EFFECT OF SUBSTRATE CONCENTRATION AND pH ON SUCCINIC ACID PRODUCTION

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Date : 27 April 2010

EFFECT OF SUBSTRATE CONCENTRATION AND pH ON SUCCINIC ACID PRODUCTION

NIK NOR AZIATI BT ABD AZIZ

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

Faculty of Chemical & Natural Resources Engineering
Universiti Malaysia Pahang

APRIL 2010

I declare that this thesis entitled “Effect of substrate concentration and pH on succinic acid production” is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.”

Signature :……

Name : Nik Nor Aziati Abd Aziz

Date : 27 April 2010

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

For all your care, support and believe in me.

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ABSTRACT

The purpose of this research is to study the effect of substrate concentration and pH on succinic acid production. Succinic acid has been produced from the fermentation process by using *Escherichia coli b-strain*. The experiments were conducted in Modified *Schott* Bottle in incubator shaker to maintain anaerobic condition, temperature and agitation speed. Fermentation were carried out at the optimum temperature of *E.coli* growth which is 37^{O}C for 72 hours. The results of HPLC analysis showed that increase in fermentation time will increase succinic acid production. The maximum production of succinic acid is at pH 6.0. Initial substrate concentration (5 to 15 g/L) will increase Succinic acid production, but after 15 g/L substrate concentration, the succinic acid production will decrease. From this study, it was observed that the substrate concentration and pH are important factor that affects both growth and growth-associated productions of molecules to increased succinic acid production.

ABSTRAK

Kajian ini dilakukan bertujuan untuk mengkaji kesan kepekatan substrat dan pH terhadap penghasilan asid suksinik. Asid suksinik dihasilkan melalui proses fermentasi dengan menggunakan *Escherichia coli b-strain*. Eksperimen ini dilaksanakan di dalam *schott* botol yang telah diubah suai dan dijalankan di dalam incubator untuk mengekalkan keadaan anaerobik, suhu dan juga halaju adukkan. Fermentasi dilakukan pada suhu optimum bagi pertumbuhan *E.coli* iaitu 37^{o}C selama 72 jam. Keputusan eksperimen menunjukkan, peningkatan masa fermentasi akan meningkatkan penghasilan asid suksinik. Penghasilan asid suksinik maksimum adalah pada pH 6.0. Penghasilan asid suksinik meningkat pada kepekatan substrat 5 hingga 15 g/L dan berkurang penghasilannya selepas 15 g/L kepekatan substrat. Hasil daripada analisa menggunakan HPLC menunjukkan kepekatan substrat dan pH merupakan faktor penting dalam pertumbuhan *E coli* bagi penghasilan asid suksinik.

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ATP	= adenosine 5'-triphosphate
C	= carbon
Ca^{2+}	= ion calcium
g	= gram
hr	= hour
H_2O_2	= hydrogen peroxide
kDa	= kilo Dalton
ml	= mililitre
Mn^{2+} and Mn^{3+}	= ion manganese
N	= nitrogen
NAD^+	= nicotinamide adenine dinucleotide
OD	= optical density
S^+	= ion sulphur
µm	= micrometer
^oC	= degree Celcius

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Succinic acid, also known as amber acid or butanedioic acid, is a dicarboxylic acid having the molecular formula of $C_4H_6O_4$. After its first purification of succinic acid from amber by Georgius Agricola in 1546, it has been produced by microbial fermentation for the use in agricultural, food and pharmaceutical industries (Menzel *et al.*, 1999).

Glycerol is very competitive with sugars used in the production of chemicals and fuels via microbial fermentation. Glycerol give the highly reduced nature of carbon atoms in glycerol, additional advantages can be realized by using glycerol instead of sugars. Fermentative metabolism would then enable higher yield of fuels and reduced chemicals from glycerol compared with those obtained from common sugars such as glucose or xylose. Anaerobic fermentation is also preferred because of its lower capital and operational costs when compared to aerobic fermentations (anaerobic fermenters are less expensive to build and operate than their aerobic counterparts and also use less energy).

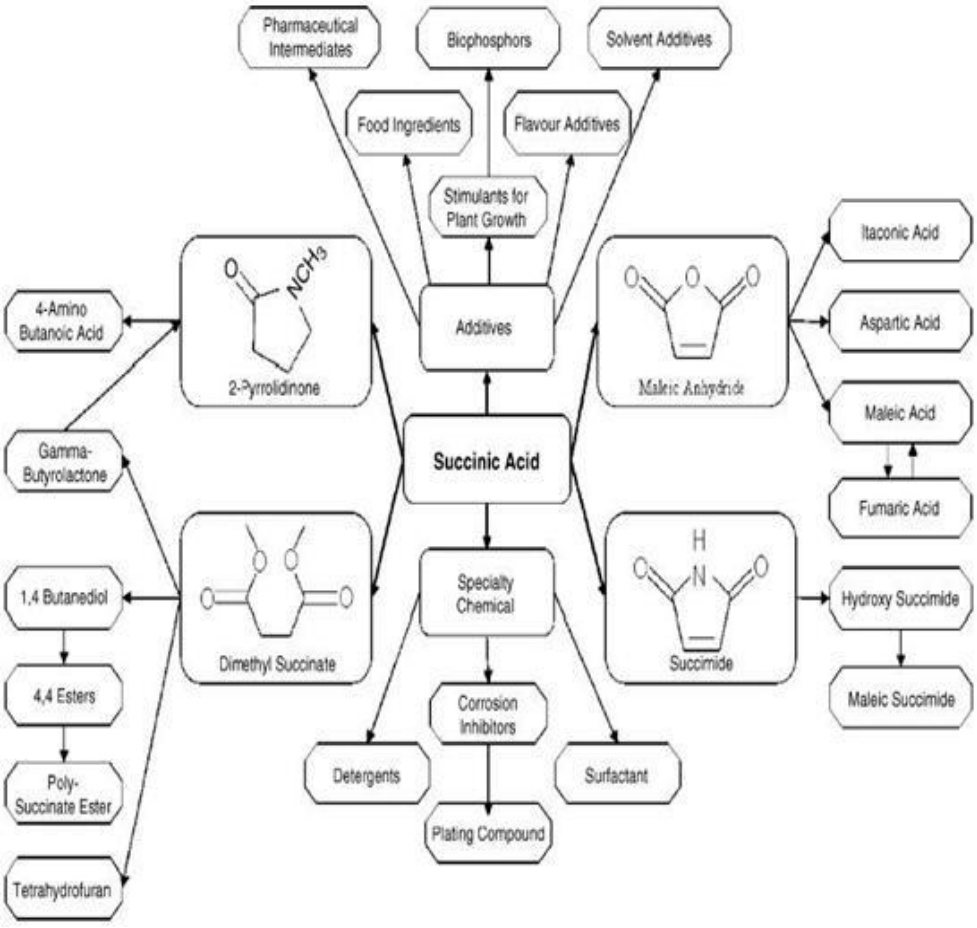

Figure 1.1: Various chemicals and products that can be synthesized from succinic acid

Furthermore, the increasing demand for succinic acid is expected as the uses of succinic acid is extended to the synthesis of glycerol. This is partially due to the high conversion cost of maleic anhydride to succinic acid by the chemical process, which limits the use of succinic acid for the wide range of applications. On the other hand, recent analysis showed that fermentative production of succinic acid from renewable resources can be more cost-effective than the petroleum-based processes (Jain *et al*., 1989).

This research study on the glycerol fermentation in order to produce succinic acid based on substrate concentration and pH. A series of batch fermentations with different initial substrate concentrations and pH were conducted in experiments and the experimental data were used to estimate parameters and also to validate the experiments. In this research also discuss the use of anaerobic fermentation processes

for the conversion of glycerol into higher value products. The experiments developed were able to successfully explain the behavior of succinic acid production with times and the fermentative characteristics of succinic acid production from glycerol fermentation process were discussed in detail. The succinic acid produced is measured by using HPLC.

1.2 Problem Statement

Production cost is a major problem for producing succinic acid in order to achieve market acceptance, secure off-take agreements and ensure that plants are operating close to capacity. Production cost of succinic acids by chemical plants is higher compare with production of fermentation process by microorganism. Second problem for chemical plants is large number of unit operations. There are a large number of unit operations such as separation processes, including extraction, absorption, membrane filtration and distillation column. Chemical plants also produce high energy (electricity) because chemical plants are designed on a large scale for continuous operation and provide most of the electrical energy used. Chemical plants highly complex because of multiple generating units. Chemical plants produce toxicity and gives effect to the environment pollution and global warming. The combustion of the plants is discharged to the air and this contains carbon dioxide and water vapor, as well as other substances such as nitrogen, nitrogen oxides, and sulfur oxides. The impact is acid rain and air pollution, and has been connected with global warming. Acid rain is caused by the emission of nitrogen oxides and sulfur dioxide into the air.

1.3 Objectives

1. To study the effect of substrate concentration and pH on succinic acid production.

1.4 Scope of Study

1. To study the effect of substrate concentration at ranges 5 to 25 g/L during glycerol fermentation.
2. To study the effect of pH at ranges 5.5 to 7.5 using sulfuric acids (H_2SO_4) and natrium Carbonat (Na_2CO_3) as a buffer during fermentation process.
3. To determine the amount of succinic acids by using HPLC.

1.5 Rationale and Significance

The microorganism's metabolic pathways are being genetically engineered so they are capable of converting different types of sugars very efficiently. The microorganism can culture in a large amount so the increase the amount of succinic acid production and the impact is expands markets for domestic agriculture and biomass crops. The production of succinic acid through fermentation processes based on sugar feedstocks has the potential to considerably reduce the chemical industry's dependence on fossil-based feedstocks, energy intensive and expensive processes and capital intensive plants. Fermentation process produce less temperature compare with chemical plant with high temperature, high pressure of the equipments. In addition, the possibilities of cost effective succinic acid production by fermentation process is reduce energy or saving the energy. The energy savings from a single combined biological and chemical plant producing chemical components could heat thousands homes for a year. Fermentation process gives the impact of environmental friendly and green friendly because carbon dioxide (CO_2) is consumed during the fermentation process. Fermentation-based production processes and technology are built around microorganisms engineered to produce such chemical products, reducing feedstock, production, plant cost and improving profitability.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Succinic acid, an important four-carbon platform chemical, is mostly being produced by chemical processes using liquefied petroleum gas or petroleum oil as a starting material. Succinic acid produced by various microorganisms can be used as a precursor of many industrially important chemicals in food, chemical and pharmaceutical industries. By using glycerol as a carbon sources the succinic acid can produced with much less by-product formation.

2.2 Background of succinic acid

Succinic Acid is another name for Amber Acid that has been used in Europe as a natural antibiotic and general curative for centuries. Succinic acid a powerful anti oxidant that helps fight toxic free radicals and disruptions of the cardiac rhythm, succinic acid has been shown to stimulate neural system recovery and bolster the immune system, and helps compensate for energy drain in the body and brain, boosting awareness, concentration and reflexes, and reducing stress (Jain *et al*.,1989).

Succinic acid has been reported to be produced and accumulated by anaerobic microorganisms as the major product of their metabolism. Thus, attempts are being

made worldwide to screen anaerobic microorganisms for succinic acid production (Zeikus *et al*., 1999).

Succinic Acid (Butanedioic Acid) is a dicarboxylic acid of four carbon atoms. It occurs naturally in plant and animal tissues. It plays a significant role in intermediary metabolism (Krebs cycle) in the body. Krebs cycle (also called citric acid cycle; tricarboxylic acid cycle) is a sequence process of enzymatic reaction which a two-carbon acetyl unit is oxidized to carbon dioxide and water to provide energy in the form of high-energy phosphate bonds. Succinic acid is a colourless crystalline solid with a melting point of 185 to 87 ^{o}C, soluble in water, slightly dissolved in ethanol, ether, acetone and glycerine, not dissolved in benzene, carbon sulfide, carbon tetrachloride and oil ether.

The common method of synthesis of succinic acid is the catalytic hydrogenation of maleic acid or its anhydride. Carboxylic acid can yield acyl halides, anhydrides, esters, amides, and nitriles for the application of drug, agriculture, and food products, and other industrial uses (Zeikus *et al*., 1999).

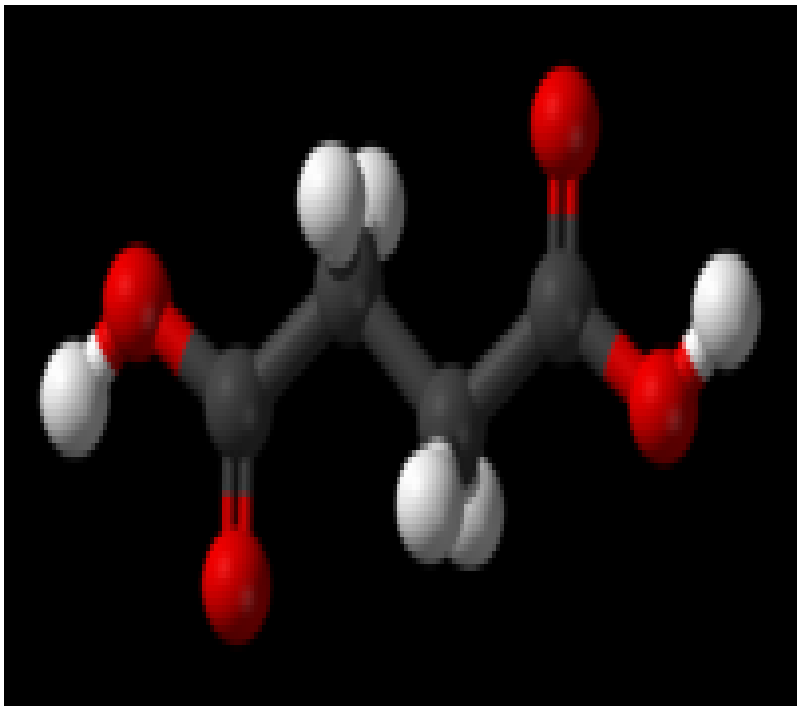

Figure 2.1: Molecule structure of succinic acid

2.3 Production of Succinic acid

2.3.1 Synthesis Process

Currently, the acid is produced commercially through chemical synthesis involving hydrolysis of petroleum products, which is associated with certain environmental hazards.

Maleic acid is converted into maleic anhydride by dehydration, to malic acid by hydration, and succinic acid by hydrogenation (ethanol / Palladium on carbon. It reacts with thionyl chloride or phosphorus pentachloride to give the maleic acid chloride (it is not possible to isolate the mono acid chloride).

Hydrogenation is the chemical reaction that results from the addition of hydrogen (H_2). The process is usually employed to reduce or saturate organic compounds. The process typically constitutes the addition of pairs of hydrogen atoms to a molecule. Catalysts are required for the reaction to be usable, non-catalytic hydrogenation takes place only at very high temperatures. Hydrogen adds to double and triple bonds in hydrocarbon (Podkovyrov and Zeikus, 1993).

Because of the importance of hydrogen, many related reactions have been developed for its use. Most hydrogenations use gaseous hydrogen (H_2), but some involve the alternative sources of hydrogen, not H_2. These processes are called transfer hydrogenations. The reverse reaction, removal of hydrogen from a molecule, is called dehydrogenation. A reaction where bonds are broken while hydrogen is added is called hydrogenolysis, a reaction that may occur to carbon-carbon and carbon-heteroatom (O, N, X) bonds. Hydrogenation differs from protonation or hydride addition: in hydrogenation, the products have the same charge as the reactants. Hydrogenation reaction is the addition of hydrogen to maleic acid to succinic acid.

