatant was removed and 1mL of ultra pure water was added into the Eppendorf tube. The tube was centrifuged again at the same rotation speed for 5 minutes. The supernatant from the second centrifugation removed and the remaining pellet dried in oven at 60°C for 48 hours. After the pellet has dried, the weight of the Eppendorf tube with the dried sample was weighed and recorded. The cell dry weight content was calculated by determining the weight difference of the empty tube with the tube with dried sample. To determine the biomass concentration, the sample cell dry weight was divided the volume of sample taken.

3.2.10 PHB Analysis

PHB analysis was performed to determine the PHB content or concentration from the fermentation product. The analysis was done by taking 1 mL of the product into an empty Eppendorf tube and centrifuged at 10,000 rpm for 5 minutes using biofuge. The supernatant was removed and the remaining pellet was dried in the oven for 24 hours. After 24 hours, 1 mL of concentrated sulphuric acid was added to the dried pellet to digest it. The Eppendorf tube was closed and it was put in the oven at 90°C for 2 hours. After 2 hours, the pellet was transferred into a test tube and 9 mL of ultra pure water was added into the test tube. The test tubes contents were mixed by using vortex mixer. Then, 1 mL sample from the test tube was taken and filtered using 0.45 μm microfilter using 1 mL needle syringe. The filtered sample was transferred from the syringe into a HPLC vial. Finally, the sample was analysed by using HPLC and the concentration of PHB was calculated based on the HPLC results as shown in the next chapter.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 INTRODUCTION

The objective of this research is to determine the optimum condition for the production of PHB biopolymer in a lab scale through fermentation using *Cupriavidus necator* and oil palm trunk sap as the medium component. Mathematical model was used to determine the optimum condition for the fermentation process. Based on the mathematical modelling results, experiments were done to test and find the actual data of the model. The experimental procedures were involved the preparation of inoculum 1 and inoculum 2, fermentation process and product analysis which were cell dry weight analysis and PHB analysis. Results obtained from the experimental procedures were analysed to determine the optimum condition for the fermentation process.

4.2 MATHEMATICAL MODELLING

The parameters values for the experimental procedures were determined by using the Method of Path of Steepest Ascent. The levels of the three studied parameters were as presented in Table 3.3 in the previous chapter.

Table 3.3: The Real Parameter Values of *n* from 1 to 10

No. of experiment, *n*	Temperature, (°C)	Agitation Speed, (rpm)	Percent Volume of OPTS, (% v/v)
1	30.3	185	19.7
2	30.5	169	24.4
3	30.8	154	29.1
4	31.1	138	33.8
5	31.3	123	38.5
6	31.6	107	43.3
7	31.9	92	48.0
8	32.1	77	52.7
9	32.4	61	57.4
10	32.7	46	62.1

Experiments were conducted based on the real parameter values as stated in Table 3.3 for *n* values of 1, 3, 5, 6, 7, 8 and 9. Cell dry weight analysis and PHB analysis were performed at the end of the experiments to determine the biomass and product concentration for each experiment.

4.3 CELL DRY WEIGHT CONTENT

Cell dry weight content for each experiment was determined by drying 1 mL of product in an Eppendorf tube. The difference of the initial and the final mass of the Eppendorf tube was used to calculate the mass of the product and divided by the amount of sample volume taken for the analysis. The biomass content of the product can be calculated by using the Equation 4.1 as below.

$$Biomass\ content, (g/l) = \frac{Final\ weight\ of\ Eppendorf\ tube, (g) - Initial\ weight\ of\ Eppendorf\ tube, (g)}{volume\ of\ sample, (l)} \quad (4.1)$$

where,

Final weight of Eppendorf tube is the weight of Eppendorf tube with dried sample,

Initial weight of Eppendorf tube is the weight of Eppendorf tube without sample, and

Volume of sample is 0.001 l.

Biomass content is used to investigate the growth of the bacteria during the fermentation process. As PHB is an intracellular product of the fermentation, the cell dry weight is important to predict the amount of PHB can be produced from the fermentation. Besides that, biomass content also can be used to determine the percentage of yield or conversion of PHB by the bacteria. Table 4.1 is shown the results for the biomass content based on the real parameter values mentioned earlier in the report.

Table 4.1: Biomass Content from Cell Dry Weight Analysis

n	**Temperature, (°C)**	**Agitation Speed, (rpm)**	**Percent Volume of OPTS, (%)**	**Biomass Content, (g/L)**
1	30.3	185	19.7	1.95
3	30.8	154	29.1	2.95
5	31.3	123	38.5	3.55
6	31.6	107	43.3	1.15
7	31.9	92	48.0	1.33
8	32.1	77	52.7	1.28
9	32.4	61	57.4	1.43

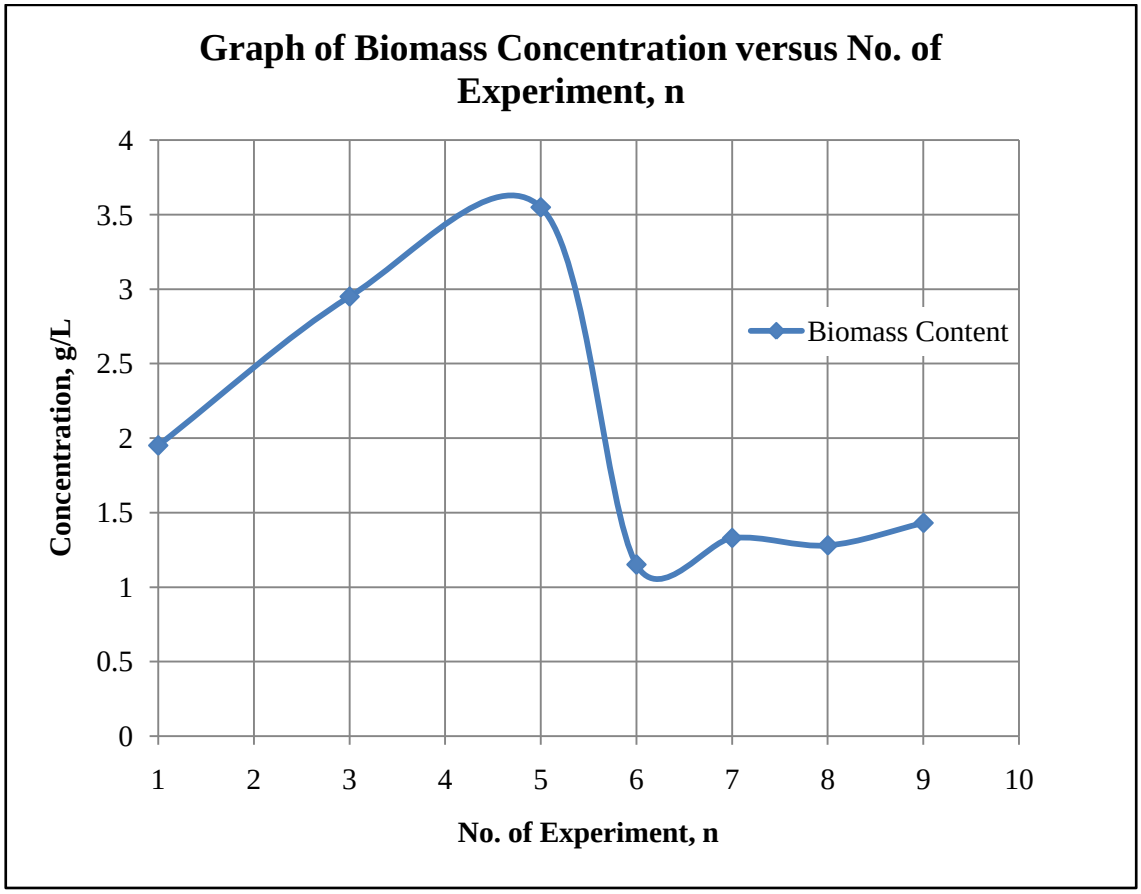


Figure 4.1: Graph of Biomass Concentration versus No. of Experiment, n

Figure 4.1 above shown the highest biomass content is at n = 5. The operating condition for the highest biomass content is at temperature of 31.3°C, 123 rpm for agitation speed and 38.5 % for percent volume of oil palm trunk sap. The lowest biomass content is at n = 6, fermented at temperature of 31.6°C, 107 rpm for agitation speed and 43.3 % for percent volume of oil palm trunk sap. The microorganism growth is at the highest rate at room temperature with moderate agitation speed and sufficient nutrient supplied into the fermentation medium. The bacteria are killed at higher temperature and faster agitation speed as the cells were died or burst due to the extreme temperature and vigorous movements of the shake flask contents. Insufficient amount of nutrient in the shake flask also resulting the bacteria growth to be limited. Therefore, the bacteria grow is best at room temperature with moderate agitation speed and sufficient nutrient supplied in the medium.

4.4 PHB CONTENT

PHB analysis was done for each experiment for *n* of 1, 3, 5, 6, 7, 8 and 9 by using HPLC. The concentration of PHB is determined by comparing the highest peak area from the graph of HPLC analysis results with the standard peak area of the product. Below equation (Equation 4.2) is used together with the results obtained from the HPLC analysis to determine the PHB content.

$$\text{PHB content, (g/L)} = \frac{area\ of\ sample}{standard\ area\ of\ PHB} \times 0.1\,\frac{g}{L} \times dilution\ factor \qquad (4.2)$$

where,
area of sample is referred to the area of the highest peak obtained from HLPC analysis graph,
standard area of PHB is referred to the area of the highest peak obtained from HLPC analysis graph, and
dilution factor of the PHB content analysis is 10.

Based on the graphs obtained from the HPLC analysis, the average standard area of PHB is 20,074.60.

The area of sample is taken from the average area of the product samples where two replications for each experiment are taken to be analysed. Then, PHB content calculations are performed by using the above equation and the average area of the samples. The results of the calculations performed is shown in Table 4.2 with respect to the real parameter values mentioned earlier in the report The HPLC results for every analysis done were attached at the end of the report in the Appendix.

Table 4.2: PHB Contents based on HPLC Analysis Data

n	Temperature, (°C)	Agitation Speed, (rpm)	Percent Volume of OPTS, (%)	PHB Content, (g/L)
1	30.3	185	19.7	0.359
3	30.8	154	29.1	0.484
5	31.3	123	38.5	0.825
6	31.6	107	43.3	0.389
7	31.9	92	48.0	0.467
8	32.1	77	52.7	0.537
9	32.4	61	57.4	0.674

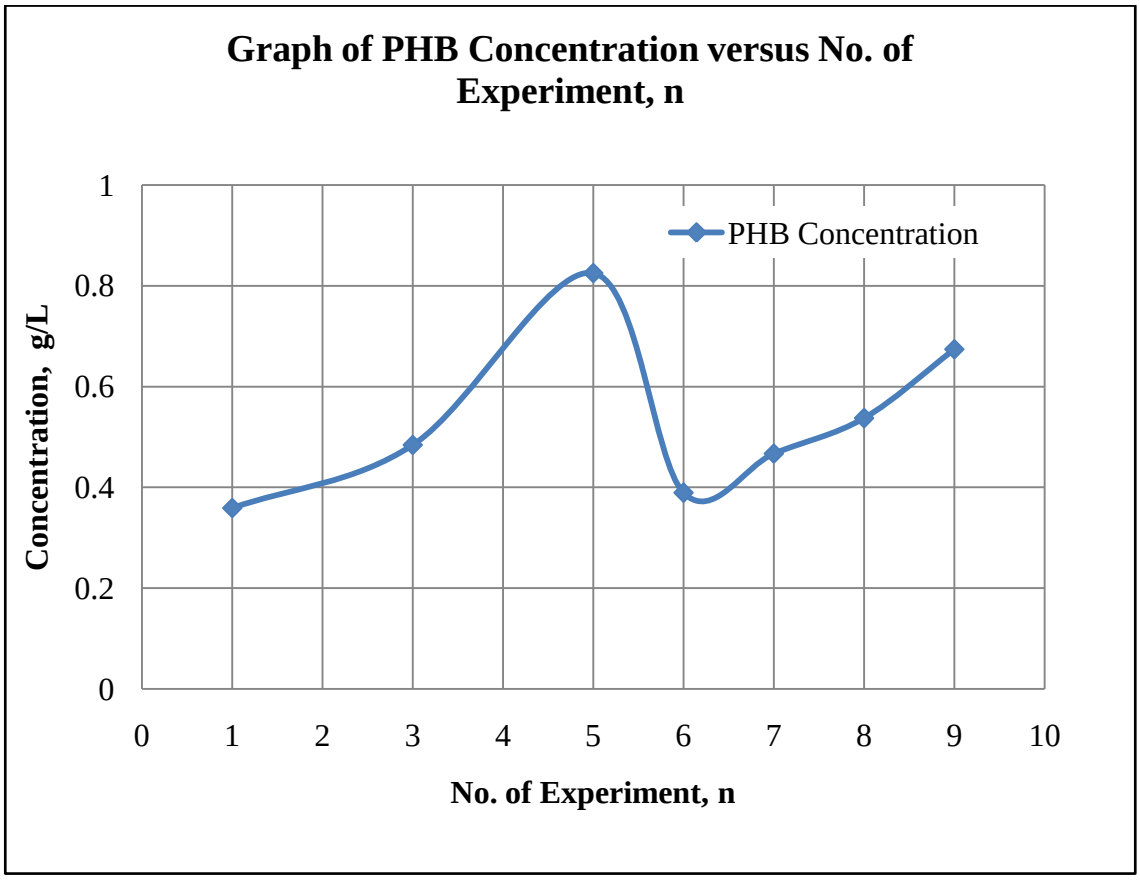


Figure 4.2: Graph of PHB Concentration versus No. of Experiment, n

Based on Figure 4.2 above, the highest concentration of PHB is obtained at $n = 5$ with 0.825 g/L of PHB and the lowest amount of PHB is produced at $n = 1$ with 0.359 g/L PHB. The highest amount of PHB is produced at temperature of 31.3°C, 123 rpm for agitation speed and 38.5 % for percent volume of oil palm trunk sap. The lowest PHB content is at temperature of 30.3, 185 rpm for agitation speed and 19.7 % for percent volume of oil palm trunk sap. The physiological stress condition which applied to the bacteria fermentation has produced the highest PHB yield with the combination of these fermentation parameters.