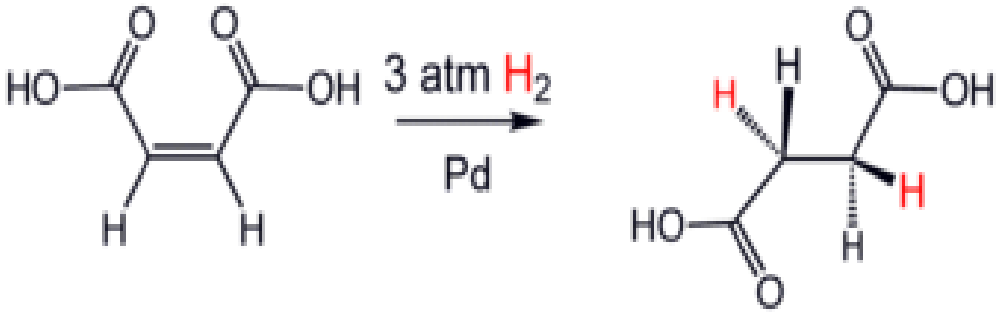

Figure 2.2: The structure for hydrogenation process

2.3.2 Fermentation Process

Fermentation, process by which the living cell is able to obtain energy through the breakdown of glucose and other simple sugar molecules without requiring oxygen. Fermentation is achieved by somewhat different chemical sequences in different species of organisms. Two closely related paths of fermentation predominate for glucose. When muscle tissue receives sufficient oxygen supply, it fully metabolizes its fuel glucose to water and carbon dioxide. However, at times of strenuous activity, muscle tissue uses oxygen faster than the blood can supply it.

During this anaerobic condition, the six-carbon glucose molecule is only partly broken down to two molecules of the three-carbon sugar called lactic acid. This process, called lactic acid fermentation, also occurs in many microorganisms and in the cells of most higher animals. In alcoholic fermentation, such as occurs in brewer's yeast and some bacteria, the production of lactic acid is bypassed, and the glucose molecule is degraded to two molecules of the two-carbon alcohol, ethanol, and to two molecules of carbon dioxide. Many of the enzymes of lactic acid and alcoholic fermentation are identical to the enzymes that bring about the metabolic conversion known as glycolysis. Alcoholic fermentation is a process that was known to antiquity.

Therefore, much attention has been focused in the past few years on fermentative production of succinic acid by anaerobic or facultative anaerobic microorganisms. This is because it is a common intermediate in the metabolic pathway of several anaerobic and facultative anaerobic microorganisms (Chotani *et al.*, 2000). This fermentation process for the production of succinic acid can be regarded as a "green technology" not only because renewable substrates are used for its production but also because CO_2, a green house gas is fixed during microbial production of succinic acid (Lee *et al*., 2000).

2.4 Factors effect of Fermentation

Fermentation is affected by several factors including the temperature, salt concentration, pH, oxygen availability and nutrient availability. The rate of fermentation can be controlled by manipulating any of these factors (Hong *et al*., 2003).

Different bacteria tolerate different temperatures. Most have an optimum of between 20 to 30°C although some prefer higher temperatures (50 to 55°C) and others colder (15 to 20°C). Most lactic acid bacteria work best at temperatures of 18 to 22°C. The *Leuconostoc* species which initiate fermentation have an optimum of 18 to 22°C. The *Lactobacillus* species have temperature optima above 22°C. The optimum temperature for pickle fermentation is around 21°C. A variation of just a few degrees from this temperature alters the activity of the microbes and affects the quality of the final product.

Lactic acid bacteria tolerate high salt concentrations, which gives them an advantage over other less tolerant species. This allows the lactic acid fermenters to begin metabolism, which produces acid, which then further inhibits the growth of non-desirable organisms. *Leuconostoc* species tolerate high salt concentrations, which makes them ideal to start the lactic acid fermentation.

Salt plays an important role in initiating the fermentation and also in the quality of the product. The addition of too much salt may inhibit the desirable bacteria and also affect the hardness of the product. The principle function of salt is to withdraw juice from the vegetables and make a favourable environment for fermentation. Salt is generally added to give a final concentration of 2.0 to 2.5%. At this concentration the *Lactobacilli* are slightly inhibited but the *Leuconostoc* are not affected.

The optimum pH for most bacteria is near the neutral point (pH 7.0). Certain bacteria are acid tolerant and will survive at reduced pH levels. Both *Lactobacillus* and *Streptococcus* species are acid tolerant. Some of the fermenters are anaerobes while others require oxygen. Some of the *lactobacilli* are microaerophilic which means they grow in the presence of reduced amounts of oxygen (Samuelov *et al*., 1991).

All bacteria require a source of nutrients for metabolism. The fermenters require carbohydrates, either simple sugars such as glucose and fructose or complex ones such as starch or cellulose. The energy requirements of microbes are very high. Limiting the amount of substrate available can reduce the rate of fermentation.

2.5 Raw material for Glycerol Fermentation

2.51 Glycerol

Glycerol is an organic compound, also called glycerin or glycerine. It is a colorless, odorless, viscous liquid that is widely used in pharmaceutical formulations. Glycerol has three hydrophilic hydroxyl groups that are responsible for its solubility in water and its hygroscopic nature. The glycerol substructure is a central component of many lipids. Glycerol is sweet-tasting and of low toxicity.

Glycerol is an attractive carbon substrate for biological conversion because it is available from renewable resources in large amounts and can be utilized by number of microorganism. Glycerols are produced as a surplus by-product in the growing oleochemical industries for the production soaps, fatty acids, waxes, and surfactants. Therefore, several environmentally friendly processes based on microbial fermentation fermentation have been proposed for glycerol utilization. Some useful chemicals have been produced from glycerol fermentation (Lee *et al*., 2001).

The simplest trihydric alcohol, with the formula $CH_2OHCHOHCH_2OH$. It is widely distributed in nature in the form of its esters, called glycerides. The glycerides are the principal constituents of the class of natural products known as fats and oils. It is completely soluble in water and alcohol but is only slightly soluble in many common solvents, such as ether, ethyl acetate, and dioxane. Glycerin is insoluble in hydrocarbons. It boils at 290°C (554°F) at atmospheric pressure and melts at 17.9°C. Its specific gravity is 1.262 at 25°C (77°F) referred to water at 25°C, and its molecular weight is 92.09. It has a very low mammalian toxicity.

Glycerin is used in nearly every industry. With dibasic acids, such as phthalic acid, it reacts to make the important class of products known as alkyd resins, which are widely used as coating and in paints. It is used in innumerable pharmaceutical and cosmetic preparations; it is an ingredient of many tinctures, elixirs, cough medicines, and anesthetics; and it is a basic medium for toothpaste.

In foods, it is an important moistening agent for baked goods and is added to candies and icings to prevent crystallization. It is used as a solvent and carrier for extracts and flavoring agents and as a solvent for food colors. Many specialized lubrication problems have been solved by using glycerin or glycerin mixtures. Many millions of pounds are used each year to plasticize various materials (Podkovyrov and Zeikus, 1993).

2.52 Yeast

Yeasts are eukaryotic organisms that exist in nature predominantly as single cells. Traditionally, they have played an important role for man, being used for thousands of years in bread-making, brewing, and making certain foods palatable and nutritious. The commercial importance of yeast has grown considerably over the pas few decades, and they are now being used in a variety of fermentative processes for the synthesis of simple sugars and ammonium nitrogen as well as certain fats, vitamins, and proteins. Although there are about 350 recognized species of yeast, only a relatively small number are important commercially, notably members of the genera *saccharomyces* and *candida*.

Species of *saccharomyces*, such as *S.cerevisiae* and *S.uvarum* (which is sometimes referred to as *S.carlsbergensis*) are used in the manufacture of breads, wines and beers, while some species of Candida are sources of animal food and fodder. One species in particular, *S.cerevisiae*, has received and enormous amount of attention from generations of biologists. This is not so much due to its commercial importance, but rather because it is amenable to most of the cultural techniques and genetic manipulations used with laboratory bacteria. It has been extremely well-characterized genetically and defined mutations in most biochemical pathways have been established.

The genetic engineering of yeast using recombinant DNA techniques has been made possible by the development, recently, of a transformation system and the construction of DNA cloning vectors which mediate the introduction of DNA into yeast cells. These technological advances, combined with classical genetic studies, have provided a wealth of knowledge of yeast molecular biology. The ability to introduce individual genes into yeast and apply selection by complementation to identify a clone carrying a particular gene now permits the isolation of virtually any yeast gene provided that a suitable recipient yeast strain is available. Furthermore, gene cloning in yeast is likely to be of great industrial importance, not only for improvement of existing strains used in fermentation processes, but also for the bulk

production of commercially important proteins not selectable in or compatible with bacterial systems (Swarna and Rao.,1993)

2.53 Trypton

Tryptone is the assortment of peptides formed by the digestion of casein by the protease trypsin. Tryptone is commonly used in microbiology to produce Lysogeny broth for the growth of *E. coli* and other microorganisms. It provides a source of amino acids for the growing bacteria. Tryptone is similar to casamino acids, both being digests of casein, but casamino acids can be produced by acid hydrolysis and typically only have free amino acids and few peptide chains

2.6 Anaerobic Fermentation

Under anaerobic conditions, the pyruvate molecule can follow other anaerobic pathways to regenerate the NAD^+ necessary for glycolysis to continue. These include alcoholic fermentation and lactate fermentation. In the absence of oxygen the further reduction or addition of hydrogen ions and electrons to the pyruvate molecules that were produced during glycolysis is termed fermentation.

This process recycles the reduced NADH to the free NAD^+ coenzyme which once again serves as the hydrogen acceptor enabling glycolysis to continue. Alcoholic fermentation, characteristic of some plants and many microorganisms, yields alcohol and carbon dioxide as its products. Yeast is used by the biotechnology industries to generate carbon dioxide gas necessary for bread-making and in the fermentation of hops and grapes to produce alcoholic beverages. Depending on the yeast variety used, the different alcohol levels realized act as a form of population control by serving as the toxic element which kills the producers. Birds have been

noted to fly erratically after they have gorged themselves on the fermenting fruit of the Pyracantha shrub (Macy *et al*., 1978).

Reduction of pyruvate by NADH to release the NAD^+ necessary for the glycolytic pathway can also result in lactate fermentation, which takes place in some animal tissues and in some microorganisms. Lactic acid-producing bacterial cells are responsible for the souring of milk and production of yogurt. In working animal muscle cells, lactate fermentation follows the exhaustion of the ATP stores. Fast twitch muscle fibers store little energy and rely on quick spurts of anaerobic activity, but the lactic acid that accumulates within the cells eventually leads to muscle fatigue and cramp (Hong *et al*., 2003).

Many anaerobic and facultative anaerobic microorganisms ferment carbohydrates to a mixture of acids, e.g. formate, acetate, lactate and succinate as end products. Phosphoenol pyruvate (PEP) is one of the central intermediates during the mixed acid fermentation. It is either converted into pyruvate resulting in the formation of the fermentation products acetate, formate, ethanol and lactate or it is converted into oxaloacetate resulting in the formation of end products succinate and propionate via the reversible arm of tricarboxylic acid (TCA) cycle (Clark, 1989).

Under anoxic conditions, the flux of PEP towards either oxaloacetate or puruvate is affected by environmental factors such as pH, temperature, hydrogen, carbon dioxide and nutritional factors as carbon, nitrogen sources and metal ions of the growth medium.

2.7 Microorganism

2.7.1 *Escherichia-coli*

Escherichia coli are a Gram negative, rod-shaped, bacterium that is commonly found in the lower intestine of warm-blooded organisms (endotherms). Most *E. coli* strains are harmless, but some, such as serotype O157:H7, can cause serious food poisoning in humans, and are occasionally responsible for product recalls. The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K_2, and by preventing the establishment of pathogenic bacteria within the intestine.

E. coli are not always confined to the intestine, and their ability to survive for brief periods outside the body makes them an ideal indicator organism to test environmental samples for fecal contamination. The bacteria can also be grown easily and its genetics are comparatively simple and easily-manipulated or duplicated through a process of metagenics, making it one of the best-studied prokaryotic model organisms, and an important species in biotechnology and microbiology (Clark, 1989).

A wild type *E. coli* primarily ferments glucose to ethanol, formic, acetic and lactic acids with only detectable amounts of succinic acid under anaerobic condition. It has been known that *E. coli* utilizes six pathways to form succinic acid, and differently from three bacteria mentioned above, the PEP carboxykinase plays a minor role (Clark ,1989). Nevertheless, metabolic flux analysis showed that the maximum achievable succinic acid molar yield in *E. coli* (Hong *et al*, 2003).

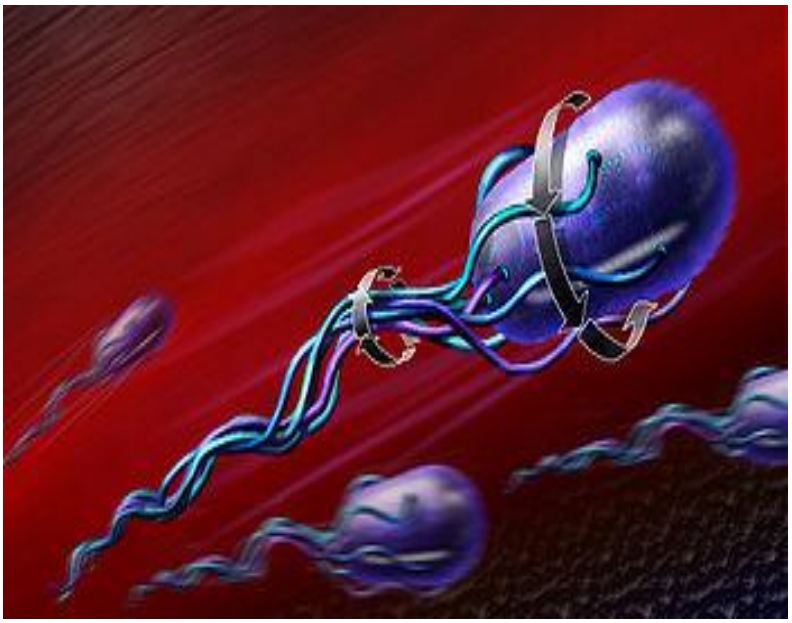

Figure 2.3: *Escherichia coli* cells propel themselves with flagella

2.7.2 Nutrition and metabolism in Bacteria

Depending on the mode nutrition, bacteria can be grouped as autotrophs or heterotrophs, majority of, which are heterotrophs, which are either parasites or saprophytes. Parasites either obligate ones, who depend entirely on living host for organic food, or they may be powerful who sometimes get food, or they may be powerful who sometimes get food from non-living organic source. On the other hand, saprophytes get nutrition from decaying organic matter by putrefaction and fermentation. The usual saprophytic bacteria responsible for various types of separation of elements in nature are rather mapping in their nutritional requirements and get nourishment from number of organic substances, namely, carbohydrates, proteins, fats and amino acids and for types of metabolism they are supplied with a number of extracellar and intracellular enzymes (Menzel *et al*.,1999).

At an elementary level, the nutritional requirements of a bacterium such as *E. coli* are revealed by the cell's elemental composition, which consists of C, H, O, N, S. P, K, Mg, Fe, Ca, Mn, and traces of Zn, Co, Cu, and Mo. These elements are

found in the form of water, inorganic ions, small molecules, and macromolecules which serve either a structural or functional role in the cells.

Table 2.1: Major elements, their sources and functions in bacterial cells.

Element	% of dry weight	Source	Function
Carbon	50	organic compounds or CO_2	Main constituent of cellular material
Oxygen	20	H_2O, organic compounds, CO_2, and O_2	Constituent of cell material and cell water; O_2 is electron acceptor in aerobic respiration
Nitrogen	14	NH_3, NO_3, organic compounds, N_2	Constituent of amino acids
Hydrogen	8	H_2O, organic compounds, H_2	Main constituent of organic compounds and cell water
Phosphorus	3	inorganic phosphates (PO_4)	Constituent of nucleic acids, nucleotides, phospholipids, LPS, teichoic acids
Sulfur	1	SO_4, H_2S, S^o, organic sulfur compounds	Constituent of cysteine, methionine, glutathione, several coenzymes
Potassium	1	Potassium salts	Main cellular inorganic cation and cofactor for certain enzymes
Magnesium	0.5	Magnesium salts	Inorganic cellular cation, cofactor for certain enzymatic reactions
Calcium	0.5	Calcium salts	Inorganic cellular cation, cofactor for certain enzymes and a component of endospores

Iron	0.2	Iron salts	Component of cytochromes and certain nonheme iron-proteins and a cofactor for some enzymatic reactions

2.7.3 Phases of growth

The growth of population follow fours phases when a sample bacteria is transferred from one to another and the growth curve takes an S-form and declines. The schematic growth curve shown below is associated with simplistic conditions known as a batch culture. It refers to a single bacterial culture, introduced into and growing in a fixed volume with a fixed (limited) amount of nutrient. Industrial situations involving MIC tend to be much more complex in nature than such a simplified model.

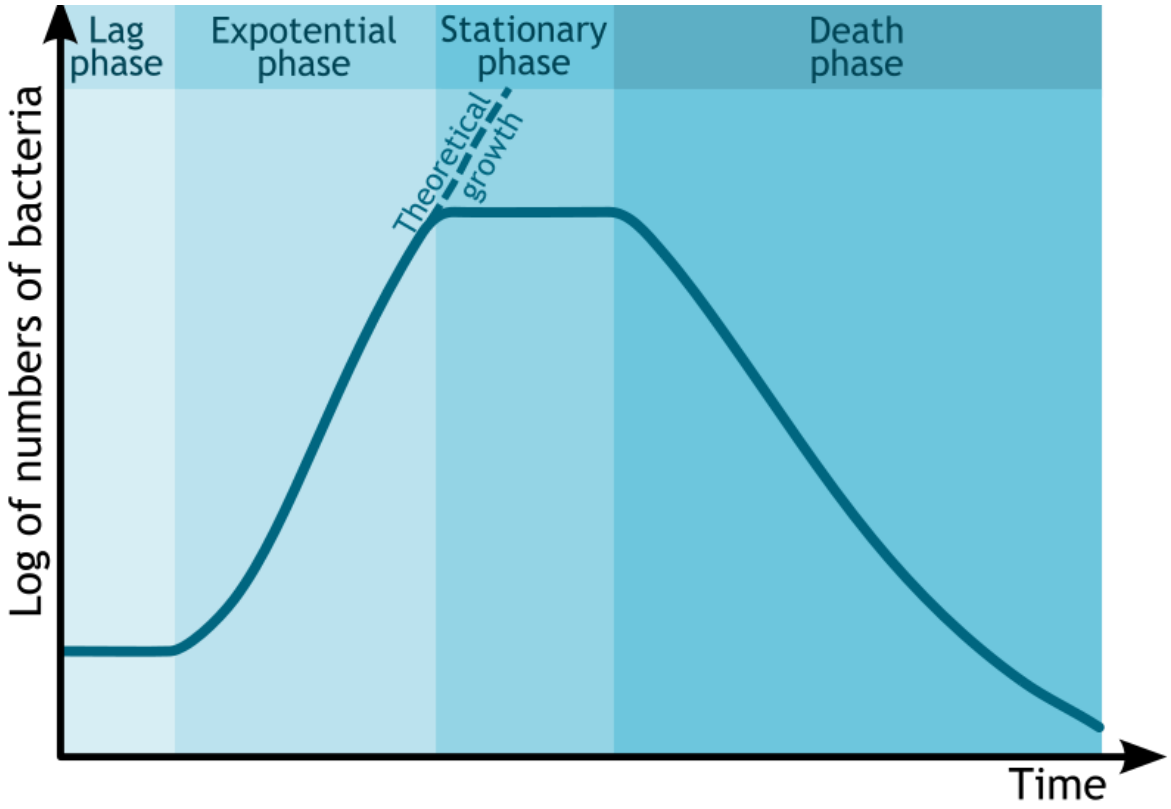

Figure 2.4: Showed the profile growth of the bacteria

2.7.3.1 Log phase

When population get a new climate at initial is followed by active growth phase follow rapid divisions called log phase or exponential phase. Then growth gradually comes down either by the use-up of nutrients of toxic metabolic products or some other growth limited factors and finally stops and this period is static and termed stationary phase, and lead the cells die and leads to loss of capacity to reproduce, however death rate differ with organisms and the environment factors. Some bacteria die and consequently few viable cells remain after 72 hours such as, *Bacillus* species, while enteric species die very slowly and may continue for months.

2.7.3.2 Exponential Growth Phase

Exponential phase (sometimes called the log phase) is a period characterized by cell doubling. The living bacteria population increases rapidly with time at an exponential growth in numbers, and the growth rate increasing with time. Conditions are optimal for growth.

The number of new bacteria appearing per unit time is proportional to the present population. If growth is not limited, doubling will continue at a constant rate so both the number of cells and the rate of population increase doubles with each consecutive time period. For this type of exponential growth, plotting the natural logarithm of cell number against time produces a straight line. The slope of this line is the specific growth rate of the organism, which is a measure of the number of divisions per cell per unit time.

The actual rate of this growth (the slope of the line in the figure) depends upon the growth conditions, which affect the frequency of cell division events and the probability of both daughter cells surviving. Exponential growth cannot continue indefinitely, however, because the medium is soon depleted of nutrients and enriched with wastes.

2.7.3.3 Stationary Phase

The third phase of bacterial cell growth is called the "stationary phase". With the exhaustion of nutrients and build-up of waste and secondary metabolic products, the growth rate has slowed to the point where the growth rate equals the death rate. Effectively, there is no net growth in the bacteria population. As resources are used, the rate of cell death begins to match the rate of cell division. Thus, the entire colony slows its growth.

The stationary phase is often referred to as a being in a state of equilibrium. This simply means that the colony of bacteria is not getting any bigger or smaller, it is simply living. In addition to limited resources, the build-up of bacterial waste products can limit the growth of a bacterial colony.

2.7.3.4 Death Phase

The living bacteria population decreases with time, due to a lack of nutrients and toxic metabolic by-products.

2.7.4 Measurement of growth

Bacterial growth can be quantified either by cell mass or cell number. Growth is an orderly increase in the quantity of cellular constituents. It depends upon the ability of the cell to form new protoplasm from nutrients available in the environment. In most bacteria, growth involves increase in cell mass and number of ribosomes, duplication of the bacterial chromosome, synthesis of new cell wall and plasma membrane, partitioning of the two chromosomes, septum formation, and cell division. This asexual process of reproduction is called binary fission.

2.7.4.1 Cell mass

Cell mass can be done directly weighing the biomass through the determination of turbidity by optical means. The turbidity in water sample due to bacterial growth is got through turbidometer where photoelectric measurement in based on the decrease in light transmission due to increase in assumed that increase in OD is related to increase in cell number, other words, OD is directly proportional to bacterial cell density, otherwise transmission of light is affected and give wrong data.

2.7.4.2 Cell number

Bacterial cell is measured by a particular amount of water sample by diluting it and directly count cells either under a microscope or through electrical particle counter (coulter counter) which finds the presence of particle causing electrical in store and displays on a digital reading where water should be free of any other debris. Cell estimation can be done by culturing a drop bacterial suspension in agar plates hatched for a fixed time and at fixed temperature and finally count the number of colonies developed on plate(colony count), where in each cell is supposed to grow into a colony after a certain period. The number of colonies is multiplied by a dilution factor. Estimation could include total number of cells living or dead, which accounts for living cell only.

2.7.5 Factor affecting bacterial growth

Variety of factors such as the nutrients, temperature, pH, oxidation reduction potential and moisture content. For example, each organism has an optimum pH for growth and for the high temperature bacteria can denatured.

2.7.5.1 Nutrient availability

Growth of bacterial population is influenced by type available nutrient like vitamins, amino acids and their proper way through the cell membrane. Nutrient which deficient mutated in a bacterial species, where particular nutrient has to be supplement for proper growth.

Nutrients in food, their kind and proportions determine the type of organism that will grow. Also, microorganisms vary in their ability to use nutrients. The presence of easily utilizable nutrients will encourage faster growth and quicker damage. For example, a food with easily utilizable sugars will allow better growth than one which contains polysaccharides. Also, the concentration of the sugars will determine the type and extent of growth, since it affects both the osmotic pressure. Generally, yeasts and molds are more resistant to high concentrations of sugar than bacteria (Clark, 1989).

Most foods contain enough peptides and amino acids that they can meet the nitrogen requirement of most organisms found in foods. Some organisms are also proteolytic and can grow on proteins found in the food. The mineral requirement of microorganisms is generally met by the food and this is not a limiting factor. Some foods may contain antibacterial substances which may prevent bacterial growth and food spoilage. For example, egg is rich in lysozyme and thus, even if the bacteria cross the outer shell (If the egg, they are destroyed by the lysozyme before they can cause any damage.

2.7.5.2 Temperature

Bacteria have varying requirements in terms of the range of temperatures in which they will grow. Those which grow at low temperatures (usually below 20°C) are called *psychrophiles* and at high temperatures (above 45°C) are thermophiles. Some spoilage bacteria fall into these categories.

Most pathogens, however, like warmth and are known as *mesophiles*. They will grow at temperatures between 5°C and 63°C, commonly referred to as the growth or 'danger' zone and have an optimum temperature for growth of about 37°C. *Listeria* bacteria will grow very slowly below 5°C, but most pathogens become inactive (dormant) at low temperatures. They start to multiply more rapidly as the temperature rises.

At a temperature of about 37°C (human body temperature) pathogens multiply most quickly but as the temperature continues to rise, their rate slows down and they will stop growing altogether above 63°C. However, in order to destroy bacteria, temperatures must rise further. A temperature of 70°C for 2 minutes is recommended as a means of killing pathogens during the normal cooking process.

2.7.5.3 pH

Most yeasts favour a pH around 4 to 4.5 while fungi can tolerate a pH much below, that. Most bacteria favour a pH around 7.0. Thus, both the growth as well as their survival in foods depends on the pH of the food material. The pH of foods varies; some may be neutral while others may be acidic. Acid foods (pH below 4.5) are not readily spoiled by bacteria but are susceptible to spoilage by yeast and molds. Foods may have a low pH either because of inherent acidity as in fruits and soft drinks or develop acidity as a result of microbial activity as in lactic acid fermentation (Latha and Rao, 1991)

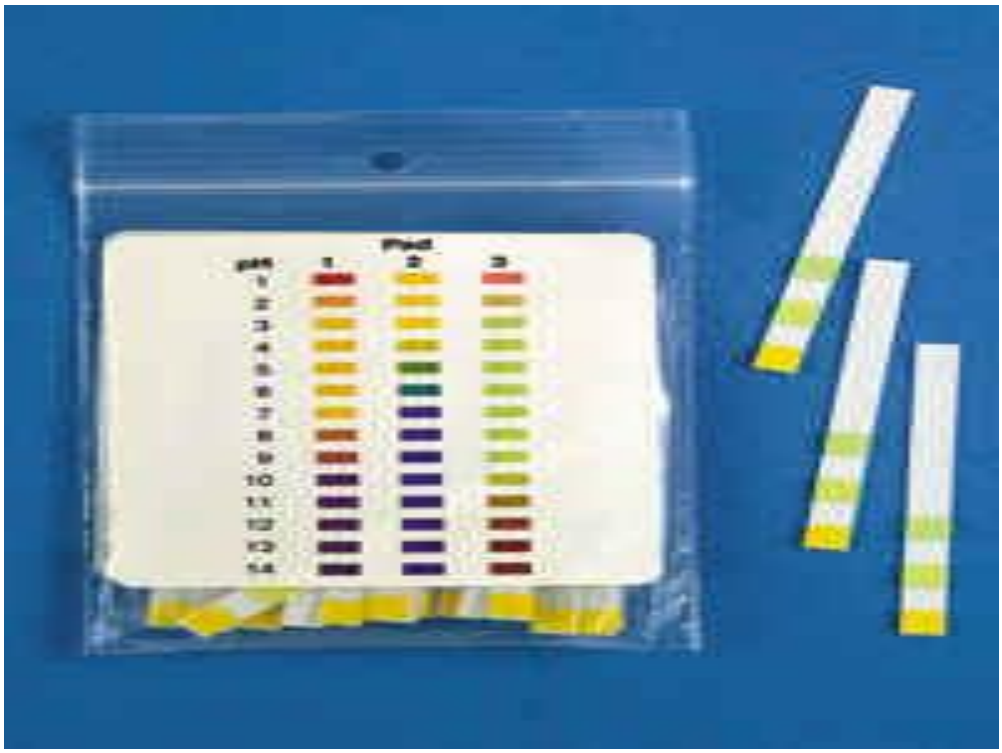

Figure 2.5: pH meter for measurement the pH

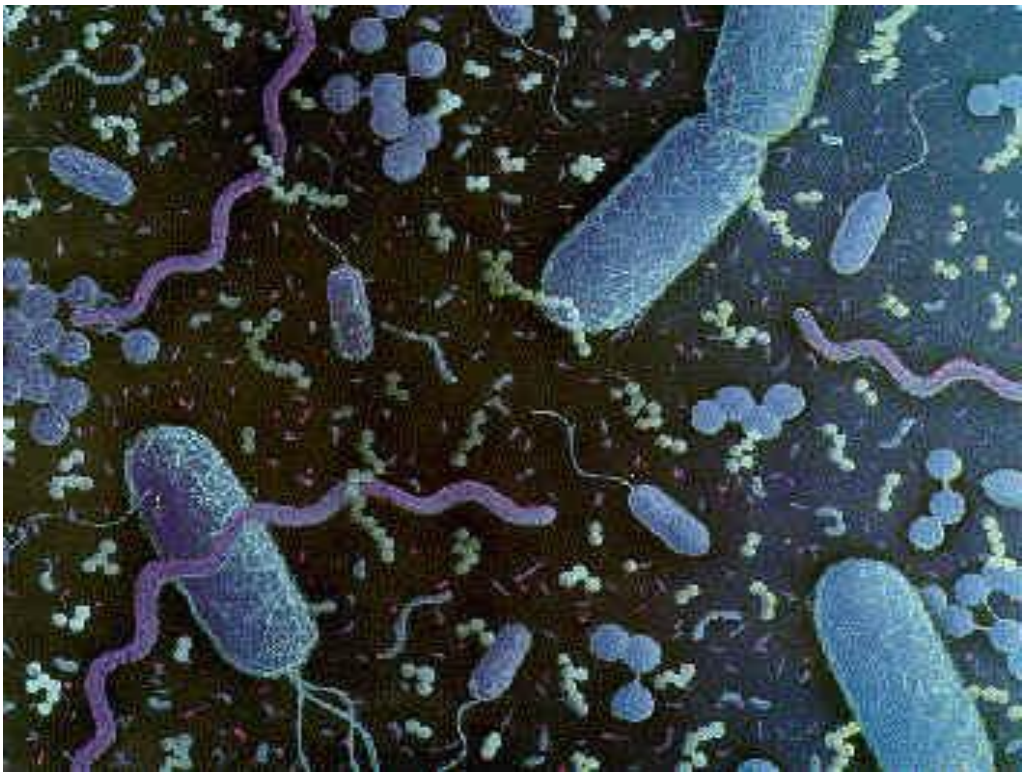

Figure 2.6: pH can have large effects on the growth rate of the organism

Table 2.2: Minimum, maximum and optimum pH for growth of certain procaryotes.