4.5 METHOD OF PATH OF STEEPEST ASCENT APPLIED TO FERMENTATION OF PHB BIOPOLYMER USING OIL PALM FRONDS JUICE AS MEDIUM COMPONENT

Based on the results obtained for both the cell dry weight analysis and PHB analysis, the results is tabulated in Table 4.3 and a graph as shown in Figure 4.1 is plotted to investigate the relationship between the concentration of biomass and the concentration of PHB produced for each experiment. From the table constructed and graph plotted, the optimum condition for the fermentation process is further discussed in this section.

Table 4.3: Results of Fermentation Process

n	Temperature, (°C)	Agitation Speed, (rpm)	Percent Volume of OPTS, (%)	Cell Dry Weight, (g/L)	PHB Content, (g/L)
1	30.3	185	19.7	1.95	0.359
3	30.8	154	29.1	2.95	0.484
5	31.3	123	38.5	3.55	0.825
6	31.6	107	43.3	1.15	0.389
7	31.9	92	48.0	1.33	0.467
8	32.1	77	52.7	1.28	0.537
9	32.4	61	57.4	1.43	0.674

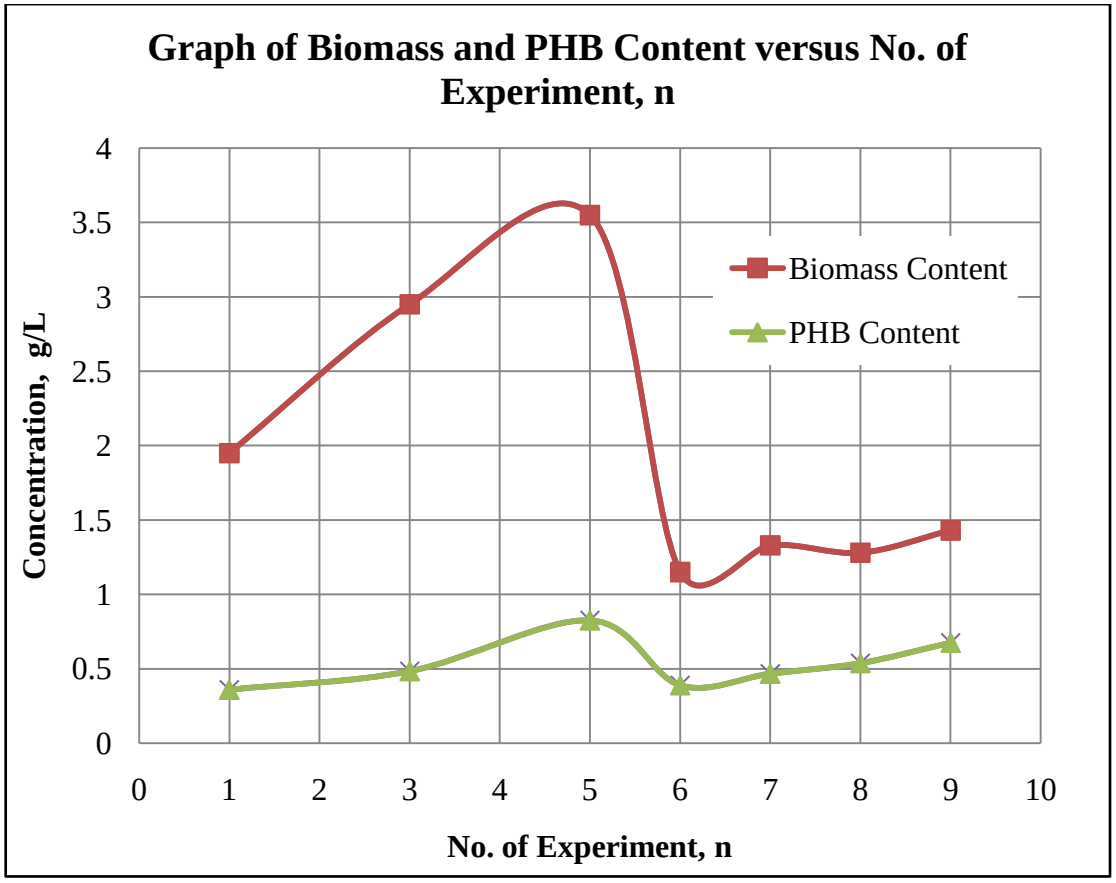


Figure 4.3: Graph of Biomass and PHB Content versus No. of Experiment, *n*

Based on the graph plotted in Figure 4.1, it can be concluded that the relationship between the biomass content is directly proportional to the PHB concentration of the fermentation. From the graph, it is shown that physiological stress conditions existed during fermentation process. In the fermentation medium, the cells grow but did not produce large amount of PHB and in other fermentation, the cells concentration is lower but yield larger amount of PHB. The physiological stress is the condition which is desired in this research. This condition will be further explained in the next sub-section in this report.

4.5.1 Optimum Temperature

Most of cells grow best at room temperature which is 37°C. However, adjusting the temperature to be lowered than 37°C is not damaging the cells but only affects to the cell growth rate. However, increasing slightly the temperature to above of 40°C will be damaging the cells. It is important to maintain the temperature lowered than 40°C.

In this research, it is believed that the optimum temperature is at range of 30-33°C based on the literature review, which is slightly lower than room temperature. Based on the temperature changes between every experiment, small temperature difference is detected which can be considered that it does not cause large effect to the fermentation process.

The highest PHB yield is at temperature of. 31.3°C meanwhile the lowest PHB produced is at 30.3°C; only 1°C differs from the optimum temperature. Since the temperature of the fermentation is shifting in range of 0.3-0.5°C, these temperatures still can be considered as at room temperature. Therefore, the growth of bacteria and the PHB produced from the fermentation process can be concluded as more depends on the agitation speed and percent volume of oil palm trunk sap.

4.5.2 Optimum Agitation Speed

The agitation speed plays important role in the fermentation process. Agitation provides mixing or blending of the sugar in the shake flask. Agitation also provides better aeration for the cells by dispersing air through the fermentation medium. Besides that, agitation also needed to maintain the suspension of cells in the medium component. The other importance of mixing is to promote heat transfer to maintaining the temperature.

Slow agitation speed may cause the possibilities of cells aggregation, making the culture medium more heterogeneous. The cell growth may be decreased and thus affecting the production of PHB. Increase of agitation speed to higher than moderate level also gives negative impact for cell growth and more importantly to the production of PHB. At higher agitation speed, the cell may be disrupted and dead. Therefore, there will be less or no PHB produced in the fermentation process.

The best condition for the fermentation is at moderate agitation speed which is optimum at 123 rpm. It gives the cells better aeration; air can be dispersed thoroughly in the media and provides mixing to the medium component besides providing better heat transfer to maintain the temperature.

4.5.3 Optimum Percent Volume of Oil Palm Trunk Sap

One of the novelties of this research is the usage of oil palm trunk sap as the medium component. As mentioned earlier in the report, oil palm trunk sap is used as carbon source for the fermentation process. The percent volume of oil palm trunk sap is varied in the research to obtain the optimum its percent volume.

Carbon source is important for the production of PHB. Bacteria take up the glucose and convert it to PHB at physiological stress conditions. To produce such condition so that the glucose is stored in form of PHB within the bacteria cells, it is important to find and control the amount of glucose supplied into the medium.

Limited amount of glucose prevents the growth of the bacteria cells instead of taking the glucose and convert it to PHB, the glucose is may be only sufficient for growth and produce small amount of PHB. Thus, the amount of cells is decreased and adversely decrease the amount of PHB produced in the fermentation process. Large amount of glucose supplied in the medium also must be avoided. Since the bacteria is only taking excessive carbon when the surroundings is in limited conditions, the bacteria will not produce large amount of PHB at glucose rich solution.

Therefore, the best condition to create the stress physiological condition for the fermentation process is by supplying optimum amount of glucose into the medium. Sufficient amount of glucose for cell growth and creating the mimic condition is needed to grow the cells and at the same time producing large amount of PHB within the bacteria cells. The optimum percent volume of the oil palm trunk sap based on the experiments is 38.5 %

4.5 CONCLUSION

Therefore, it is concluded that the highest yield of PHB is produced under physiological stress condition at $n = 5$ with 0.825 g/L PHB and the biomass content is 3.55 g/L. The optimum condition for the production of PHB in shake flask is at temperature of temperature of 31.3°C, agitation speed of 123 rpm and 38.5 % of percent volume of oil palm trunk sap.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 INTRODUCTION

The purpose of this chapter is to provide a conclusion related to the study on the optimization for production of PHB biopolymer in shake flask using oil palm trunk sap as the medium component. Relevant recommendations were also provided in this chapter for improvement of the research.

5.2 CONCLUSION

As a conclusion from the research that has been conducted on the optimization for the production of PHB biopolymer in shake flask using oil palm trunk sap, the optimum conditions is at temperature of 31.3°C , agitation speed of 123 rpm and 38.5 % percent volume of oil palm trunk sap. The optimum condition yields 3.55 g/L biomass and 0.825 g/L PHB. The results obtained from the hypothesis of the optimum condition for fermentation is slightly different from the predicted optimum condition for temperature and agitation speed but largely differs for the percent volume of palm oil trunk sap. The predicted optimum condition is at temperature of 32°C, agitation speed of 150 rpm and 20% for percent volume of palm oil trunk sap. The temperature difference of the fermentation result can be neglected because it is too small. To provide physiological stress condition of the fermentation, agitation speed and percent volume of oil palm trunk sap have to be adjusted and results large difference of the agitation speed and percent volume of the oil palm trunk sap.

5.3 RECOMMENDATIONS

There are several relevant recommendations that have been made to improve the study.

Firstly, to improve the results of the research, the researcher have to carry out the research further with respect to the second order polynomial regression for better results of optimization. There are several weaknesses with regard to the linear regression which may deviate from the actual optimum value.

Secondly, it is important for the researcher to do replication in fermentation process to obtain the average data value so that the results will be more accurate compared to a single data.

Consideration must be done on the equipments used in the research as accurate data can be obtained with the usage of good equipments. In case of limited equipments, the methodology must be revised to get better results.

It is recommended that the researcher to fully understand the research methodologies before conducting the experiments to avoid any mistakes that may contribute to errors in the results. The experiments procedures must be followed step-by-step to avoid of doing any mistakes.

Finally, the researcher must emphasize the importance of sterility during experiment to avoid contamination of the fermentation medium. Besides that, it is important for the researcher to use the right methods and techniques for the experiments to obtain the actual experimental results. Measuring skills for example must be accurate especially in collecting data.

REFERENCES

Arai, Y., Shikanai, T., Doi, Y., Yoshida, S., Yamaguchi, I., Nakashita, H. (2004). Production of Polyhydroxybutyrate by Polycistronic Expression of Bacterial Genes in Tobacco Plastid. Plant Cell Physiology, 45(9): 1176-1184

Aremu, M.O., Aransiola, E.F., Layokun, S.K., Solomon, B.O. (2011). Production of Polyhydroxybutyrate (PHB) by *Psuedomonas Putida* Strain KT2440 on Cassava Hydrolysate Medium. Research Journal of Chemical Sciences, 1(4): 67-73

Chaijamrus, S., Udpuay, N. (2008). Production and Characterization of Polyhydroxybutyrate from Molasses and Corn Steep Liquor produced by Bacillus megaterium ATCC 6748". Agricultural Engineering International: the CIGR Ejournal.