Organism	Minimum pH	Optimum pH	Maximum pH
Thiobacillus thiooxidans	0.5	2.0-2.8	4.0-6.0
Sulfolobus acidocaldarius	1.0	2.0-3.0	5.0
Bacillus acidocaldarius	2.0	4.0	6.0
Zymomonas lindneri	3.5	5.5-6.0	7.5
Lactobacillus acidophilus	4.0-4.6	5.8-6.6	6.8
Staphylococcus aureus	4.2	7.0-7.5	9.3
Escherichia coli	4.4	6.0-7.0	9.0
Clostridium sporogenes	5.0-5.8	6.0-7.6	8.5-9.0
Erwinia caratovora	5.6	7.1	9.3
Pseudomonas aeruginosa	5.6	6.6-7.0	8.0
Thiobacillus novellus	5.7	7.0	9.0
Streptococcus pneumoniae	6.5	7.8	8.3
Nitrobacter spp	6.6	7.6-8.6	10.0

2.7.5.4 Oxygen

The oxygen tension or partial pressure of oxygen and the reducing and oxidizing power of the food (O-R potential) influences the growth of organisms. In relation to oxygen, bacteria can be aerobic, anaerobic or facultative, while fungi are mostly aerobic. Yeast are aerobic or facultatively anaerobic. A high O-R potential favours the growth of aerobic and facultative organisms.

Sometimes growth of an aerobe may reduce the O-R potential of food to restrain the growth of other organisms. Most fresh animal and plant foods have a low O-R potential in their interior but have a higher O-R outside. Thus, a fresh piece of meat could support the growth of aerobic organisms in the exterior and the growth of anaerobic organisms inside.

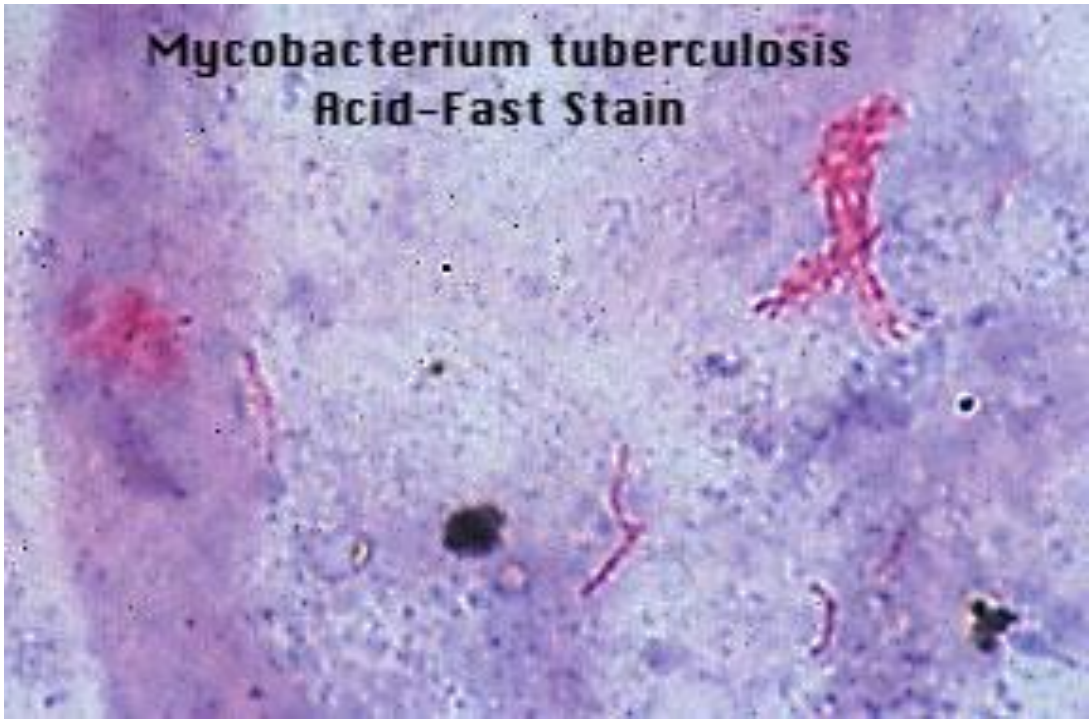

Figure 2.7: *Mycobacterium*, will grow only in presence of free oxygen

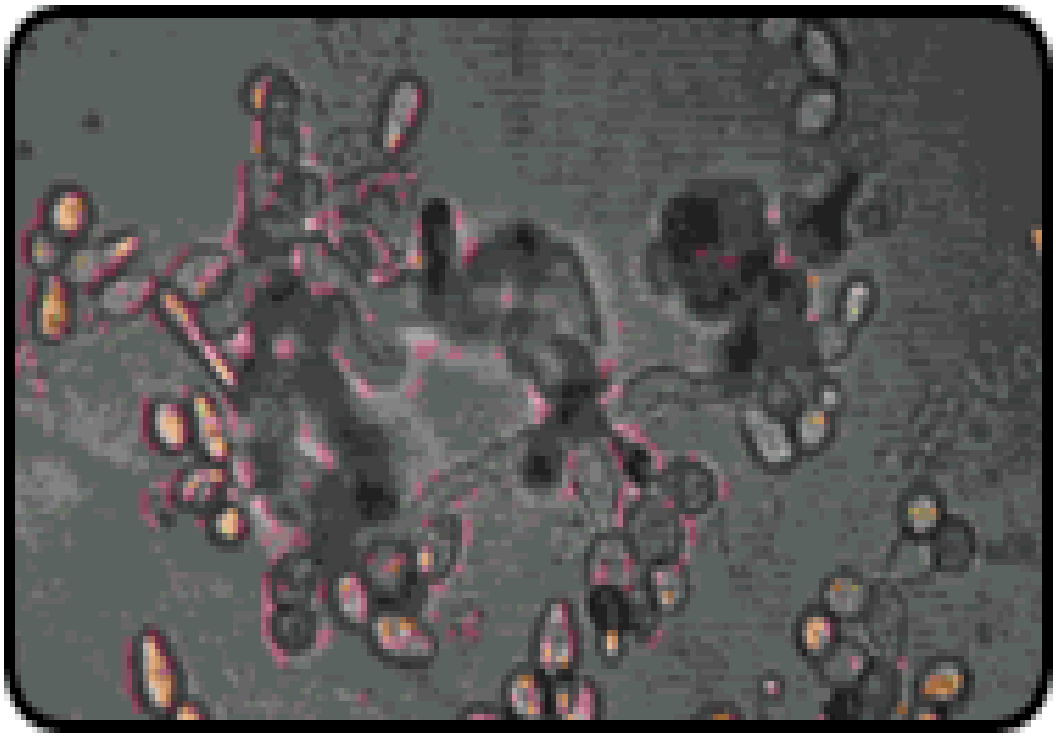

Figure 2.8: *Saccharomyces* (yeast) will grow in the absence of oxygen, but more slowly than if oxygen was present.

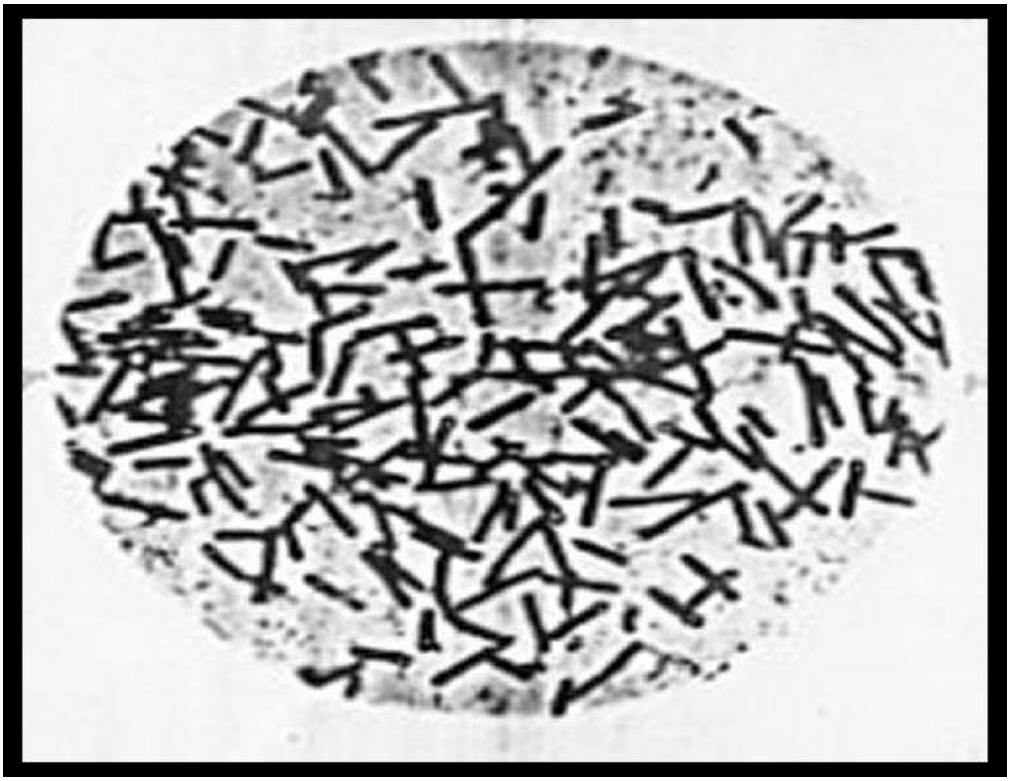

Figure 2.9: *Clostridium* will grow only in absence of oxygen which is toxic to them

2.8 Spectrophotometer

A spectrophotometer is employed to measure the amount of light that a sample absorbs. The instrument operates by passing a beam of light through a sample and measuring the intensity of light reaching a detector.

The beam of light consists of a stream of photons, represented by the purple balls in the simulation. When a photon encounters an analyte molecule (the analyte is the molecule being studied), there is a chance the analyte will absorb the photon. This absorption reduces the number of photons in the beam of light, thereby reducing the intensity of the light beam. The light source is set to emit 10 photons per second. The intensity of the light reaching the detector is less than the intensity emitted by the light source.

Figure 2.10: The spectrophotometer to detect the optical density

2.9 High Performance Liquid Chromatography

Techniques such as H.P.L.C. (High Performance Liquid Chromatography) use columns, narrow tubes packed with stationary phase, through which the mobile phase is forced. The sample is transported through the column by continuous addition of mobile phase. This process is called elution. The average rate at which an analyte moves through the column is determined by the time it spends in the mobile phase.

Chromatography involves a sample (or sample extract) being dissolved in a mobile phase (which may be a gas, a liquid or a supercritical fluid). The mobile phase is then forced through an immobile, immiscible stationary phase. The phases are chosen such that components of the sample have differing solubilities in each phase. A component which is quite soluble in the stationary phase will take longer to travel through it than a component which is not very soluble in the stationary phase but very soluble in the mobile phase. As a result of these differences in mobilities, sample components will become separated from each other as they travel through the stationary phase.

Figure 2.11: The example of high performance liquid chromatography (HPLC)

CHAPTER 3

METHODOLOGY

3.1 Introduction

This chapter will discuss about methodology that has been used in succinic acids productions with manipulated the substrate concentration and pH. The experiment methods will discuss detail in this chapter.