Choi J. and Lee S.Y., (2001). Factors affecting the economics of polyhydroxyalkaonoate production by bacterial fermentation. *Appl. Microbiol. Biotechnol.* 51, 13-21

Dawes, E.A. (1988). Polyhydroxybutyrate: an Intriguing Biopolymer. Bioscience Reports, 8(6): 537-547

Desouky, A.M., AlMa'adeed, M.A., Al-Thani, N. (2007). Physical and Chemical Properties of Polyhydroxyalkanotes Biodegradable Polymers Produced in Transgenic Yeasts. Global Journal of Environment Research 1(2): 69-73

El-sayed, A.A., Abdel Hafez, A.M., Hemmat, Abdelhady, M., Khodair, T.A. (2009) Production of Polyhydroxybutyrate (PHB) using Batch and Two-stage Batch Culture Strategies. Australian Journal of Basic and Applied Sciences, 3(2): 617-627

Gouda MK, Swellam AE, Omar SH (2001). Production of PHB by a *Bacillus megaterium* strain using sugarcane molasses and corn steep liquor as sole carbon and nitrogen source. Microbiol. Res. J. 156: 201-204.

Gunaratne, L.N., Wasantha, K., Shanks, R.A. (2008). Miscibility, melting, and crystallization behavior of poly(hydroxybutyrate) and poly(D, L-lactic acid) blends. Polymer Engineering and Science, 1-3

Ha, C.S. and W.J. Cho., (2002). Miscibility, properties, and biodegradability of microbial polyester containing blends. Prog. Polym. Sci. 27, 759-809.

Karen, B., Ilse, B., Alexander, S., Gilbert, T., Lothar, W. (2002). Constitutive Expression of the β-Ketothiolase Gene in Transgenic Plants. A Major Obstacle for obtaining Polyhydroxybutyrate-Producing Plants. Plant Physiology, 128: 1282-1290

Klinke, S., Ren, Q., Witholt, B., Kessler, B. (1999). Production of Medium Chain Length Poly(3-Hydroxyalkanoates) from Gluconate by Recombinant *Escherichia Coli*. Applied and Environmental Microbiology, 65(2): 540-548

Kumaravel, S., Hema, R., Lakshmi, R. (2010). Production of Polyhydroxybutyrate (Bioplastic) and its Biodegradation by *Pseudomonas Lemoignei* and Aspergillus *Niger*. E-Journal of Chemistry, 7(1): 536-542

Lee S. Y., (1996). Bacterial Polyhydroxyalkanoates. Biotechnol. Bioeng. 49: 1-14.

Lee B, Pometto III AL, Fratzke A, Bailey TB (1991). Biodegradation of degradable plastic polyethylene by *Phanerochaete* and *Streptomyces* species. Appl. Environ. Microbiol. 57: 678-685.

Lee SY, Chang H.N (1994). Effect of complex nitrogen source on the synthesis and accumulation of poly93-hydroxybutyric acid) by recombinant *Escherichia coli* in flask and fed-batch cultures. J. Environ. Polymer Degrad. 2: 169-176.

Lemoigne M (1926). Produit de déshydratation et de polymérisation de l'acide β-oxybutyrique. Bull. Soc. Chim. Biol. 8: 770-782.

Madison, L. L., Huisman, G. W. (1999). Metabolic Engineering of Poly(3-Hydroxyalkanoates): From DNA to Plastic. Microbiology and Molecular Biology Reviews, 63(1): 21-53

Mahapatra, K., Kumar, M.S., Vaidya, A.N., Chakrabarti, T. (2007). Production and Recovery Process of Polyhydroxybutyrate (PHB) from Waste Activated Sludge. Journal of Environmental Science and Enggineering, 49(3): 164-169

Mahmoudi, M., Sharifzadeh Baei, M., Najafpour, G.D., Tabandeh, F., Eisazadeh, H. (2010). Kinetic model for polyhydroxybutyrate (PHB) production by *Hydrogenophaga pseudoflava* and verification of growth conditions. African Journal of Biotechnology, 9(21): 3151-3157

Maliyakai, E.J., Greg, K. (1996). Metabolic pathway engineering in cotton: Biosynthesis of Polyhydroxybutyrate in fiber cells. Applied Biological Sciences, 93: 12768-12773

Marand, H., R. Alizadeh, R. Farmer, R. Desai and V. Velikov. (2000). Influence of structural and topological constraints on the crystallization and melting behaviour of polymers. Macromolecules. 33, 3392-3403.

Mariya, N.S., Kristi, D.S., Julie, B., Oliver, P.P., Bradley, G., Nii, P. (2009). Production of Polyhydroxybutyrate in Switchgrass. Patent application, 20090271889

Ojumu, T.V., Yu, J., Solomon, B.O. (2004). Production of Polyhydroxyalkanoates, a bacterial biodegradable polymer. African Journal of Biotechnology, 3(1): 18-24

Paramjit, S., Nitika, P. (2011). Isolation and characterization of two novel Polyhydroxybutyrate (PHB)-producing bacteria. African Journal of Biotechnology 10(24): 4907-4919

Petrasovits, L.A., Purnell, M.P., Nielsen, L.K., Brumbley, S.M. (2007). Production of Polyhydroxybutyrate in sugarcane. Plant Biotechnology Journal, 5(1): 162-172

Poirier Y, Nawrath C, Somerville C (1995). Production of polyhydroxyalkanoates, a family of Biodegradable plastics and elastomers, in bacterial and plant. Biotechnol. 13: 142-150.

Ramchander, M., Girisham, S., Reddy, S.M. (2010). Production of PHB (Polyhydroxybutyrate) by *Rhodopseodomonas Palustris* KU003 under nitrogen limitation. International Journal of Applied Biology and Pharmaceutical Technology, 1(2): 676-678

Rodman, T.H.C., Christopher, J.G., Helder, M., Robert, A.R., Peter, J.H., Foster, L.J.R. (2011). Manipulation of Polyhydroxybutyrate Properties through Blending with Ethyl-Cellulose for a Composite Biomaterial. International Journal of Polymer Science, 651549

Sangkharak, K., Prasertsan, P. (2007). Nutrient optimization for production of Polyhydroxybutyrate from halotolerant photosynthetic bacteria cultivated under aerobic-dark condition. Electronic Journal of Biotechnology, 11(3): 1-12

Steinbüchel, A. (1995). Use of biosynthetic, biodegradable thermoplastics and elastomers from renewable resources, The pros and cons. JMS Pure Appl. Chem. 32, 653-660.

Steinbüchel, A. and B. Füchtenbusch. (1998). Bacterial and other biological systems for polyester production. Trends Biotechnol. 16, 419-427.

Suzuki T., Yamane T. and Shimizu S., (2001). Mass Production of Poly-β-hydroxybutyric Acid by Fully Automatic Fed-batch Culture of Methylotroph. *Appl, Microbiol, Biotechnol,* 23, 322-329.

Thakor, S.N., A.M. Patel, B.U. Trivedi and C.K. Patel. (2003). Production of poly(β-hydroxybutyrate) by *Comamonas testosteroni* during growth on naphthalene. World Journal of Microbiology & Biotechnology. 19, 185-189.

Vaskova, I., Alexy, P., Bugaj, P., Nahalkova, A., Feranc, J., Mlynsky, T. (2008). Biodegradable polymer packaging materials based on polycaprolactone, starch and Polyhydroxybutyrate. Acta Chimica Slovaca, 1(1): 301-308

Wei, Y.H., Chen, W.C., Wu, H.S., Janarthanan, O.M. (2011). Biodegradable and Biocompatible Biomaterial, Polyhrdoxybutyrate Produced by an Indigenous *Vibrio sp*. BM-1 Isolated from Marine Environment. Marine Drugs Journal, 9: 615-624

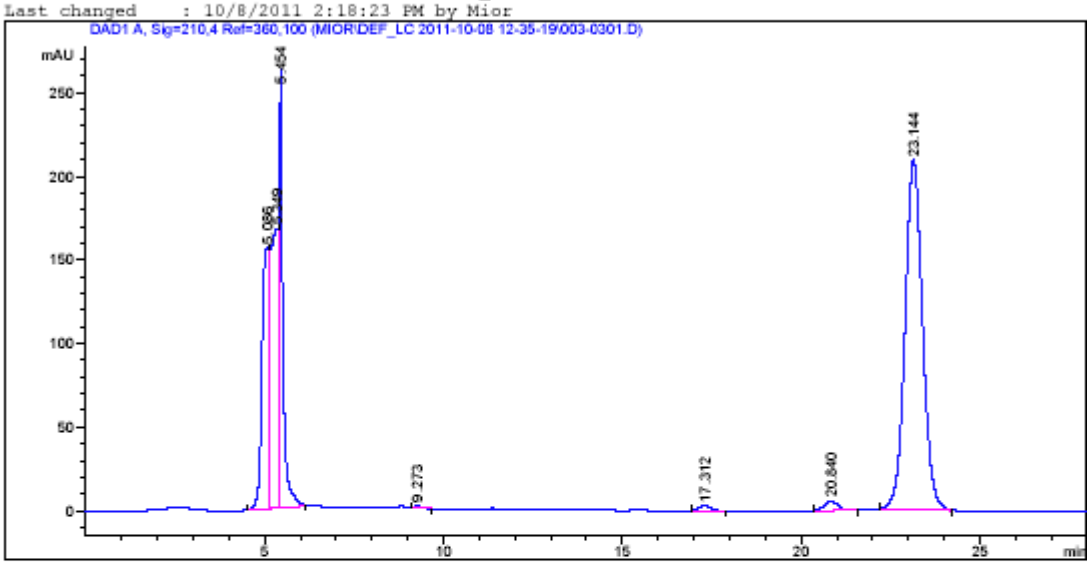
APPENDICES

APPENDIX A1

SAMPLE n = 1(a)

Data File C:\CHEM32\1\DATA\MIOR\DEF_LC 2011-10-08 12-35-19\003-0301.D
Sample Name: J1(a)

```
==================================================================
Acq. Operator   : Mior                          Seq. Line :   3
Acq. Instrument : Instrument 1                   Location : Vial 3
Injection Date  : 10/8/2011 1:41:58 PM              Inj :   1
                                                Inj Volume : 20 µl
Acq. Method     : C:\CHEM32\1\DATA\MIOR\DEF_LC 2011-10-08 12-35-19\MIOR (ORGANIC ACID, AMINEX).M
Last changed    : 10/8/2011 12:35:12 PM by Mior
Analysis Method : C:\CHEM32\1\DATA\MIOR\DEF_LC 2011-10-08 12-35-19\MIOR (ORGANIC ACID, AMINEX).M
Last changed    : 10/8/2011 2:18:23 PM by Mior
```




```
==================================================================
                        Area Percent Report
==================================================================

Sorted By             :      Signal
Multiplier:                   :      1.0000
Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=210,4 Ref=360,100
```

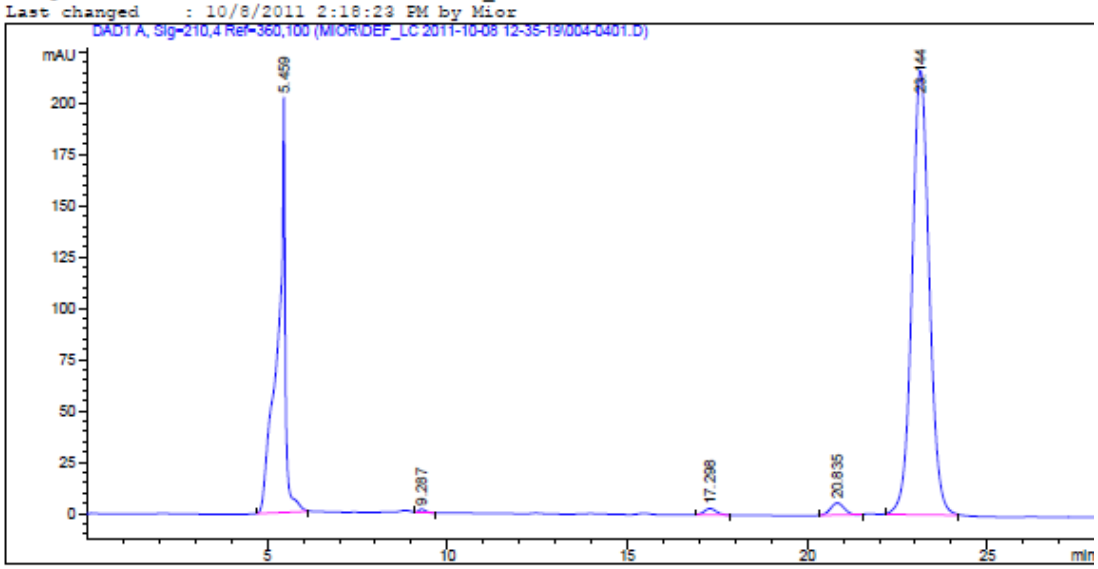
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	5.086	BV	0.2265	2283.36182	156.19647	16.5165
2	5.349	VV	0.1938	2263.30542	167.33298	16.3715
3	5.454	VB	0.0987	1952.90149	262.86725	14.1262
4	9.273	BB	0.1711	16.05162	1.40673	0.1161
5	17.312	BB	0.3599	70.13428	3.00936	0.5073
6	20.840	BB	0.4104	144.20926	5.56354	1.0431
7	23.144	BB	0.5215	7094.73242	209.28859	51.3193
Totals :				1.38247e4	805.66492	

```
==================================================================
                        *** End of Report ***

Instrument 1 10/8/2011 8:10:18 PM Mior                    Page   1 of 1
```