3.2 Flow diagram Process

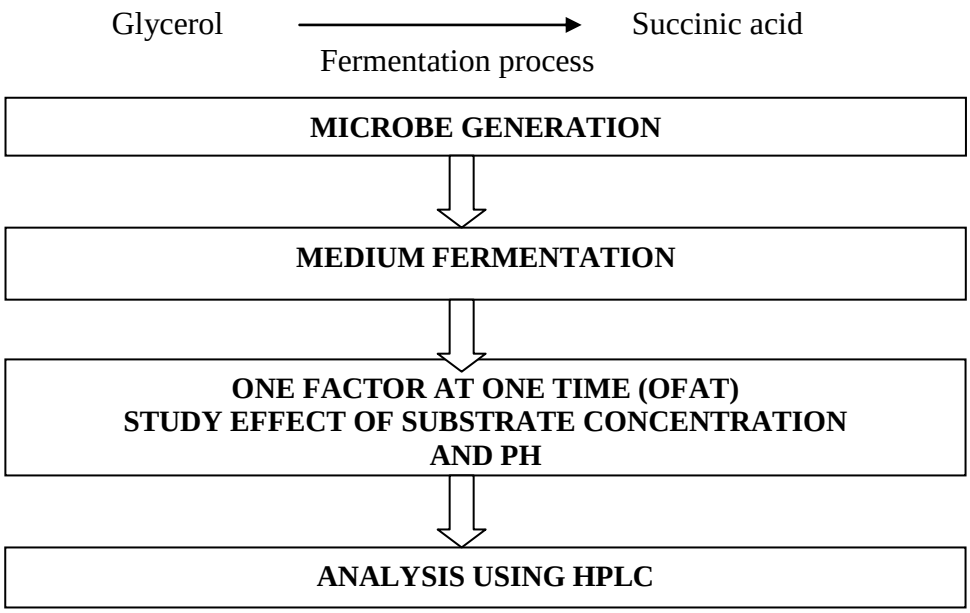

Figure 3.1: Process flow of the succinic acid fermentation

3.3 Methodology Summaries

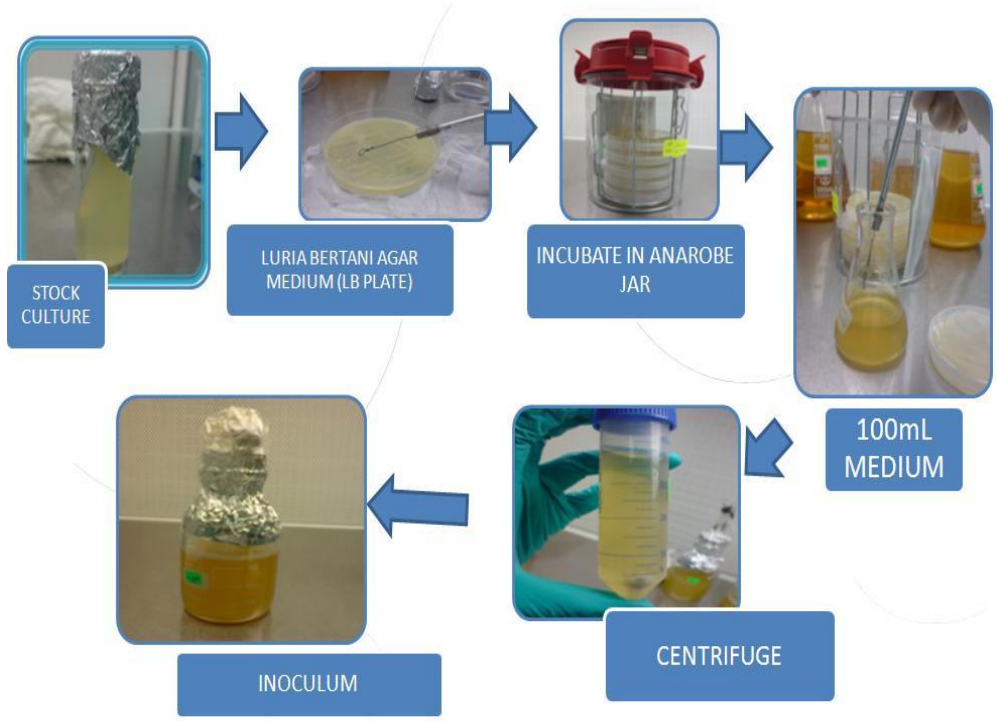

Figure 3.2: Method for pre-culture the *E-coli*

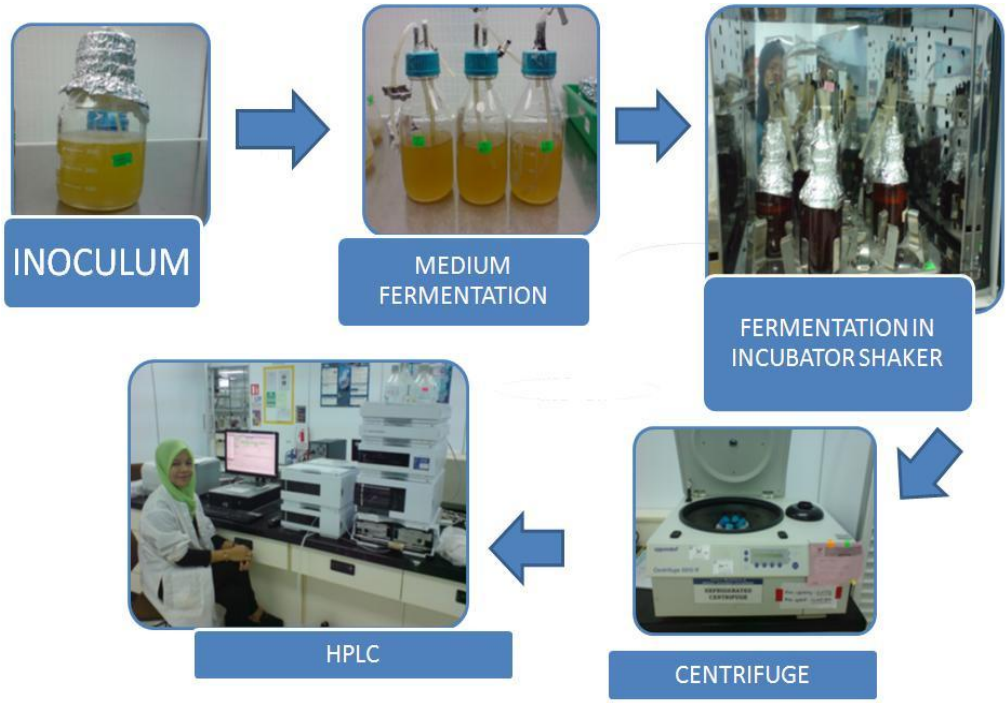

Figure 3.3: Method for fermentation process

3.4 Methodology

3.4.1 Preparation of Luria bertani Agar medium (LB)

Luria-Bertani agar broth, also known as LB agar broth or LB agar medium, is the most common solid medium used to grow bacteria (*E. coli)* by creates plates for streaking out bacterial colonies. Before start the experiment all the apparatus are autoclave at temperature 121^{o}C for 20 minutes to kill the bacteria.

Measure 10 grams of tryptone, 5 grams of yeast extract, and 10 grams of sodium chloride by using measuring weight and dissolve in 950 ml of deionized water. Heat the solution on the hot plate heater and mix properly by using stirrer. Simply add 15 grams of bacteriological agar until dissolve. A solid form of medium used to create plates for streaking out bacterial colonies. The pH of the solution was adjusted to 7.0 using 5M NaoH. The pH meter is used to measure the pH of the solution.

For this procedure were using an autoclave. The medium was autoclave at 121°C for 20 minutes to sterilize the medium and apparatus. An autoclave is designed to produce temperatures and pressures that will completely sterilize objects. It is important that using gloves designed for use with the autoclave because its produce high temperature.

Place in the beaker a glass stirring rod to be sterilized. The stirring rod will be used after autoclaving. Agar will not dissolve into solution until the solution has been heated. Cover the beakers with aluminum foil (shiny side facing the inside of the beaker), and put the nutrient agar in an autoclave. The time necessary to sterilize the solution depends on your particular autoclave. Place autoclave tape on beakers if it is available. Autoclave tape has stripes on it that are originally light colored but turn black when exposed to the temperature and pressure needed for sterilization. The function is can assured that the sterilization process was successful if the stripes on the autoclave tape change color.

After sterilization is complete, remove the beakers wearing autoclave gloves. The autoclave operates at 121 ^{o}C (250 ^{o}F), and a steam pressure of 15 lbs per square inch (psi). The solution which comes out is very hot. After removal from the autoclave, allow the beakers to cool enough that they can be comfortably handled while wearing vinyl gloves.

After autoclave the mixture are letting cool down about 50 to 60°C and the solution was pours into petri dishes in laminar flow. The laminar flow was sterilized with 70% alcohol to kill and prevent contamination of microorganism and other bacteria. Wait for about an hour for the broth to solidify. The plates were stored at 4°C in plastic bags in freezer.

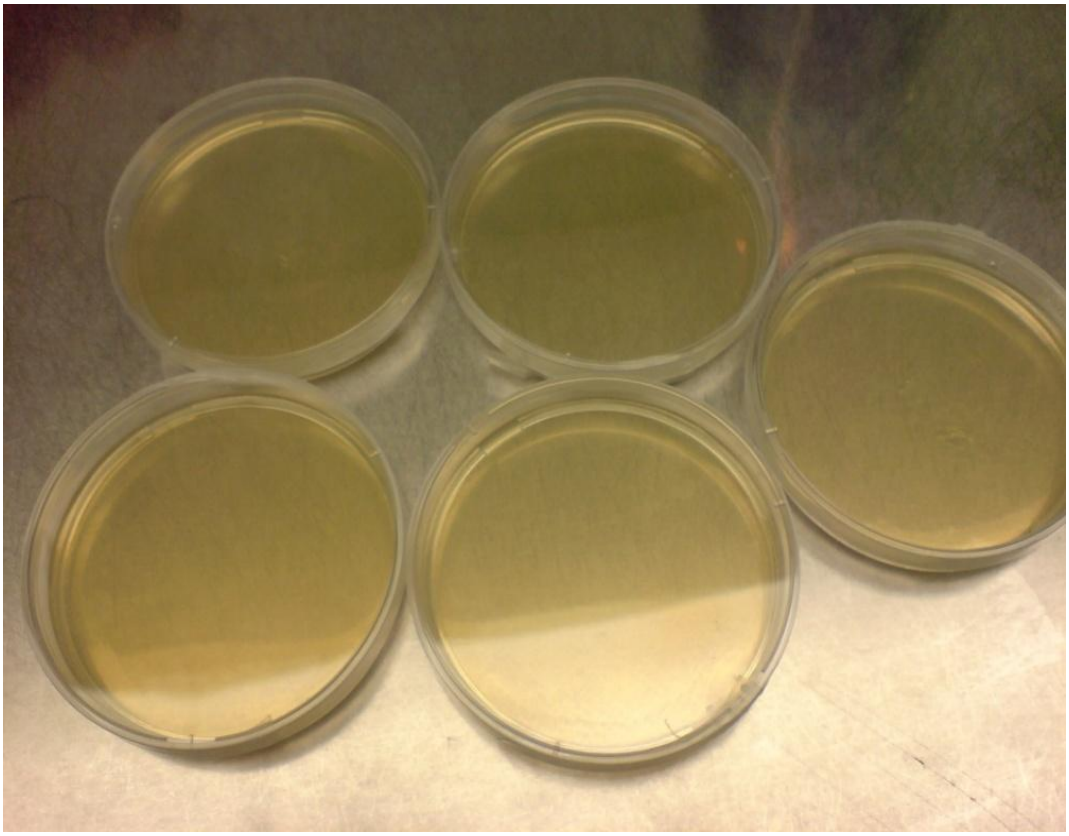

Figure 3.4: Luria bertani agar medium (LB) in the plates.

3.4.2 Medium preparation

All the apparatus are autoclave before started the experiment at temperature 121 ^{o}C for 20 minutes. Measured 10 g tryptone, 5 g yeast extract, 3 kg K_2HPO_4, 1 g NaCl, 1 g $(NH_4)_2SO_4$, 0.2 g $CaCl_2.2H_2O$, 0.2 g $MgCl_2.6H_2O$ and 1 g Na_2CO_3 by using measuring weight and mix in the 1000 ml distilled water in the 1L conical flask. Heated and stirred the medium by using hot plate heater.

The medium and the appropriate volume of glycerol are autoclave to kill the bacteria for 20 minutes. After autoclave, 20 g/L Glycerol are measured by using measuring cylinder in laminar flow and in hot condition the medium and the glycerol are mix together. Medium are those that contain the minimum nutrients possible for colony growth, generally without the presence of amino acids, and are often used by microbiologists and geneticists to grow microorganisms like *Escherichia coli*.

Figure 3.5: The medium after mix with glycerol in laminar flow

3.4.3 Culture medium

All experiment is conducted in the laminar flow. The stock cultures of *e-coli* (stored as glycerol stocks at -80^{o}C) were incubates 24 hours to growth the *Escherichia coli*. Touch the needle end of a sterile loop to a well isolated *E. coli* colony. Take one loops of *Escherichia coli* and streaked onto LB plates. Then inoculate the plate in three parallel lines about 5mm distance from each others. After striking the bacteria on Petri dish seal the Petri dish with parafilm.

The plates were incubated overnight at 37 ^{o}C in an anaerobic jar with the anaerogen for the generation of anaerobic condition. The anaerobic jar is place in incubator at 37^{o}C until the *Escherichia coli* growth on the luria bertani or LB plate.

Figure 3.6: *Escherichia coli b-strain (E. coli)* was culture in Luria Bertani agar medium in Anaerobic Jar.

A single colony of *E. coli* was used to inoculate completely filled with 50 ml medium (Medium supplemented with 10 g/L tryptone, 5 g/L yeast extract, and 5 g/L glycerol) in 100 ml conical flask. One loop of *Escherichia coli* are taken from the

last point streak because at this point the concentration of *Escherichia coli* are low so it's good for the *Escherichia coli* growth in the medium.

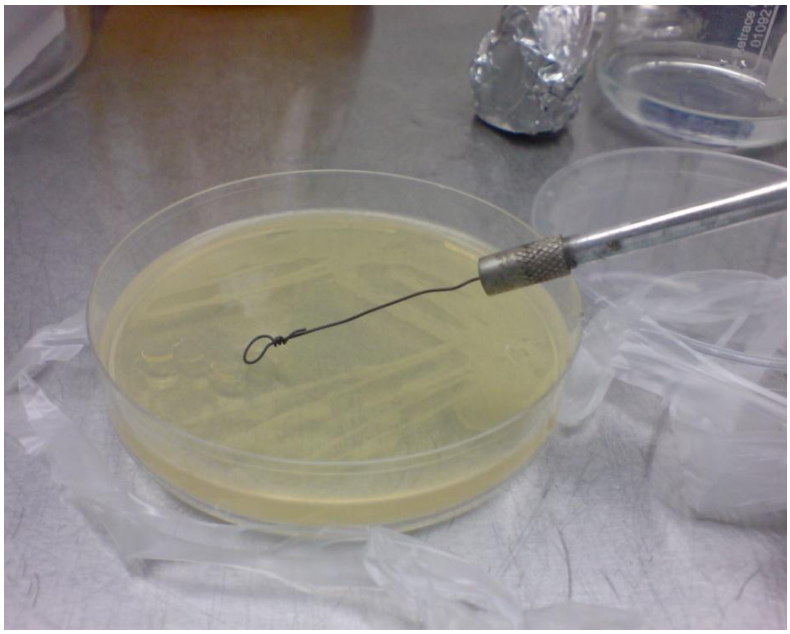

Figure3.7: One loop of *E. coli* was taken to inoculate 50ml medium culture.

The medium were incubated at 37.degree. C. until an OD with wavelength 550 about 0.4 was reached. The medium were conducted in incubator shaker with speed 200 rpm. An appropriate volume of this actively growing pre-culture was centrifuged and the pellet washed and used to inoculate 350 mL of medium with the target starting optical density of 0.05 at 550 nm.

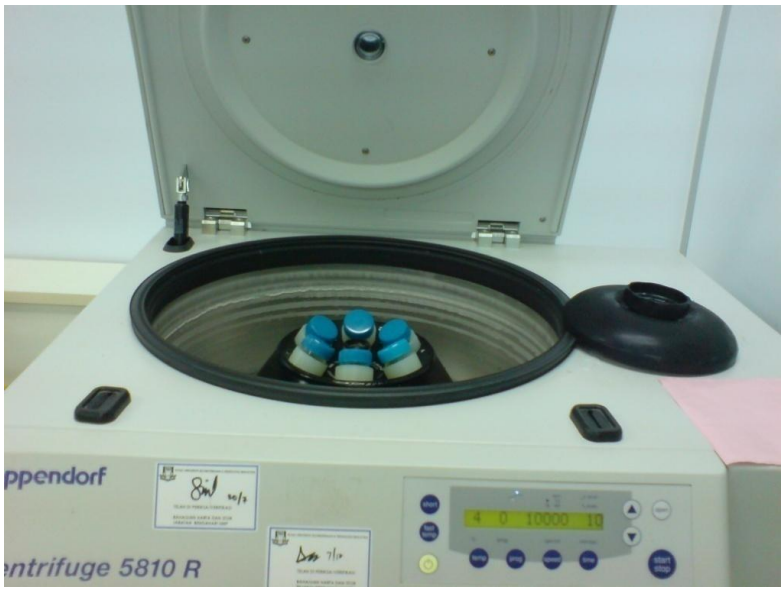

Figure 3.8: An appropriate volume of the sample was centrifuge.

3.4.4 Fermentation process.

Fermentations were conducted in a modified schott bottle 500 ml with 300 ml working volume and control of temperature and stirrer speed (200 rpm) under anaerobic conditions. All the schott bottles are autoclaves before filled with the medium at temperature 121^{o}C for 20 minutes. The medium are transfer in laminar flow. The laminar flow was sterile with 70% alcohol to kill and prevent the contamination of microorganism.

Figure 3.9: Modification of schott bottle for anaerobic condition.

All experiment was conducted triplicates for each pH and substrate concentration (glycerol concentration) and conducted in anaerobic condition. 10 % of the inoculums from the working volume are about 30 ml are added to the 300 ml medium.

The pH was controlled at 6.5 with using sulfuric acids (H_2SO_4) and natrium Carbonat (Na_2CO_3) as a buffer and the substrate concentration (glycerol) is 20 g/L. The medium were spurge with nitrogen about 10 minutes to provide anaerobic condition and the medium were conducted in incubator shaker with speed 200 rpm and temperature 37 ^{O}C.

Figure 3.10: The fermentation was conducted in incubator shaker.

After 72 hours the sample are taken and measure the optical density. The optical density was measured at 550 nm and used as an estimate of cell concentration. The samples are taken and centrifuge for 10 minutes with speed 10000rpm and temperature 4^{o}C. After centrifugation, the supernatant was stored at -20 ^{o}C for HPLC analysis. Fermentation were conducted for 72 hours or 3 days and every 6 hour the sample are taken to check at what time succinic acid can produce with high productivity.

The procedures were repeated with the pH 5.5, 6.0, 6.5, 7.0 and 7.5 using sulfuric acids (H_2SO_4) and natrium Carbonat (Na_2CO_3) as a buffer during fermentation process with maintain substrate concentration 20 g/L and repeat the procedures with the different substrate concentration 5, 10, 15, 20 and 25 g/L with maintain pH 6.5. For pH and substrate concentration the samples are taken only after 72 hours fermentation.

3.4.5 Estimation of Succinic Acids.

Succinic acid produced was estimated on high performance liquid chromatography (HPLC, Shimadzu) with using column HPX-87H organic acid column (BIO-RAD.RTM., Hercules, Calif.). The mobile phase using is 0.05 H_2SO_4 and temperature of the column is 42^oC. The standard used to detect succinic acid in the sample is the pure solution of succinic acid. The calibration curve for succinic acid were prepare for 1 g/L, 2g/L,4g/L,6g/L,8 g/L and 10g/L.

For this the sample was centrifuged at 10000 rpm for 10 minutes and temperature 4^oC to separate the pallet and supernatant of the sample. The succinic acid contain in the supernatant of the sample. The supernatant were filtered by using 0.45 micro meter filter and the samples are filled in the vials and each sample was run on HPLC.

Figure 3.11: The sample was determined by using High Performance Liquid Chromatography (HPLC).

CHAPTER 4

RESULTS AND DISCUSSION

From the experiment *Escherichia coli b-strain* (*E.coli*) was culture using glycerol as a carbon source under anaerobic condition with difference pH and substrate concentration. The results for this experiment are:

4.1 Culture of *Escherichia coli (E. coli)*

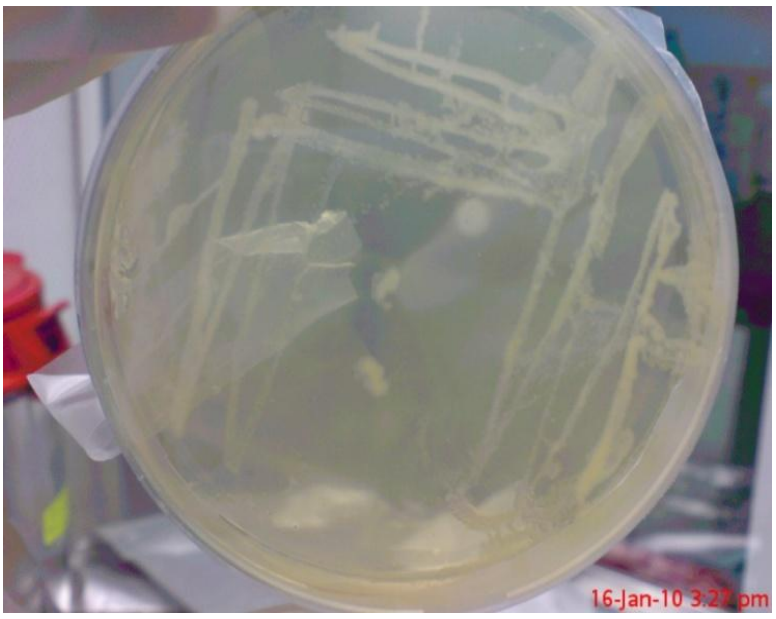

Figure 4.1: *E. coli* growth on the LB medium plate.

After 18 to 24 hours, the plates are removed from the incubator. From the observation white color appeared on the surface luria bertani agar plate. The characteristic of the *Escherichia coli* type b-strain are white colorless colonies when growth on the LB plate. From the comparison, it does can identify that the microorganism were growth on the plate are *Escherichia coli* type b-strain.

Escherichia coli can growth on the Luria agar bertani agar medium because this medium contains composition of ingredients used to promote growth. Peptides and peptones are provided by trypton. Vitamins and certain trace elements are provided by yeast extract. Sodium ions for transport and osmotic balance are provided by sodium chloride. Tryptone is used to provide essential amino acids to the growing bacteria, while the yeast extract is used to provide a plethora of organic compounds helpful for bacterial growth.

Figure 4.1 show the growths of *Escherichia coli* b-strain in Luria bertani medium plate after 24hour incubate in Incubator at temperature 37^{o}C. Others factors that help *Escherichia coli* growth are the pH of the medium (7.0) and temperature of the microbe surrounding (37^{o}C) that were controlled.

The conditions for optimal growth-temperature, pH, salt concentrations and nutrient sources are differences according to species. Refrigeration retards food spoilage because most microorganisms grow only very slowly at such low temperatures. Temperature acts as a catalyst for many things, and it helps speed up many chemical processes. 37^{o}C is the optimal condition for *E. coli* and that *E. coli* has a doubling time of about 20 minutes at this temperature.

4.2 Pre-culture the *E. coli*

Figure 4.2: The culture of *Escherichia-coli* after 18 hours

The inoculums were culture for 18 to 20 hours depended on the optical density reached about 0.8. From the observation and the measurement of optical density shown the *Escherichia coli* growth well in the medium of preculture *E. coli* grows and divides through asexual reproduction.

Growth will continue until all nutrients are depleted and the wastes rise to a toxic level. This is demonstrated by the Log of the number of cells per unit volume versus Time growth curve. Temperature is the most influential factor of growth in bacteria. The optimal temperature of *E.coli* is 37^{o}C, which was maintained throughout the experiment.

Aside from temperature, the pH of the organisms environment exerts the greatest influence on its growth. The pH limits the activity of enzymes with which an organism is able to synthesize new protoplasm. The optimum pH of *E. coli* growing in a culture at 37^{o}C is 6.0 to 7.0. It has a minimum pH level of 4.4 and a maximum level of 9.0 required for growth.

Bacteria obtains it nutrients for growth and division from their environment, thus any change in the concentration of these nutrients would cause a change in the growth rate. Drugs or antibiotics are another very common tool in molecular biology used to inhibit a specific process.

4.3 Observation on Pellet

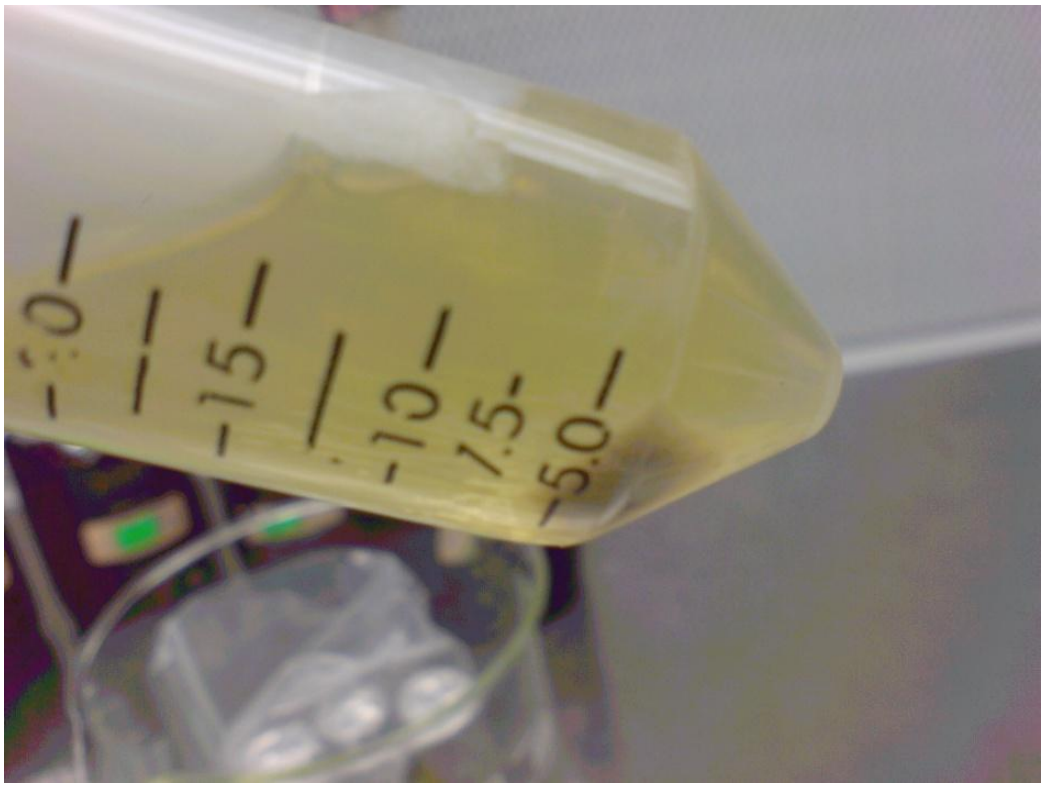

Figure 4.3: The pellet observe after centrifuge

After 72 hours fermentation the samples were taken and centrifuge. After centrifuge the solid form occur on the bottom of the centrifuge tube. The solid form are called pellet and the liquid form are called supernatant. The color of the pellet is white color. Pellet is produce by *Escherichia coli* from the fermentation of glycerol. The pellet produce can determine the growth of *E. coli* in the medium.

Centrifugation is the most widely used procedure to separated the homogenate into different parts, or fractions. The homogenate is placed in the tubes and rotated at high speed in the centrifuge. Centrifuges are rotate 10000 rpm speed or 10000 revolutions per minute and produce enormous forces. At such speeds, centrifuge chambers must be refrigerated and evacuated so that friction does not heat up the homogenate.

Pellet is a solid and visible mass of molecules or particles (biomass cells), which is typically obtained from a liquid medium in a conical tube by centrifugation. Biomass is a renewable energy source and biological material derived from living, or recently living organisms. Supernatant is the liquid phase a top of the pellet after centrifugation. Pellet is formed after centrifugation at the bottom because large and more density component and the supernatant are the top of pallet because less and small density component. Supernatant liquid is the upper layer of fluid found after a mixture has been centrifuged. Because of its lower density, the fluid and the components in it have a lesser tendency to migrate to the bottom of a centrifuge tube.

4.4 Comparison the color of medium before and after fermentation

Figure 4.4: The medium before fermentation

Figure 4.5: The medium after fermentation

The medium after fermentation contain the nutrient for *Escherichia coli* growth. The observation of medium is clear before fermentation but after fermentation the medium change to the cloudy. This observation has shown *Escherichia coli* can gowth well in the medium.

Escherichia coli also can change the glycerol into succinic acid after 72 hours fermentation. This is proved by the reading of optical density. The optical density, which measures with a transmission densitometer, is a representation of a material's light blocking ability. The optical density scale is unitless and logarithmic, and it enhances the data resolution for materials that transmit only a small fraction of incident light.

4.5 Profile growth

Table 4.1: The data for the times and the Average Optical Density

Time (hour)	Average Optical Density
0	0.009
6	0.436
12	0.489
18	0.568
24	0.732
30	0.890
36	0.895
42	0.943
48	0.963
54	0.976
60	0.975
66	0.945
72	0.958

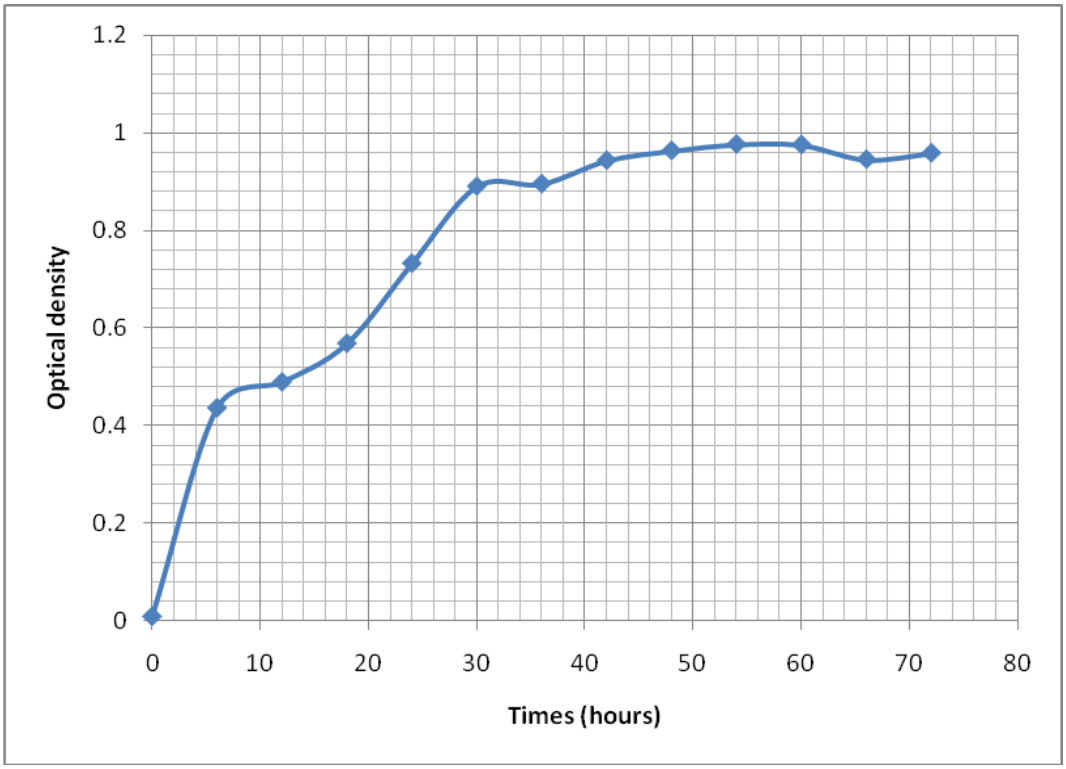

Figure 4.6: Show the optical density versus time (hours)

The Growth profile of *Escherichia coli* type b-strain grown in a Scott Bottle is shown in Figure 4.6. *Eschericha colis* was cultured using 20 g/L glycerol as a carbon source under anaerobic condition. The effects on growth curves of strains of *Escherichia coli* were investigated using the spectrophotometer instrument or uv-vis. The results showed that the optical density obtain every six hours were observed by the profile growth of the *Escherichia coli* in the medium fermentation.

Figure above shows a typical fermentation profile for *Escherichia coli* type b-strain in a medium supplemented with 10 g/L trypton. The starting optical density at 0 times is about 0.09. Cells grew to an OD (wavelength 550) of 0.732 at 24 hour and thereafter cell concentration increased gradually. Exponential growth was observed for a period of 30 hour, starting when the culture was about 6 hour and ending around 30 hour.

From that graph, the bacteria *Escherichia coli* in medium are growth actively from 0 to 36 hours. From time 0 to 36 hours, the phase that call is exponential phase (sometimes called the log phase) is a period characterized by cell doubling. The number of new bacteria appearing per unit time is proportional to the present population.

Based on the result, after 36 to 60 hour the growth rate bacteria are low. This phase is reached as the bacteria begin to exhaust the resources that are available to them. This phase is call as stationery phase. This phase is a constant value as the rate of bacterial growth is equal to the rate of bacterial death. Then, after 60 to 72 hour, the growth rate of bacteria *Escherichia coli* are not active where the bacteria run out of nutrients and die. This phase is call death phase.

Maximal increase in optical density in the residual growth phase was found to be concentration dependent for substrate concentration, pH, temperature and the ratio carbon used. In the construction and use of culture media, one must always consider the optimum pH for growth of a desired organism and incorporate buffers in order to maintain the pH of the medium in the changing milieu of bacterial waste products that accumulate during growth.