APPENDIX A2

SAMPLE n = 1(b)

```
Data File C:\CHEM32\1\DATA\MIOR\DEF_LC 2011-10-08 12-35-19\004-0401.D
Sample Name: J1(b)

==================================================================
Acq. Operator    : Mior                          Seq. Line :   4
Acq. Instrument : Instrument 1                    Location : Vial 4
Injection Date  : 10/8/2011 2:14:14 PM               Inj :   1
                                                 Inj Volume : 20 µl
Acq. Method     : C:\CHEM32\1\DATA\MIOR\DEF_LC 2011-10-08 12-35-19\MIOR (ORGANIC ACID, AMINEX).M
Last changed    : 10/8/2011 12:35:12 PM by Mior
Analysis Method : C:\CHEM32\1\DATA\MIOR\DEF_LC 2011-10-08 12-35-19\MIOR (ORGANIC ACID, AMINEX).M
Last changed    : 10/8/2011 2:18:23 PM by Mior
```



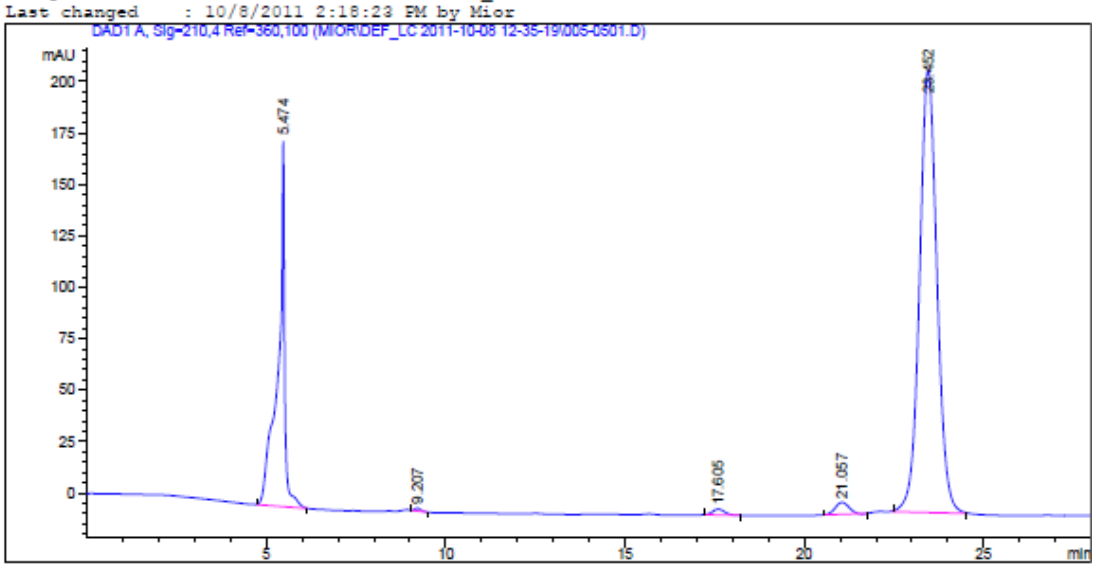

```
==================================================================
                        Area Percent Report
==================================================================

Sorted By             :      Signal
Multiplier:                   :      1.0000
Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=210,4 Ref=360,100

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]    [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   5.459 BB    0.1879 3019.40259  202.59708  28.8264
   2   9.287 BB    0.1743   18.85826    1.63880   0.1800
   3  17.298 BB    0.3472   69.53866    3.05940   0.6639
   4  20.835 BB    0.4015  149.36992    5.81764   1.4260
   5  23.144 BB    0.5107 7217.26123  216.62427  68.9036

Totals :                  1.04744e4   429.73739

==================================================================
                        *** End of Report ***

Instrument 1 10/8/2011 8:13:45 PM Mior                    Page   1 of 1
```

APPENDIX A3

SAMPLE n = 1(c)

Data File C:\CHEM32\1\DATA\MIOR\DEF_LC 2011-10-08 12-35-19\005-0501.D
Sample Name: J1(c)

```
==================================================================
Acq. Operator   : Mior                          Seq. Line :   5
Acq. Instrument : Instrument 1                   Location : Vial 5
Injection Date  : 10/8/2011 2:46:38 PM             Inj :   1
                                                 Inj Volume : 20 µl
Acq. Method     : C:\CHEM32\1\DATA\MIOR\DEF_LC 2011-10-08 12-35-19\MIOR (ORGANIC ACID, AMINEX).M
Last changed    : 10/8/2011 12:35:12 PM by Mior
Analysis Method : C:\CHEM32\1\DATA\MIOR\DEF_LC 2011-10-08 12-35-19\MIOR (ORGANIC ACID, AMINEX).M
Last changed    : 10/8/2011 2:18:23 PM by Mior
```



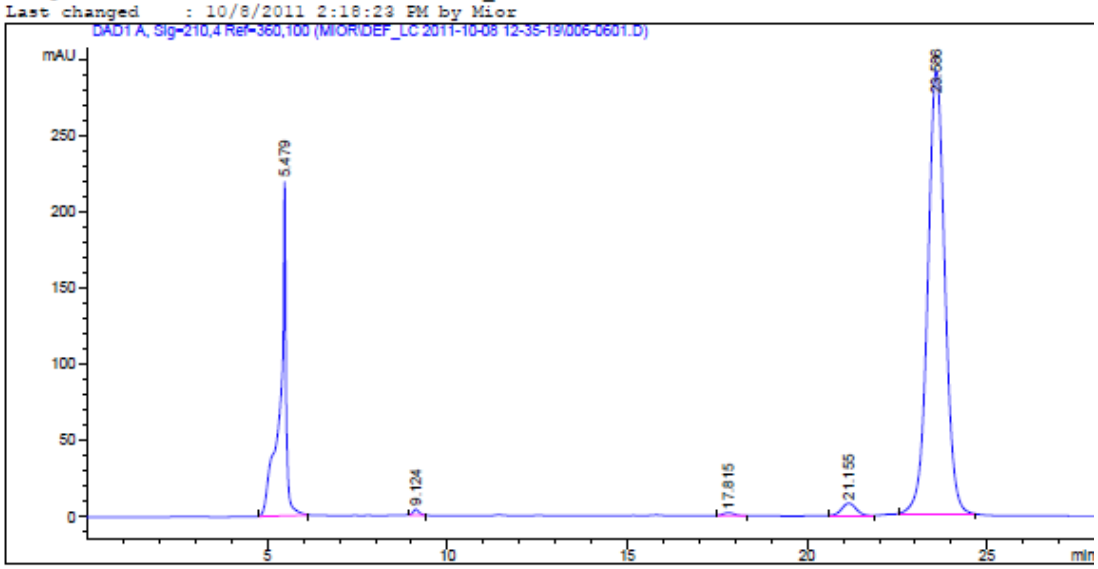

```
==================================================================
                      Area Percent Report
==================================================================

Sorted By             :      Signal
Multiplier:                   :      1.0000
Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=210,4 Ref=360,100

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   5.474 BB    0.1654 2314.88916  178.43431  23.5633
   2   9.207 BB    0.1619   15.26251    1.43674   0.1554
   3  17.605 BB    0.3568   65.75337    2.85336   0.6693
   4  21.057 BB    0.4103  152.04945    5.79117   1.5477
   5  23.452 BB    0.5191 7276.18262  214.87161  74.0643

Totals :                  9824.13711  403.38720

==================================================================
                        *** End of Report ***
```

APPENDIX A4

SAMPLE n = 3(a)

Data File C:\CHEM32\1\DATA\MIOR\DEF_LC 2011-10-08 12-35-19\006-0601.D
Sample Name: J3(a)

```
==================================================================
Acq. Operator   : Mior                          Seq. Line :   6
Acq. Instrument : Instrument 1                   Location : Vial 6
Injection Date  : 10/8/2011 3:18:58 PM             Inj :   1
                                                 Inj Volume : 20 µl
Acq. Method     : C:\CHEM32\1\DATA\MIOR\DEF_LC 2011-10-08 12-35-19\MIOR (ORGANIC ACID, AMINEX).M
Last changed    : 10/8/2011 12:35:12 PM by Mior
Analysis Method : C:\CHEM32\1\DATA\MIOR\DEF_LC 2011-10-08 12-35-19\MIOR (ORGANIC ACID, AMINEX).M
Last changed    : 10/8/2011 2:18:23 PM by Mior
```



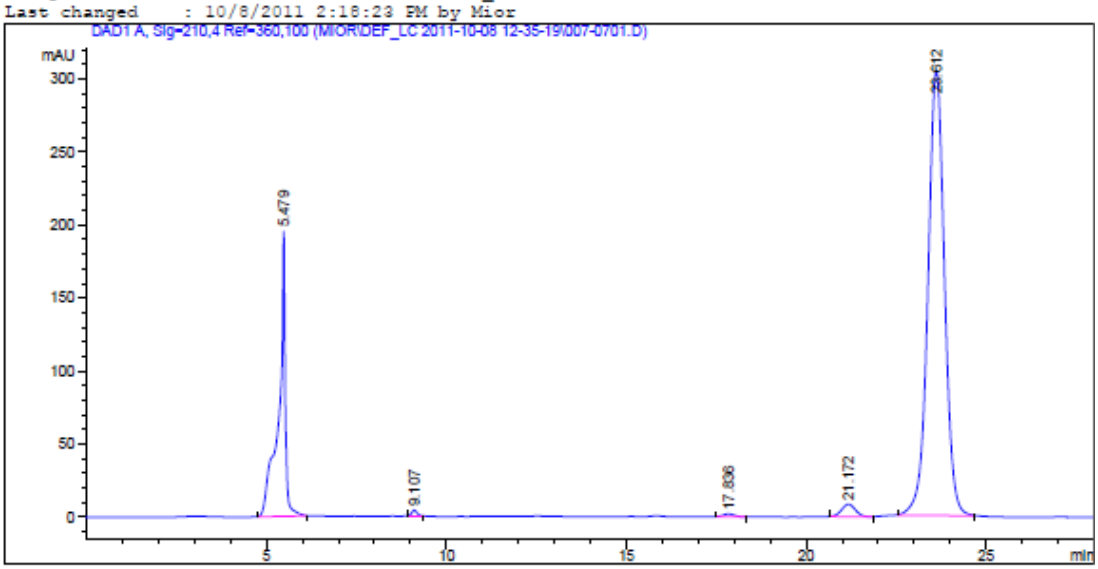

```
==================================================================
                        Area Percent Report
==================================================================

Sorted By             :      Signal
Multiplier:                   :      1.0000
Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=210,4 Ref=360,100

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   5.479 BB    0.1465 2527.09717  219.20001  20.2477
   2   9.124 BB    0.1701   45.02188    3.91663   0.3607
   3  17.815 BB    0.3288   40.41964    1.86571   0.3239
   4  21.155 BB    0.4234  219.40898    8.06427   1.7580
   5  23.586 BB    0.5088 9648.97168  291.00598  77.3098

Totals :                  1.24809e4   524.05260

==================================================================
                        *** End of Report ***
```

APPENDIX A5

SAMPLE n = 3(b)

Data File C:\CHEM32\1\DATA\MIOR\DEF_LC 2011-10-08 12-35-19\007-0701.D
Sample Name: J3(b)

```
==================================================================
Acq. Operator    : Mior                          Seq. Line :   7
Acq. Instrument : Instrument 1                    Location : Vial 7
Injection Date  : 10/8/2011 3:51:19 PM                 Inj :   1
                                                 Inj Volume : 20 µl
Acq. Method     : C:\CHEM32\1\DATA\MIOR\DEF_LC 2011-10-08 12-35-19\MIOR (ORGANIC ACID, AMINEX).M
Last changed    : 10/8/2011 12:35:12 PM by Mior
Analysis Method : C:\CHEM32\1\DATA\MIOR\DEF_LC 2011-10-08 12-35-19\MIOR (ORGANIC ACID, AMINEX).M
Last changed    : 10/8/2011 2:18:23 PM by Mior
```



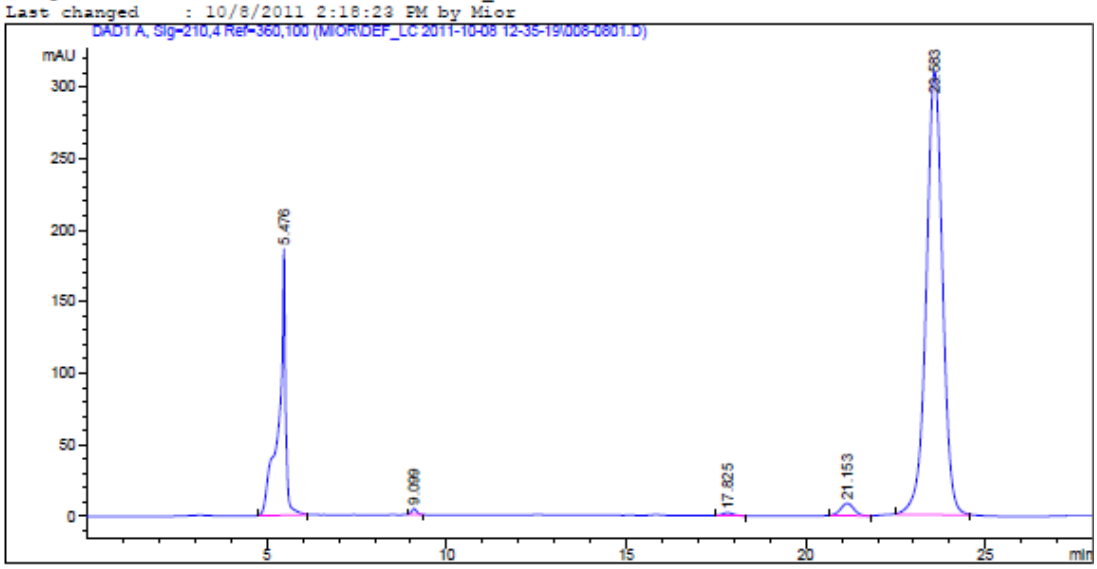

```
==================================================================
                      Area Percent Report
==================================================================

Sorted By             :      Signal
Multiplier:                   :      1.0000
Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=210,4 Ref=360,100

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   5.479 BB    0.1588 2456.14575  195.14244  19.6141
   2   9.107 BB    0.1580   43.69118    4.11144   0.3489
   3  17.836 BB    0.3165   40.05190    1.94281   0.3198
   4  21.172 BB    0.4066  222.78256    8.47708   1.7791
   5  23.612 BB    0.4896 9759.64746  304.74561  77.9380

Totals :                  1.25223e4   514.41938

==================================================================
                        *** End of Report ***
```

APPENDIX A6

SAMPLE n = 1(c)

Data File C:\CHEM32\1\DATA\MIOR\DEF_LC 2011-10-08 12-35-19\008-0801.D
Sample Name: J3(c)

```
==================================================================
Acq. Operator    : Mior                          Seq. Line :   8
Acq. Instrument : Instrument 1                    Location : Vial 8
Injection Date  : 10/8/2011 4:23:36 PM               Inj :   1
                                                 Inj Volume : 20 µl
Acq. Method     : C:\CHEM32\1\DATA\MIOR\DEF_LC 2011-10-08 12-35-19\MIOR (ORGANIC ACID, AMINEX).M
Last changed    : 10/8/2011 12:35:12 PM by Mior
Analysis Method : C:\CHEM32\1\DATA\MIOR\DEF_LC 2011-10-08 12-35-19\MIOR (ORGANIC ACID, AMINEX).M
Last changed    : 10/8/2011 2:18:23 PM by Mior
```



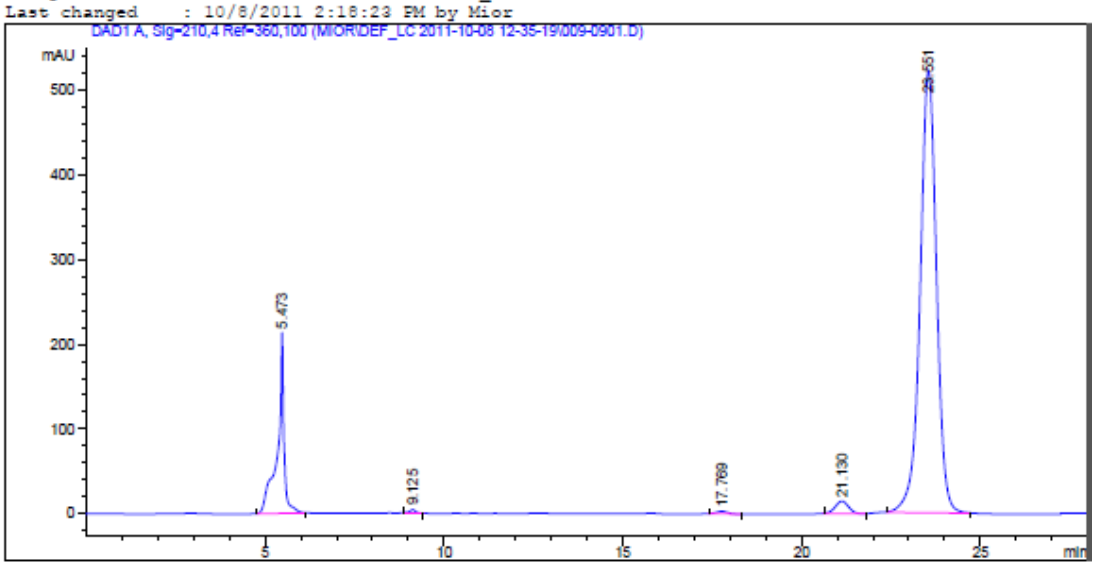

```
==================================================================
                        Area Percent Report
==================================================================

Sorted By             :      Signal
Multiplier:                   :      1.0000
Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=210,4 Ref=360,100

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]    [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   5.476 BB    0.1663 2427.92432  185.96568  19.4408
   2   9.099 BB    0.1542   43.87318    4.25800   0.3513
   3  17.825 BB    0.3083   39.69221    1.97671   0.3178
   4  21.153 BB    0.3929  221.96295    8.60478   1.7773
   5  23.583 BB    0.4831 9755.33398  310.00021  78.1127

Totals :                  1.24888e4   510.80539

==================================================================
                        *** End of Report ***
```

APPENDIX A7

SAMPLE n = 5(a)

Data File C:\CHEM32\1\DATA\MIOR\DEF_LC 2011-10-08 12-35-19\009-0901.D
Sample Name: J5(a)

```
==================================================================
Acq. Operator    : Mior                          Seq. Line :   9
Acq. Instrument : Instrument 1                    Location : Vial 9
Injection Date  : 10/8/2011 4:56:01 PM              Inj :   1
                                                 Inj Volume : 20 µl
Acq. Method     : C:\CHEM32\1\DATA\MIOR\DEF_LC 2011-10-08 12-35-19\MIOR (ORGANIC ACID, AMINEX).M
Last changed    : 10/8/2011 12:35:12 PM by Mior
Analysis Method : C:\CHEM32\1\DATA\MIOR\DEF_LC 2011-10-08 12-35-19\MIOR (ORGANIC ACID, AMINEX).M
Last changed    : 10/8/2011 2:18:23 PM by Mior
```



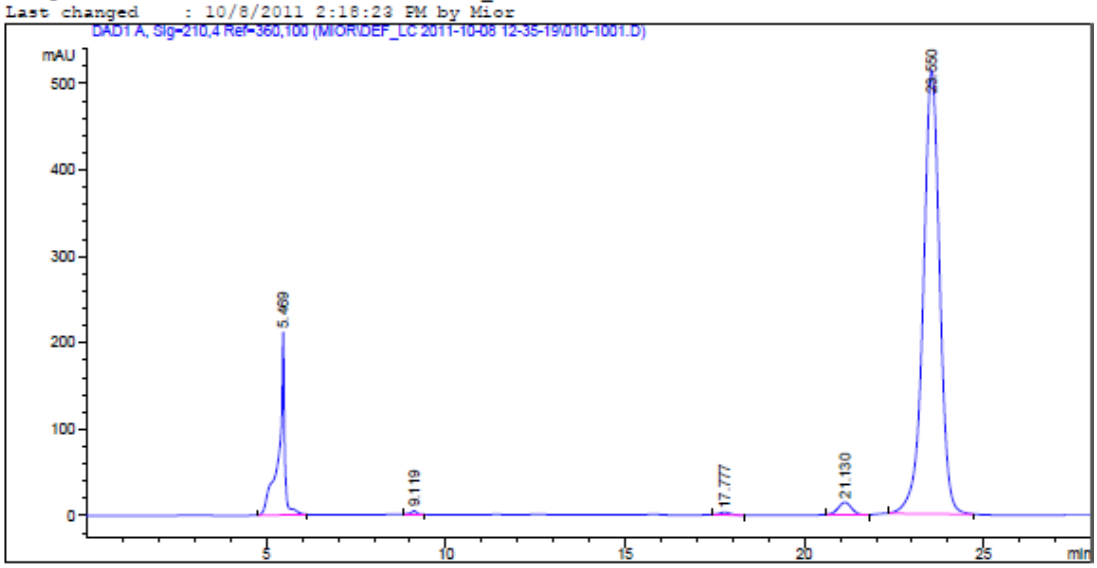

```
==================================================================
                        Area Percent Report
==================================================================

Sorted By             :      Signal
Multiplier:                   :      1.0000
Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=210,4 Ref=360,100

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   5.473 BB    0.1606 2681.62085  213.39012  13.5237
   2   9.125 BB    0.1808   52.81020    4.19731   0.2663
   3  17.769 BB    0.3231   64.18710    3.08099   0.3237
   4  21.130 BB    0.3994  382.95215   14.72459   1.9313
   5  23.551 BB    0.4856 1.66474e4   522.64105  83.9550

Totals :                  1.98290e4   758.03406

==================================================================
                        *** End of Report ***
```

APPENDIX A8

SAMPLE n = 5(b)

Data File C:\CHEM32\1\DATA\MIOR\DEF_LC 2011-10-08 12-35-19\010-1001.D
Sample Name: J5(b)

```
==================================================================
Acq. Operator    : Mior                          Seq. Line :  10
Acq. Instrument : Instrument 1                    Location : Vial 10
Injection Date  : 10/8/2011 5:28:21 PM                 Inj :  1
                                                 Inj Volume : 20 µl
Acq. Method     : C:\CHEM32\1\DATA\MIOR\DEF_LC 2011-10-08 12-35-19\MIOR (ORGANIC ACID, AMINEX).M
Last changed    : 10/8/2011 12:35:12 PM by Mior
Analysis Method : C:\CHEM32\1\DATA\MIOR\DEF_LC 2011-10-08 12-35-19\MIOR (ORGANIC ACID, AMINEX).M
Last changed    : 10/8/2011 2:18:23 PM by Mior
```



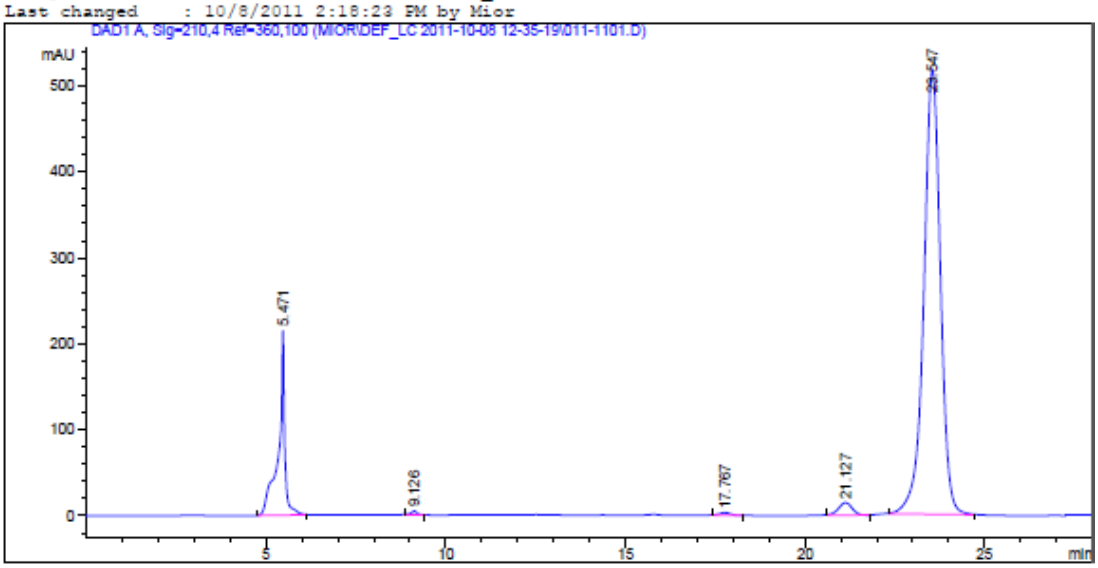

```
==================================================================
                      Area Percent Report
==================================================================

Sorted By             :      Signal
Multiplier:                   :      1.0000
Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=210,4 Ref=360,100

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   5.469 BB    0.1478 2427.31104  211.67821  12.5498
   2   9.119 BB    0.1833   54.90730    4.29124   0.2839
   3  17.777 BB    0.3171   61.91615    3.02074   0.3201
   4  21.130 BB    0.4031  374.49765   14.41703   1.9362
   5  23.550 BB    0.4895 1.64228e4   512.94489  84.9099

Totals :                  1.93414e4   746.35211

==================================================================
                      *** End of Report ***
```