4.6 Effect of Fermentation time on Succinic Acid Production

Table 4.2: The data for the times and the Amount of succinic acid

Time (hr)	Amount succinic acid (g/L)
6	0.009833846
12	0.00986299
18	0.009882064
24	0.009958876
30	0.009993572

36	0.010045816
42	0.010142536
48	0.010297761
54	0.010315568
60	0.01044105
66	0.010441982
72	0.01044251

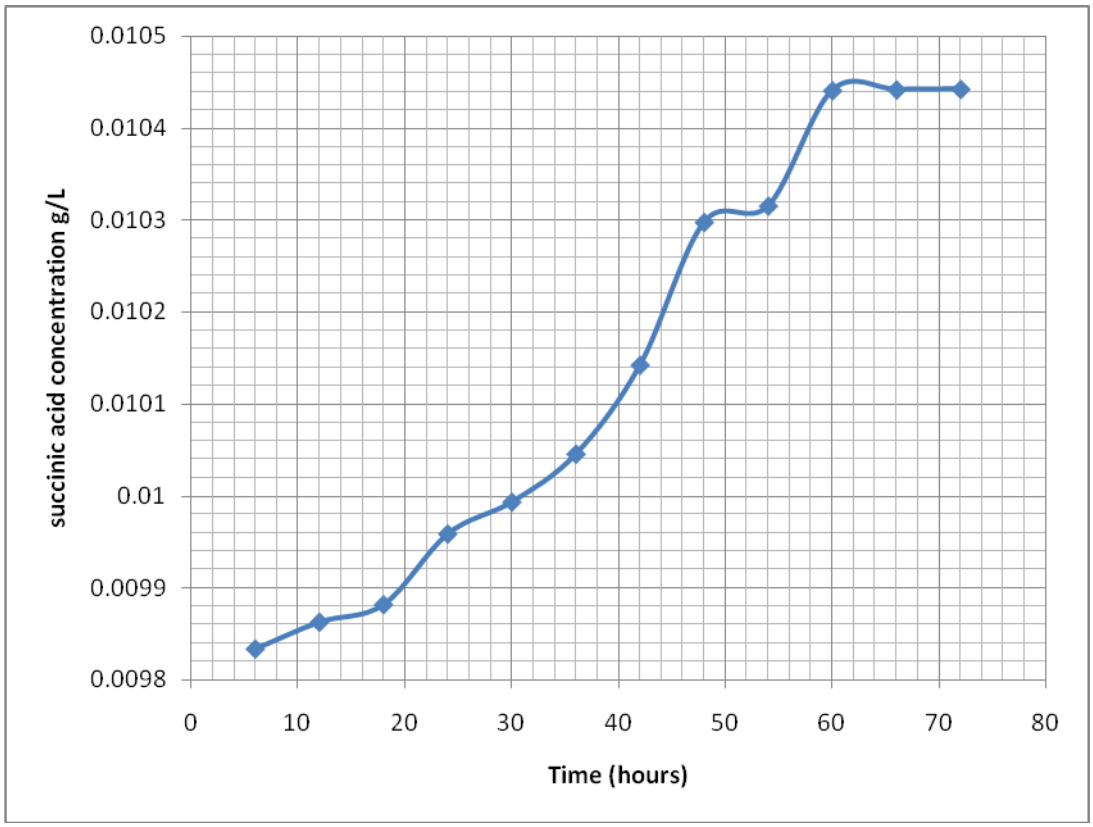

Figure 4.7: The production of succinic acid versus time (hours)

Succinic can produce in a high productivity with increase the time. Times are important to determine the productivity of succinic acid production. The graph showed the succinic acid production increase with time until at 60 hour the succinic acid production started contant. At 60 hours the succinic acid production is about 0.01044105 g/L.

The *Escherichia coli* can growth well in media are those that contain the minimum nutrients possible for colony growth, generally without the presence of amino acids, and are often used by microbiologists and geneticists to grow *Escherichia coli*. The medium fermentation contains a carbon source for bacterial growth, which a sugar such as glycerol, various salts, which may vary among bacteria species and growing conditions, these generally provide essential elements such as magnesium , nitrogen , phosphorus, and sulfur to allow the bacteria to synthesize protein and nucleic acid and water.

Among the various organic nitrogen sources tested, tryptone maximally enhanced the production of both succinic acid as well enzyme activities. The most probable reason for the maximum enzyme activities and succinic acid yield could be the availability of hydrogen along with the nitrogen source. Among different temperatures, maximum succinic acid production was achieved at 37 °C. However, the organism failed to grow at 50 °C. Similarly *Escherichia coli* grew best and produced succinic acid at 37 °C in 72 hour. The effect of temperature on the activities of the enzymes involved in the production of succinic acid (via the reverse TCA cycle) are effect the produvtivity of succinic acid. The best activities of these enzymes are maximum at 37°C.

By using the glycerol as a carbon sources the productivity of succinic acid increase. Metal ions are known to play an important role in maintaining cellular metabolism and enzyme activities. The activities of the enzymes of the reverse TCA cycle were also high in $MgCl_2 6H_2O$. The reason could be that Mg^{2+} is a cofactor for most of the enzymes involved in the anaerobic pathway. This is in accordance to the findings of Meyer *et al* who reported that in *E. coli* the rate of PPC activity for substituted cation Mg^{2+} ions was higher than for metal ions like Mn^{2+}, Co^{2+}, Zn^{2+}. Mg^{2+} has also been reported as the cofactors for the activity of PPC.

Production of 0.01044251 g/L of succinic acid was obtained in 72 hour when the medium was inoculated with 10% (v/v) of inoculums. There was no increase in amount of succinic acid produced on subsequent increase in inoculums density. This is because the inoculums density of 10% (v/v) shortened the lag period and increased

the final cell concentration resulting in reduction of total fermentation time. However, further increase in the inoculums density beyond this did not enhance the production of succinic acid, because there was competition of nutrients and certain essential nutrients became limiting much faster. The quantity of inoculums was no increase in production of succinic acid

4.7 Effect of Substrate concentration on Succinic Acid Production

Table 4.3: The data for the optical density and the succinic acid production with difference substrate concentration

Concentration substrate (g/L)	Trials	Optical density (wavelength)	Succinic acid production (g/L)
5	1	0.876	0.010567192
	2	0.986	0.010180445
	3	0.875	0.010100502
10	1	0.768	0.0105938
	2	0.786	0.01046859
	3	0.754	0.010442455
15	1	0.865	0.0113588
	2	0.784	0.011209794
	3	0.877	0.011696606
20	1	0.678	0.0113588
	2	0.878	0.010802346
	3	0.921	0.011222154
25	1	0.876	0.010509529
	2	0.637	0.010737017
	3	0.738	0.010617782

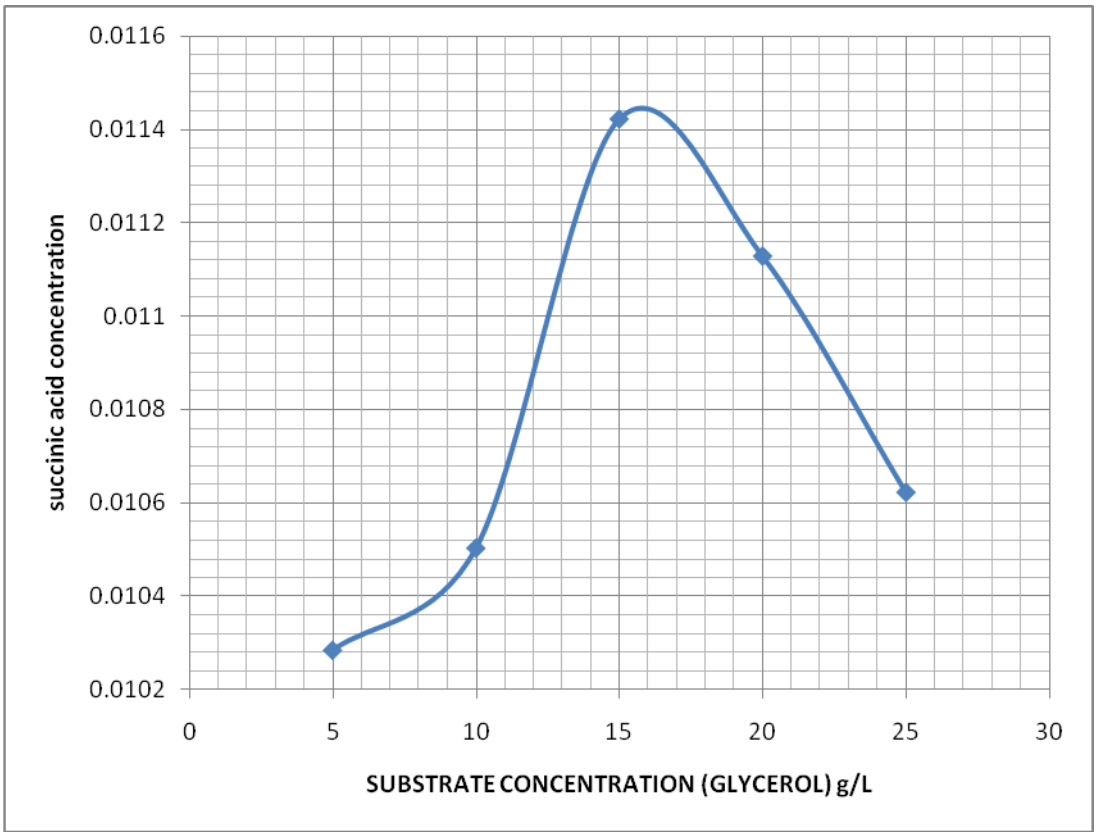

Figure 4.8: Effect of different substrate concentration (glycerol) on succinic acid production.

In order to investigate the effect of substrate concentration on the anaerobic fermentation of glycerol, batch fermentations were carried out using the medium supplemented with varying concentrations (5, 10, 15, 20, and 25 g L−1) of glycerol. All the experiments were conducted in triplicates and the result are presented in graphs. The result for optical density with wavelength 550 and the sample are taken after 72 hours fermentation.

From the graph at the production of succinic acid increase with the substrate concentration (glycerol) until at 15 g/L glycerol the succinic acid production decrease with 0.011421733 g/L succinic acid production. The optimum substrate concentration is at 15 g/L with 0.011421733 g/L succinic acid production. The optical density measure for the optimum is at ranges 0.7 to 0.9 with wavelength 550.

One factor effects the production of succinic acid are effects of yeast extract and trypton on cell growth and glycerol consumption, which is the ratio of the yeast extract and the trypton added to the glycerol as a carbon sources. Some nutritional components present in complex nitrogen sources can be essential for the growth of cells. Therefore, the effects of varying the concentrations of yeast extract, trypton and glycerol on cell growth are effect succinic acid production.

When more yeast was supplemented, both cell growth and succinic acid production were enhanced. On the other hand, the addition of trypton alone did not support cell growth and succinic acid production when glycerol as a carbon source. Only when yeast extract was also supplemented did trypton exert positive effects on cell growth and succinic acid production. Trypton could enchance cell growth and succinic acid production as much as yeast extract when glycerol used as a carbon source. Therefore, it seems that unknown nutritional components present in yeast extract are important for glycerol utilization in *Escherichia coli*.

The ratio of glycerol, yeast, and trypton are important to produce high productivity of succinic acid. When the ratio of glycerol is to large to the ratio of yeast extract and trypton, it's cannot enchance cell growth and the production of succinic acid were decrease.

4.8 Effect of pH on Succinic Acid Production

Table 4.4: The data for the optical density and the succinic acid production with difference pH

pH	Trials	Optical density (wavelength 550)	Succinic acid production (g/L)
5.5	1	0.837	0.010316534
	2	0.838	0.010769549
	3	0.739	0.0010374198

6.0	1	0.837	0.009834778495
	2	0.828	0.011592269
	3	0.809	0.009846005102
6.6	1	0.726	0.010078112
	2	0.729	0.010141458
	3	0.789	0.010106679
7.0	1	0.972	0.010176244
	2	0.728	0.00999429508
	3	0.838	0.010022841
7.5	1	0.982	0.009851604493
	2	0.892	0.009935887512
	3	0.680	0.00987268742

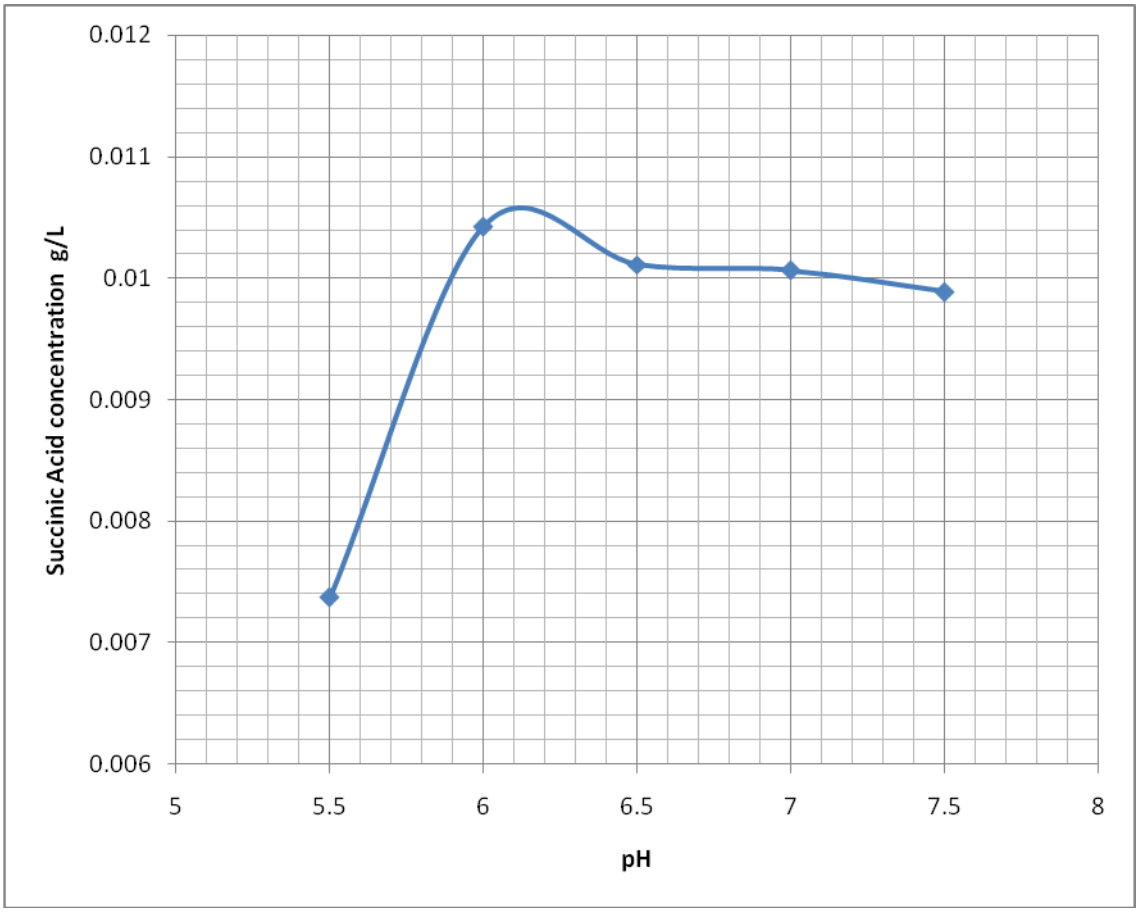

Figure 4.9: Effect of different pH on succinic acid production.

The pH is an important factor that affects both growth and growth-associated production of molecules. The samples were taken after 72 hours fermentation with different pH. Each pH is conducted in triplicates and the pH is measure by using pH meter with do the calibration before used. The pH, or hydrogen ion concentration, $[H^+]$, of natural environments varies from about 0.5 in the most acidic soils to about 10.5 in the most alkaline lakes. Appreciating that pH is measured on a logarithmic scale, the $[H^+]$ of natural environments varies over a billion-fold and some microorganisms are living at the extremes.

From the experiment pH are increase with the succinic acid production but when pH 6.0 the production of succinic acid are decrease with 0.010424 g/L succinic acid produce and the optical density in range 0.8 to 0.85. It was observed that *Escherichia coli* was capable of producing succinic acid in the wide pH with maximum production at pH 6.0 (0.010424 g/L in 72 hour) and there was a subsequent decrease in the amount of succinic acid produced with the increasing pH.

The activity of reverse TCA cycle enzymes was also high at pH 6.0. Thus, indicating that in the cells grown at pH 6.0, both succinic acid production as well as the activities of the enzymes involved in its production are maximum. Similar has been reported by Samuelov *et al.* who studied the influence of pH on the level of fermentative enzymes responsible for end-product formation and found that in the cells grown at pH 6.2, both the PPCK activity and succinic acid production was high.

pH affects bacteria the same way it affects all living things. Extremes of pH affect the function of enzyme systems by denaturing them. However, bacteria become adapted over time to their surroundings. Bacteria that live in acid conditions are adapted to them. If they are moved to an environment that is neutral or basic they will probably die and it depends on the bacteria and what its natural environment can growth.