APPENDIX A9

SAMPLE n = 5(c)

Data File C:\CHEM32\1\DATA\MIOR\DEF_LC 2011-10-08 12-35-19\011-1101.D
Sample Name: J5(c)

```
==================================================================
Acq. Operator    : Mior                          Seq. Line :  11
Acq. Instrument : Instrument 1                    Location : Vial 11
Injection Date  : 10/8/2011 6:00:41 PM                 Inj :  1
                                                 Inj Volume : 20 µl
Acq. Method     : C:\CHEM32\1\DATA\MIOR\DEF_LC 2011-10-08 12-35-19\MIOR (ORGANIC ACID, AMINEX).M
Last changed    : 10/8/2011 12:35:12 PM by Mior
Analysis Method : C:\CHEM32\1\DATA\MIOR\DEF_LC 2011-10-08 12-35-19\MIOR (ORGANIC ACID, AMINEX).M
Last changed    : 10/8/2011 2:18:23 PM by Mior
```



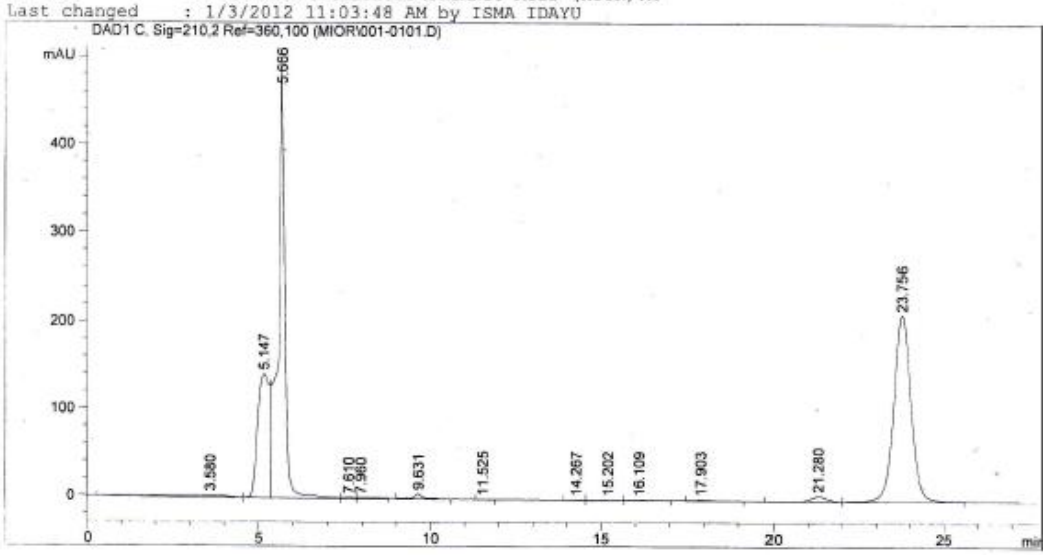

```
==================================================================
                        Area Percent Report
==================================================================

Sorted By             :      Signal
Multiplier:                   :      1.0000
Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=210,4 Ref=360,100

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]    [mAU*s]    [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   5.471 BB    0.1525 2594.83740  215.53329  13.1539
   2   9.126 BB    0.1827   56.09698    4.40086   0.2844
   3  17.767 BB    0.3153   63.41205    3.04083   0.3215
   4  21.127 BB    0.4026  378.46475   14.49495   1.9185
   5  23.547 BB    0.4915 1.66339e4   516.79749  84.3217

Totals :                  1.97267e4   754.26742

==================================================================
                        *** End of Report ***
```

APPENDIX A10

SAMPLE n = 6(a)

Data File C:\Chem32\1\DATA\MIOR\001-0101.D
Sample Name: J6,1

Injection Date	: 1/3/2012 11:45:37 AM	Seq. Line	: 1
Sample Name	: J6,1	Location	: Vial 1
Acq. Operator	: MIOR	Inj	: 1
Acq. Instrument	: Instrument 1	Inj Volume	: 40 µl
Sequence File	: C:\CHEM32\1\SEQUENCE\KAMAL_PSM_03012012.S		
Method	: C:\CHEM32\1\METHODS\ORGANIC ACID (MIOR).M		
Last changed	: 1/3/2012 11:03:48 AM by ISMA IDAYU		



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1 C, Sig=210,2 Ref=360,100

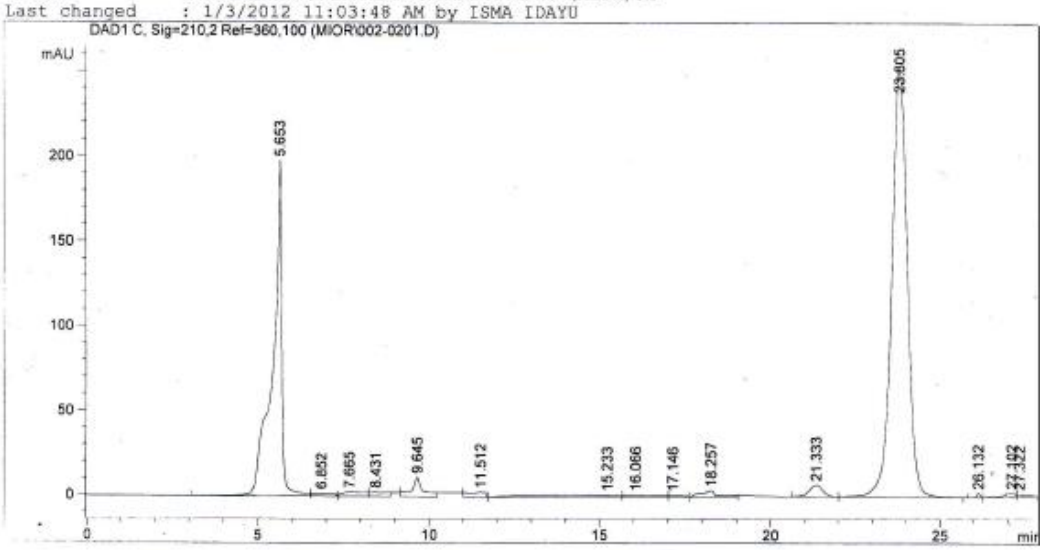
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	3.580	BV	1.3022	291.03543	2.65634	1.6008
2	5.147	VV	0.3701	3323.01929	141.41899	18.2775
3	5.666	VB	0.2002	6561.41406	477.78787	36.0896
4	7.610	BV	0.3008	30.17665	1.30972	0.1660
5	7.960	VB	0.5962	56.83865	1.15880	0.3126
6	9.631	BV	0.3108	133.59908	5.93130	0.7348
7	11.525	VV	0.3394	7.80775	3.12576e-1	0.0429
8	14.267	BV	0.4332	12.63515	3.74928e-1	0.0695
9	15.202	VV	0.6249	24.66796	4.78957e-1	0.1357
10	16.109	VB	0.6900	32.68991	6.01526e-1	0.1798
11	17.903	BV	0.5273	38.29839	9.32206e-1	0.2107
12	21.280	BV	0.4783	176.38510	5.61731	0.9702
13	23.756	VV	0.5395	7492.35498	212.44157	41.2100
Totals :				1.81809e4	851.02210	

APPENDIX A11

SAMPLE n = 6(b)

Data File C:\Chem32\1\DATA\MIOR\002-0201.D
Sample Name: J6,2

Injection Date	: 1/3/2012 12:17:48 PM	Seq. Line :	2
Sample Name	: J6,2	Location :	Vial 2
Acq. Operator	: MIOR	Inj :	1
Acq. Instrument	: Instrument 1	Inj Volume :	40 µl
Sequence File	: C:\CHEM32\1\SEQUENCE\KAMAL_PSM_03012012.S		
Method	: C:\CHEM32\1\METHODS\ORGANIC ACID (MIOR).M		
Last changed	: 1/3/2012 11:03:48 AM by ISMA IDAYU		



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1 C, Sig=210,2 Ref=360,100

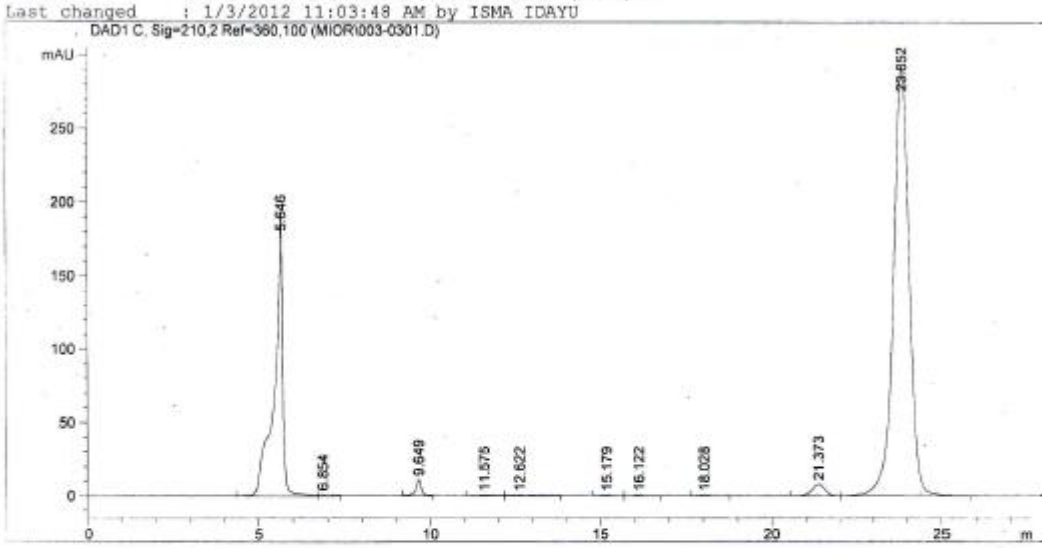
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	5.653	BB	0.2395	3651.98340	197.69205	27.8875
2	6.852	BV	0.4696	63.27970	1.71866	0.4832
3	7.665	VV	0.5754	114.23315	2.45258	0.8723
4	8.431	VB	0.4669	87.35308	2.38733	0.6671
5	9.645	BB	0.3049	253.00078	11.14344	1.9320
6	11.512	BV	0.4728	100.45332	2.65886	0.7671
7	15.233	VV	2.0947	180.15218	1.01896	1.3757
8	16.066	VB	0.7931	75.79733	1.16193	0.5788
9	17.146	BV	0.3846	27.78570	8.79355e-1	0.2122
10	18.257	VB	0.4789	123.23824	3.46532	0.9411
11	21.333	BV	0.4098	183.81142	6.66417	1.4036
12	23.805	VB	0.4961	8136.41162	252.39633	62.1317
13	26.132	BV	0.1027	15.41915	2.54524	0.1177
14	27.102	VV	0.3623	56.51601	2.04604	0.4316
15	27.322	VBA	0.2382	25.98849	1.47067	0.1985

APPENDIX A12

SAMPLE n = 7(a)

Data File C:\Chem32\1\DATA\MIOR\003-0301.D
Sample Name: J7,1

Injection Date	: 1/3/2012 12:49:57 PM	Seq. Line	: 3
Sample Name	: J7,1	Location	: Vial 3
Acq. Operator	: MIOR	Inj	: 1
Acq. Instrument	: Instrument 1	Inj Volume	: 40 µl
Sequence File	: C:\CHEM32\1\SEQUENCE\KAMAL_PSM_03012012.S		
Method	: C:\CHEM32\1\METHODS\ORGANIC ACID (MIOR).M		
Last changed	: 1/3/2012 11:03:48 AM by ISMA IDAYU		



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1 C, Sig=210,2 Ref=360,100

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	5.646	BV	0.4114	3217.77368	177.61353	24.3566
2	6.854	VB	0.3733	34.91853	1.22223	0.2643
3	9.649	BB	0.1829	140.93243	10.89534	1.0668
4	11.575	VB	0.5084	26.03913	6.83585e-1	0.1971
5	12.622	BB	0.9664	44.06409	5.59767e-1	0.3335
6	15.179	BV	0.4409	18.75537	5.45773e-1	0.1420
7	16.122	VB	0.4928	21.80115	5.61735e-1	0.1650
8	18.028	BB	0.3358	16.66006	6.39814e-1	0.1261
9	21.373	BV	0.4200	216.81013	7.80796	1.6411
10	23.852	VB	0.4997	9473.35449	291.07394	71.7075
Totals :				1.32111e4	491.60367	

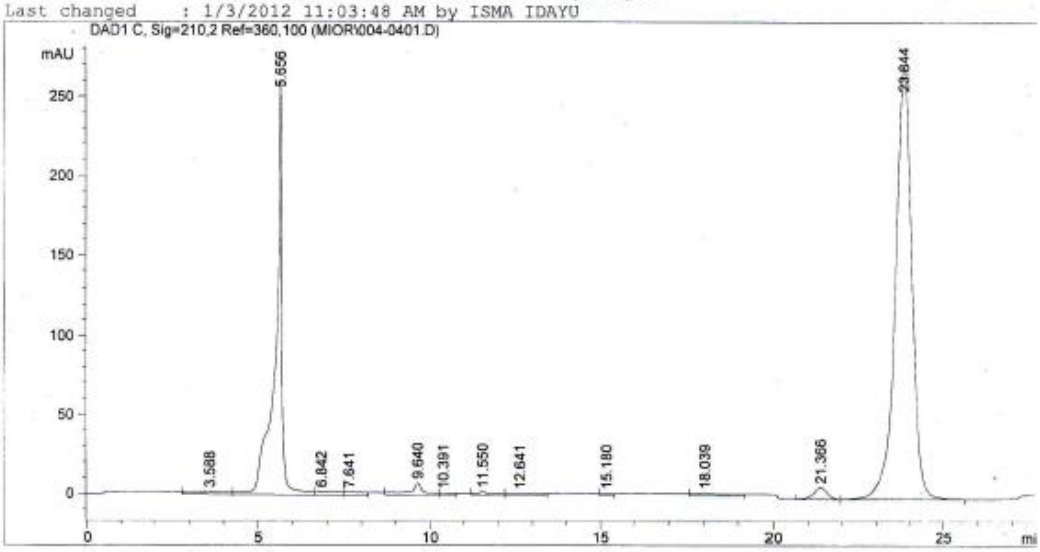
*** End of Report ***

APPENDIX A13

SAMPLE n = 7(b)

Data File C:\Chem32\1\DATA\MIOR\004-0401.D
Sample Name: J7,2

Injection Date	: 1/3/2012 1:22:07 PM	Seq. Line	: 4
Sample Name	: J7,2	Location	: Vial 4
Acq. Operator	: MIOR	Inj	: 1
Acq. Instrument	: Instrument 1	Inj Volume	: 40 µl
Sequence File	: C:\CHEM32\1\SEQUENCE\KAMAL_PSM_03012012.S		
Method	: C:\CHEM32\1\METHODS\ORGANIC ACID (MIOR).M		
Last changed	: 1/3/2012 11:03:48 AM by ISMA IDAYU		



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1 C, Sig=210,2 Ref=360,100

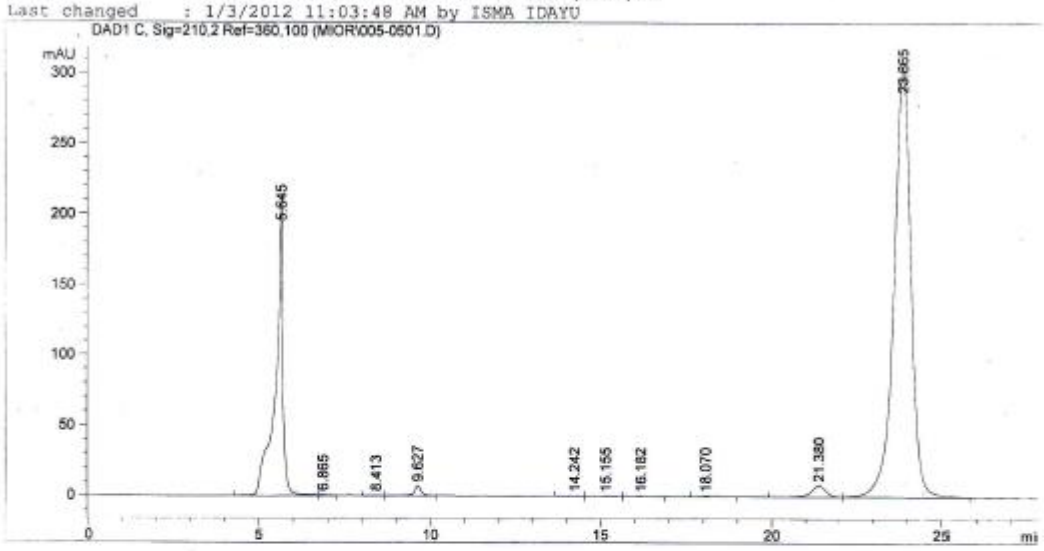
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	3.588	BV	1.0081	146.19647	1.73322	1.0585
2	5.656	VB	0.1820	3438.87231	253.90152	24.8988
3	6.842	BB	0.5545	91.85114	2.08296	0.6650
4	7.641	BB	0.4737	67.00100	1.80235	0.4851
5	9.640	BV	0.3659	212.90576	7.62083	1.5415
6	10.391	VB	0.3344	35.66925	1.41353	0.2583
7	11.550	BV	0.5528	82.03502	1.95788	0.5940
8	12.641	VB	0.8172	93.36400	1.37326	0.6760
9	15.180	BV	0.3181	35.67654	1.45498	0.2583
10	18.039	BB	0.8927	139.36356	1.88908	1.0090
11	21.366	BV	0.4366	206.56229	7.42453	1.4956
12	23.844	VB	0.5179	9261.91895	271.56497	67.0599
Totals :				1.38114e4	554.21912	

APPENDIX A14

SAMPLE n = 8(a)

Data File C:\Chem32\1\DATA\MIOR\005-0501.D
Sample Name: J8,1

Injection Date	: 1/3/2012 1:54:17 PM	Seq. Line	: 5
Sample Name	: J8,1	Location	: Vial 5
Acq. Operator	: MIOR	Inj	: 1
Acq. Instrument	: Instrument 1	Inj Volume	: 40 µl
Sequence File	: C:\CHEM32\1\SEQUENCE\KAMAL_PSM_03012012.S		
Method	: C:\CHEM32\1\METHODS\ORGANIC ACID (MIOR).M		
Last changed	: 1/3/2012 11:03:48 AM by ISMA IDAYU		



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1 C, Sig=210,2 Ref=360,100

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	5.645	BV	0.3884	3106.98828	192.39972	21.8346
2	6.865	VB	0.2716	14.26225	7.18533e-1	0.1002
3	8.413	BV	0.3980	9.44997	3.14995e-1	0.0664
4	9.627	VB	0.2056	100.84788	7.10289	0.7087
5	14.242	BV	0.3514	6.78941	2.41901e-1	0.0477
6	15.155	VV	0.4097	9.70207	2.93450e-1	0.0682
7	16.182	VB	0.3385	7.41840	2.89979e-1	0.0521
8	18.070	BB	0.3135	11.39194	5.16060e-1	0.0801
9	21.380	BV	0.4587	247.91177	8.15316	1.7422
10	23.865	VB	0.5312	1.07149e4	304.01379	75.2997
Totals :				1.42296e4	514.04448	

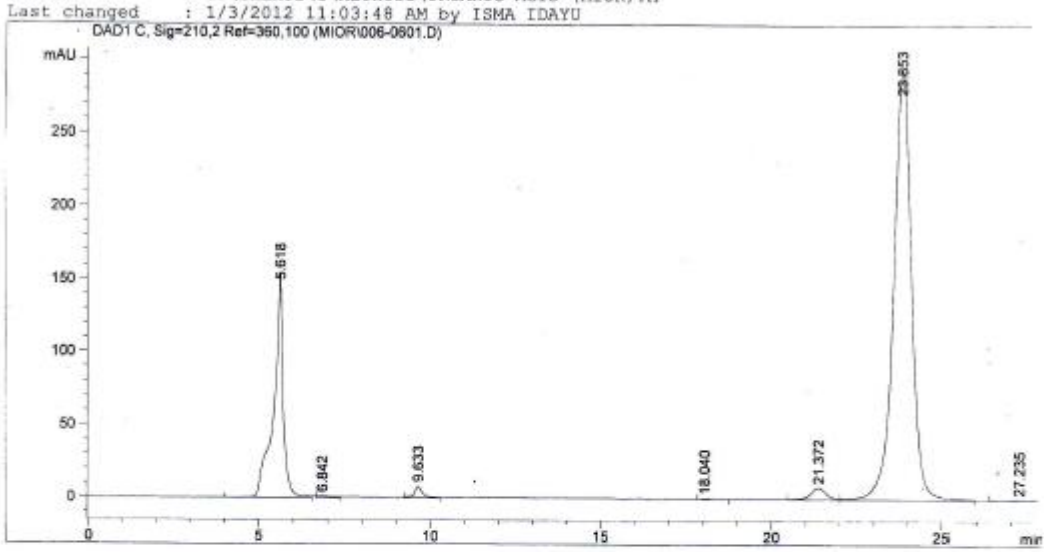
*** End of Report ***

APPENDIX A15

SAMPLE n = 8(b)

Data File C:\Chem32\1\DATA\MIOR\006-0601.D
Sample Name: J8,2

Injection Date	: 1/3/2012 2:26:27 PM	Seq. Line :	6
Sample Name	: J8,2	Location :	Vial 6
Acq. Operator	: MIOR	Inj :	1
Acq. Instrument	: Instrument 1	Inj Volume :	40 µl
Sequence File	: C:\CHEM32\1\SEQUENCE\KAMAL_PSM_03012012.S		
Method	: C:\CHEM32\1\METHODS\ORGANIC ACID (MIOR).M		
Last changed	: 1/3/2012 11:03:48 AM by ISMA IDAYU		



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1 C, Sig=210,2 Ref=360,100

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	5.618	BB	0.2626	2902.13477	146.84752	20.4566
2	6.842	BB	0.3785	32.25970	1.11187	0.2274
3	9.633	BB	0.2265	126.11169	7.88754	0.8889
4	18.040	BB	0.2348	8.91861	4.75640e-1	0.0629
5	21.372	BV	0.4994	259.79059	7.98850	1.8312
6	23.853	VB	0.5582	1.08380e4	293.91693	76.3951
7	27.235	BBA	0.8380	19.55586	2.91762e-1	0.1378
Totals :				1.41868e4	458.51976	

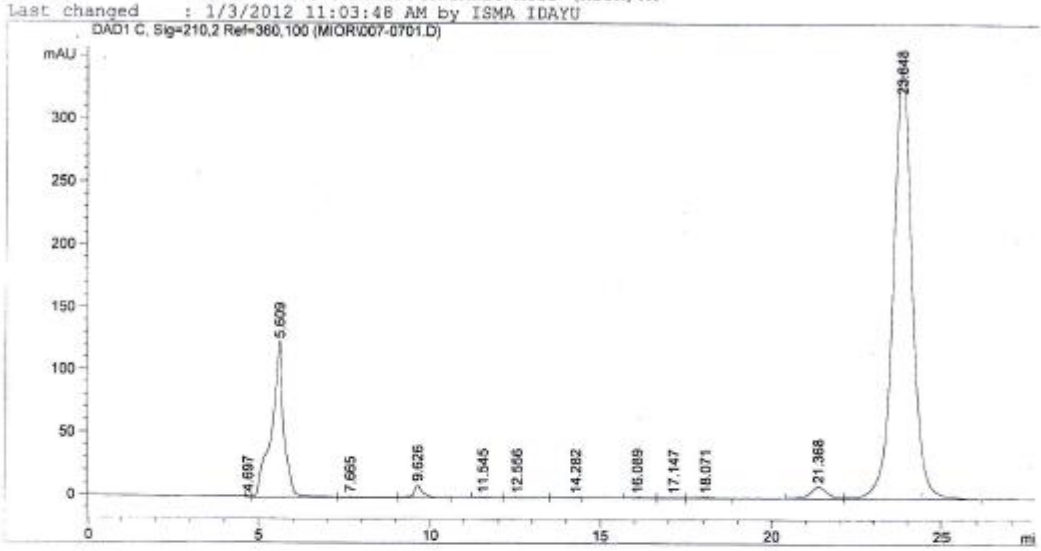
*** End of Report ***

APPENDIX A16

SAMPLE n = 9(a)

Data File C:\Chem32\1\DATA\MIOR\007-0701.D
Sample Name: J9,1

Injection Date	: 1/3/2012 2:58:37 PM	Seq. Line :	7
Sample Name	: J9,1	Location :	Vial 7
Acq. Operator	: MIOR	Inj :	1
Acq. Instrument	: Instrument 1	Inj Volume :	40 µl
Sequence File	: C:\CHEM32\1\SEQUENCE\KAMAL_PSM_03012012.S		
Method	: C:\CHEM32\1\METHODS\ORGANIC ACID (MIOR).M		
Last changed	: 1/3/2012 11:03:48 AM by ISMA IDAYU		



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1 C, Sig=210,2 Ref=360,100

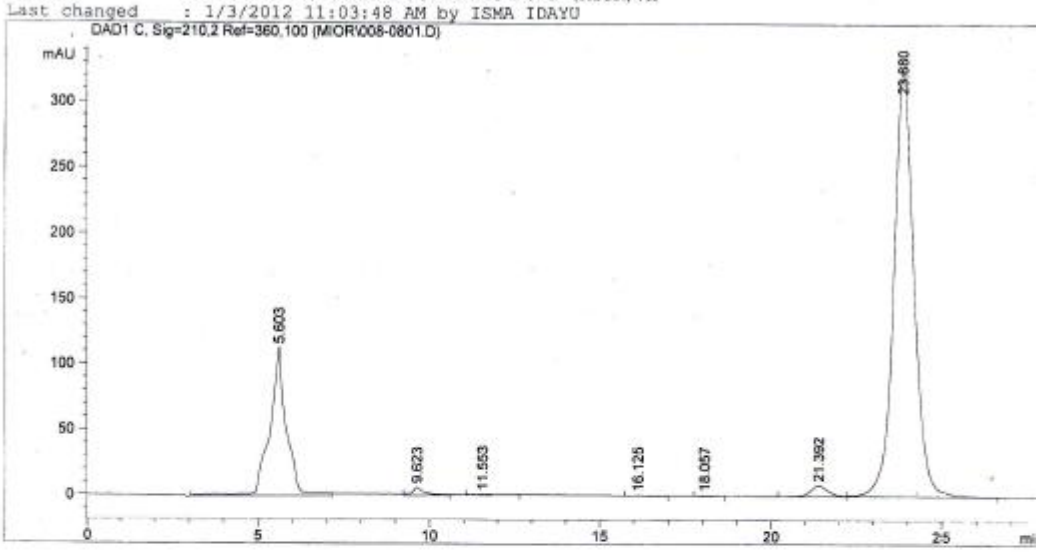
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	4.697	BV	0.1545	7.83283	8.45019e-1	0.0456
2	5.609	VB	0.3324	3093.43726	123.42622	18.0207
3	7.665	BV	0.9734	36.53359	4.60605e-1	0.2128
4	9.626	VB	0.2547	167.09950	9.43108	0.9734
5	11.545	BB	0.4270	11.97272	3.60927e-1	0.0697
6	12.556	BB	0.7876	12.58516	2.66331e-1	0.0733
7	14.282	BV	0.4523	11.08353	3.07664e-1	0.0646
8	16.089	BV	0.4140	14.67234	4.30134e-1	0.0855
9	17.147	VV	0.4388	9.38980	2.59071e-1	0.0547
10	18.071	VB	0.3957	21.29713	6.98458e-1	0.1241
11	21.368	BV	0.5276	325.74023	9.14320	1.8976
12	23.848	VB	0.5954	1.34544e4	341.71390	78.3779
Totals :				1.71660e4	487.34261	

APPENDIX A17

SAMPLE n = 9(b)

Data File C:\Chem32\1\DATA\MIOR\008-0801.D
Sample Name: J9,2

Injection Date	: 1/3/2012 3:30:47 PM	Seq. Line :	8
Sample Name	: J9,2	Location :	Vial 8
Acq. Operator	: MIOR	Inj :	1
Acq. Instrument	: Instrument 1	Inj Volume :	40 µl
Sequence File	: C:\CHEM32\1\SEQUENCE\KAMAL_PSM_03012012.S		
Method	: C:\CHEM32\1\METHODS\ORGANIC ACID (MIOR).M		
Last changed	: 1/3/2012 11:03:48 AM by ISMA IDAYU		



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1 C, Sig=210,2 Ref=360,100

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	5.603	BB	0.4310	3784.10718	112.90777	21.1246
2	9.623	BB	0.3314	151.30518	6.05757	0.8447
3	11.553	BB	0.6989	31.55549	5.45467e-1	0.1762
4	16.125	BB	0.4410	9.78648	2.73802e-1	0.0546
5	18.057	BB	0.3379	15.29067	5.83236e-1	0.0854
6	21.392	BV	0.5437	312.14169	8.43482	1.7425
7	23.880	VB	0.6315	1.36090e4	325.65778	75.9720
Totals :				1.79132e4	454.46044	

*** End of Report ***

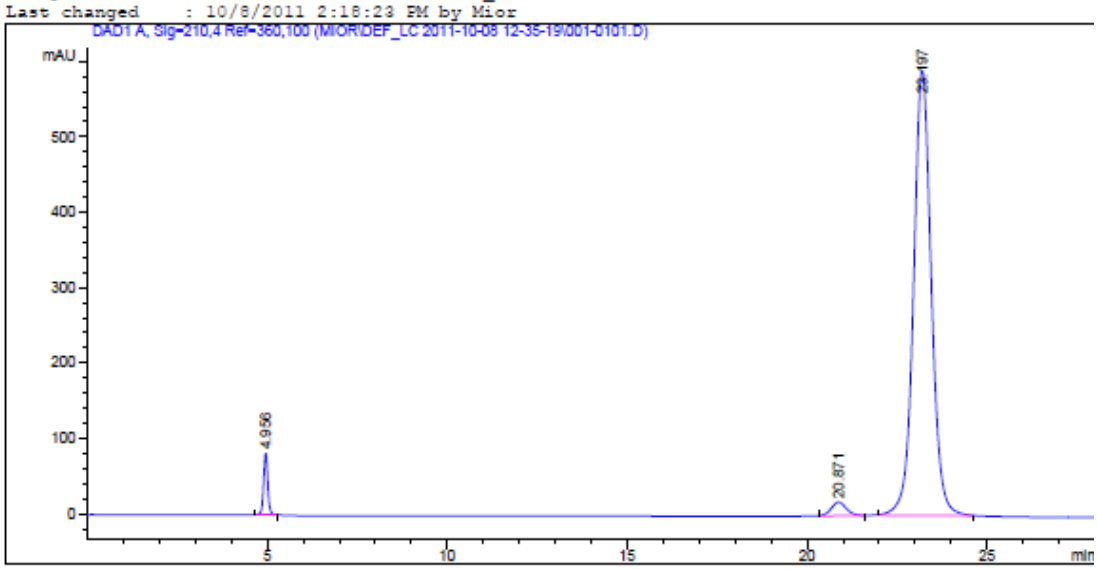
Instrument 1 1/3/2012 3:59:19 PM MIOR Page 1 of 1

APPENDIX A18

SAMPLE STANDARD A

Data File C:\CHEM32\1\DATA\MIOR\DEF_LC 2011-10-08 12-35-19\001-0101.D
Sample Name: Std PHB 1

```
=====================================================================
Acq. Operator    : Mior                          Seq. Line :   1
Acq. Instrument : Instrument 1                    Location : Vial 1
Injection Date  : 10/8/2011 12:37:20 PM              Inj :   1
                                                 Inj Volume : 20 µl
Acq. Method     : C:\CHEM32\1\DATA\MIOR\DEF_LC 2011-10-08 12-35-19\MIOR (ORGANIC ACID, AMINEX).M
Last changed    : 10/8/2011 12:35:12 PM by Mior
Analysis Method : C:\CHEM32\1\DATA\MIOR\DEF_LC 2011-10-08 12-35-19\MIOR (ORGANIC ACID, AMINEX).M
Last changed    : 10/8/2011 2:18:23 PM by Mior
```

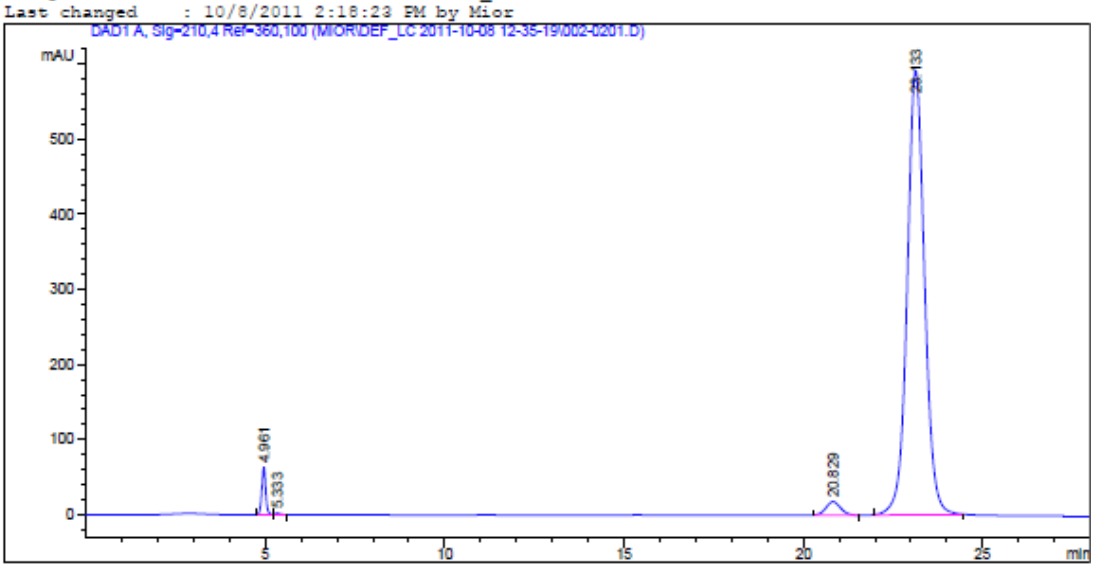



```
=====================================================================
                        Area Percent Report
=====================================================================

Sorted By             :      Signal
Multiplier:                   :      1.0000
Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=210,4 Ref=360,100

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   4.956 BB    0.1264  676.74475   81.91727   3.1648
   2  20.871 BB    0.4408  505.56754   17.72462   2.3643
   3  23.197 BB    0.5236 2.02012e4   589.79791  94.4709

Totals :                  2.13835e4   689.43980


=====================================================================
                        *** End of Report ***
```

APPENDIX A19

SAMPLE STANDARD B

```
Data File C:\CHEM32\1\DATA\MIOR\DEF_LC 2011-10-08 12-35-19\002-0201.D
Sample Name: Std PHB 2

    ======================================================================
    Acq. Operator    : Mior                          Seq. Line :   2
    Acq. Instrument : Instrument 1                    Location : Vial 2
    Injection Date  : 10/8/2011 1:09:37 PM               Inj :   1
                                                    Inj Volume : 20 µl
    Acq. Method     : C:\CHEM32\1\DATA\MIOR\DEF_LC 2011-10-08 12-35-19\MIOR (ORGANIC ACID, AMINEX).M
    Last changed    : 10/8/2011 12:35:12 PM by Mior
    Analysis Method : C:\CHEM32\1\DATA\MIOR\DEF_LC 2011-10-08 12-35-19\MIOR (ORGANIC ACID, AMINEX).M
    Last changed    : 10/8/2011 2:18:23 PM by Mior
```




```
    ======================================================================
                              Area Percent Report
    ======================================================================

    Sorted By             :      Signal
    Multiplier:                   :      1.0000
    Dilution:                     :      1.0000
    Use Multiplier & Dilution Factor with ISTDs


    Signal 1: DAD1 A, Sig=210,4 Ref=360,100

    Peak RetTime Type  Width     Area      Height     Area
      #   [min]        [min]   [mAU*s]     [mAU]       %
    ----|-------|----|-------|----------|----------|--------|
       1   4.961 BV    0.1173  486.14603   63.48924   2.3182
       2   5.333 VB    0.1613   24.23305    2.25723   0.1156
       3  20.829 BB    0.4413  512.82422   18.05989   2.4454
       4  23.133 BB    0.5153 1.99480e4   591.71289  95.1209

    Totals :                  2.09712e4   675.51925

    ======================================================================
                              *** End of Report ***


Instrument 1 10/8/2011 8:09:52 PM Mior                          Page   1 of 1
```