Bacteria need a physiological pH inside their cells, just like all other living organisms. Their ability to survive in extreme pH (either high or low) depends on

their ability to correct for the difference between inside and out. One example of a bacterium that can live in acidic environments is Helicobacter pylori which live in the stomach. It produces high amounts of urease which is an enzyme that degrades urea, and by doing so decreases the acidity (raises the pH). Imagine the bacteria produce a 'cloud' of neutral pH around them to protect them from the acidic environment. There are other bacteria that are specialized to live in basic pH, which pH is lethal for the bacteria depend on the species. Their defense is to keep the protons or OH- ions out. Would they not succeed, then their proteins would rapidly denature. That is the lethal toxicity of non-physiological pH.

pH affects the ionization and therefore the binding and interaction of a myriad of molecular processes. This includes very basic things such as nutrient availability. For example, depending on the pH certain metals will take on different ionization states and therefore will or will not be able to be utilized. pH also affects the solubility of many substances that bacteria need. There is also no certain pH level for maximum growth for bacteria in general since they all differ slightly in their evolution.

Other factors that effect the production of succinic acid decrease after pH 6.0 is the inhibitory effect. The inhibitory effects can be two-fold including pH-based inhibition and an anion specific effect on metabolism. It has been reported that high concentrations of ammonia inhibit *E.coli* growth and damage cell membranes. In order to limit toxic and inhibitory effects different neutralising agents were tested.

Optimal pH used with Na_2CO_3 as buffer to control the pH can effect the production of succinic acid. It was observed that cells lost viability during the cause anaerobic phase. It resulted in decreasing succinic acid productivities. It is believed that the viability decrease is a combined effect of organic acids concentration and the osmolarity of the medium.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

In conclusion, the effects of substrate concentration and pH on succinic acid production from glycerol fermentation were determined. Increase in fermentation time, will increase succinic acid production. The maximum production of succinic acid is at pH 6.0. Initial substrate concentration (5 to15 g/L) will increase Succinic acid production, but after 15 g/L substrate concentration, the succinic acid production will decrease. The substrate concentration and pH are important factor that affects both growth and growth-associated productions of molecules to increased production of succinic acid. Succinic acid produced with much less by-product formation by using glycerol as a carbon sources. Glycerol is the best carbon source for production of succinic acid. This fermentation-derived succinic acid has the potential to become a large volume commodity chemical that would form the basis for supplying many important intermediate and specialty chemicals for the consumer product industries.

5.2 Recommendation

From the experimental results, the following recommendations are proposed:

1. For the next research, the continuous culture should be used to eliminate the preparation time and lag phase. The products will inhibition as well as down time for cleaning, sterilizing and filling.

2. For fermentation process the modified schott bottle and the tubes must always check to ensure that the fermentation still in anaerobic condition.

3. During the inoculums process, the subcultures that are done must be decrease to avoid the decreasing of inoculums productivity

4. Provide more safety in the experiment because this process involved the bacteria.

5. For hplc analysis must choose the right column to detect the succinic acid. The procedures and the condition of the hplc must study to avoid column from damage. For the mobile phase make sure used the right mobile phase and the right concentration mobile phase and the mobile phase must be clear from the particles to avoid column clogging.

6. Development of strategies for strain improvement, fermentation and purification.

7. The strain development process needs to be integrated with the fermentation process development.

8. Development of an efficient downstream process for the purification of succinic acid.

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APPENDIX A

Appendix A.1: UV-Visible Single Beam Spectrophotometer (Model U-1800)

Appendix A.2: Incubator Shaker

Appendix A.3: Refrigerated Centrifuge 5810 R

Appendix A.4.: Laminar air Flow Cabinet (Model AHC-4A1)

APPENDIX B

STANDARD CURVE FOR SUCCINIC ACID

Signal 1: DAD1 A, Sig=210,4 Ref=360,100
Signal 2: RID1 A, Refractive Index Signal

RetTime [min]	Sig	Lvl	Amount [ng/ul]	Area	Amt/Area	Ref	Grp	Name
11.460	2	1	1.00000e-1	2.19329e4	4.55936e-6			
		2	2.00000e-1	4.24246e4	4.71424e-6			
		3	4.00000e-1	8.22717e4	4.86194e-6			
		4	6.00000e-1	1.18798e5	5.05057e-6			
		5	8.00000e-1	1.59584e5	5.01304e-6			

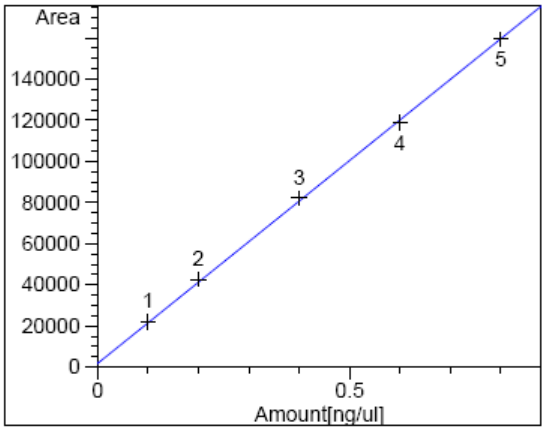
1 Warnings or Errors :

Warning : Compound has no name

Peak Sum Table

No Entries in table

Calibration Curves



at exp. RT: 11.460
RID1 A, Refractive Index Signal
Correlation: 0.99976
Residual Std. Dev.: 1505.65667
Formula: y = mx + b
m: 197429.44428
b: 1734.94808
x: Amount
y: Area

APPENDIX C

RESULTS FOR HPLC ANALYSIS

```
Injection Date  : 3/6/2010 7:28:48 PM                Inj :   1
                                                  Inj Volume : 20 µl
Acq. Method     : C:\CHEM32\1\DATA\SUCCINIC ACID HIDAWATI\ATIE 2010-03-06 17-01-20\NORHIDAWATI.M
Last changed    : 3/5/2010 5:49:50 PM by Hidawati
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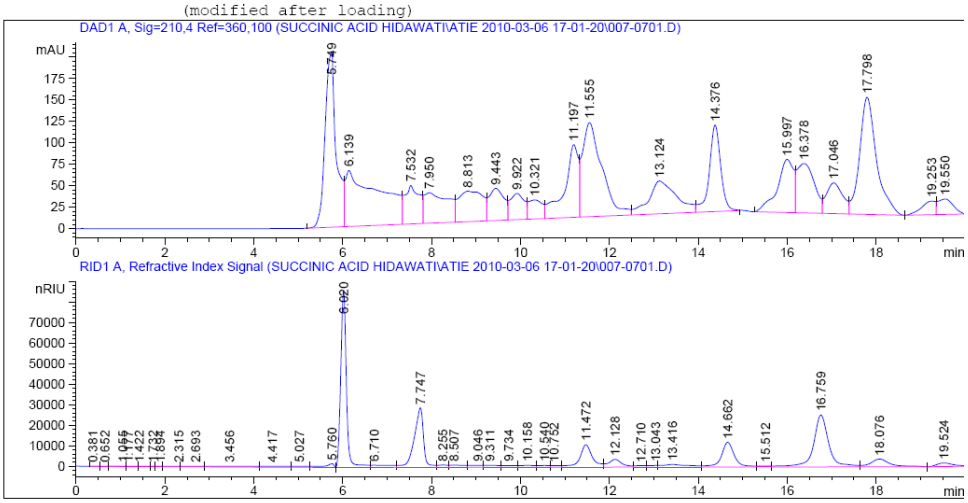
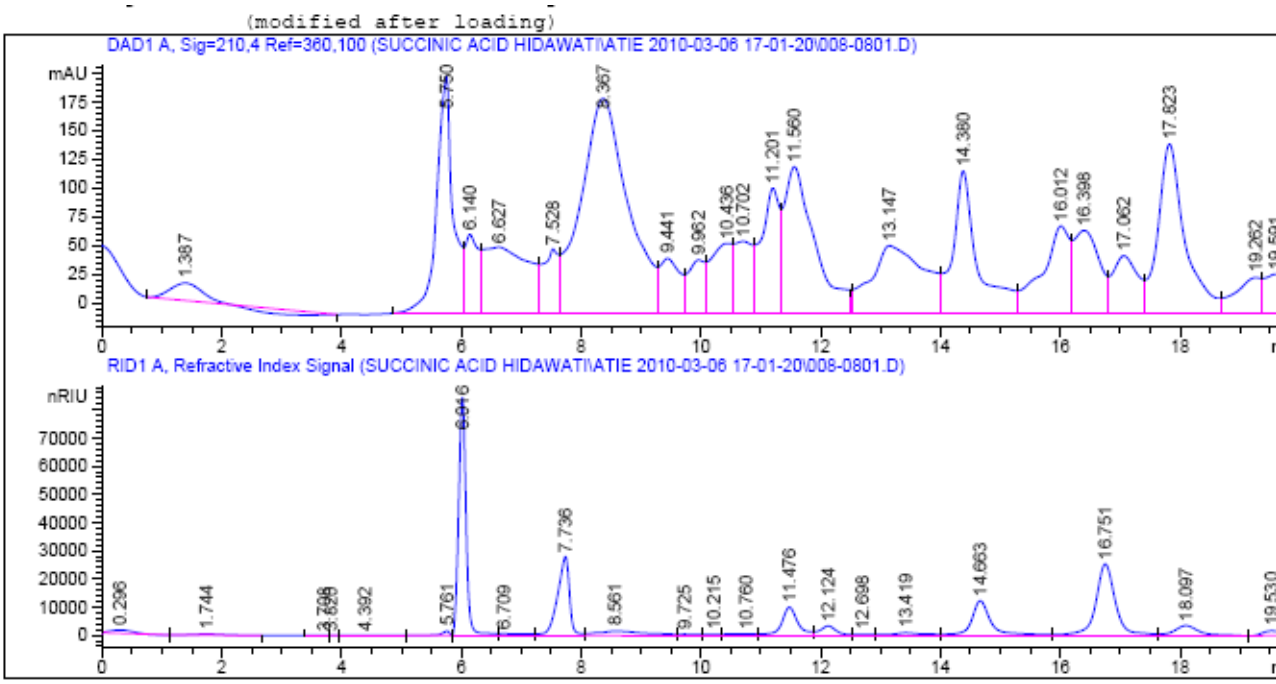


Signal 2: RID1 A, Refractive Index Signal

Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	0.381	BV	0.0670	60.60744	15.56404	2.130e-3
2	0.652	VV	0.1019	103.91460	12.70617	3.652e-3
3	1.055	VV	0.2248	1107.89624	61.01087	0.0389
4	1.177	VV	0.2123	1223.10852	72.35766	0.0430
5	1.422	VV	0.2147	1560.50952	87.45123	0.0548
6	1.732	VV	0.0910	757.90271	116.51288	0.0266
7	1.894	VV	0.1201	1264.80688	135.04120	0.0444
8	2.315	VV	0.2480	3875.16284	185.51057	0.1362
9	2.693	VV	0.3914	7444.33545	236.22124	0.2616
10	3.456	VV	0.8480	2.36506e4	327.87442	0.8312
11	4.417	VV	0.5223	1.74794e4	407.28278	0.6143
12	5.027	VV	0.3153	1.16513e4	452.89847	0.4095
13	5.760	VV	0.2044	2.70661e4	1835.82947	0.9512
14	6.020	VV	0.1326	7.56745e5	8.62586e4	26.5943
15	6.710	VV	0.4074	3.46011e4	1076.55115	1.2160
16	7.747	VV	0.2090	4.23102e5	2.90178e4	14.8691
17	8.255	VV	0.2140	1.70988e4	1139.23218	0.6009
18	8.507	VV	0.3022	2.51111e4	1103.57520	0.8825
19	9.046	VV	0.2794	1.97590e4	941.60040	0.6944
20	9.311	VV	0.3106	2.16092e4	974.38977	0.7594
21	9.734	VV	0.3235	1.92561e4	840.22675	0.6767
22	10.158	VV	0.3433	2.07293e4	861.75085	0.7285
23	10.540	VV	0.2510	1.48168e4	823.20850	0.5207
24	10.752	VV	0.2105	1.21878e4	798.16119	0.4283
25	11.472	VV	0.2843	2.13532e5	1.09193e4	7.5042
26	12.128	VV	0.2990	7.93183e4	3811.30298	2.7875
27	12.710	VV	0.2459	1.24286e4	715.37634	0.4368
28	13.043	VV	0.2130	1.08610e4	755.87708	0.3817
29	13.416	VV	0.5263	5.21774e4	1306.76978	1.8337
30	14.662	VV	0.3027	2.45370e5	1.20321e4	8.6230
31	15.512	VV	0.2274	9242.43848	488.16177	0.3248
32	16.759	VV	0.3491	6.00682e5	2.52291e4	21.1098
33	18.076	VV	0.4397	1.15134e5	3802.15576	4.0462
34	19.524	VBA	0.3897	4.45043e4	1749.30322	1.5640
Totals :				2.84551e6	1.88591e5	



Signal 2: RID1 A, Refractive Index Signal

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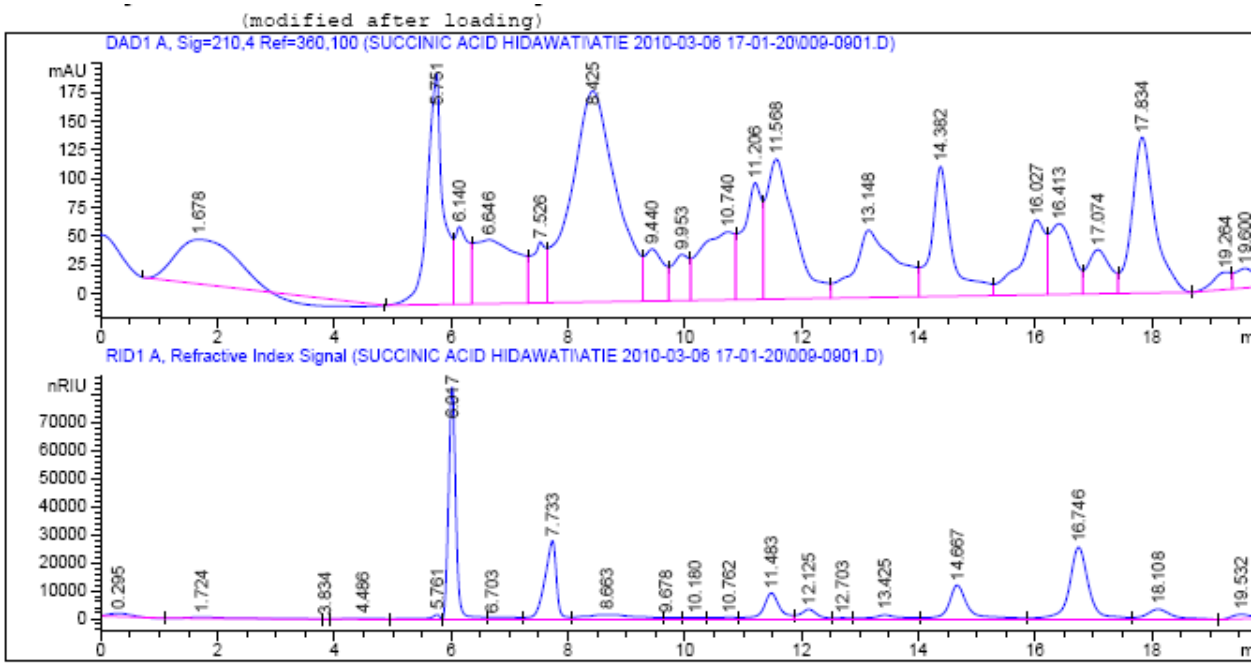
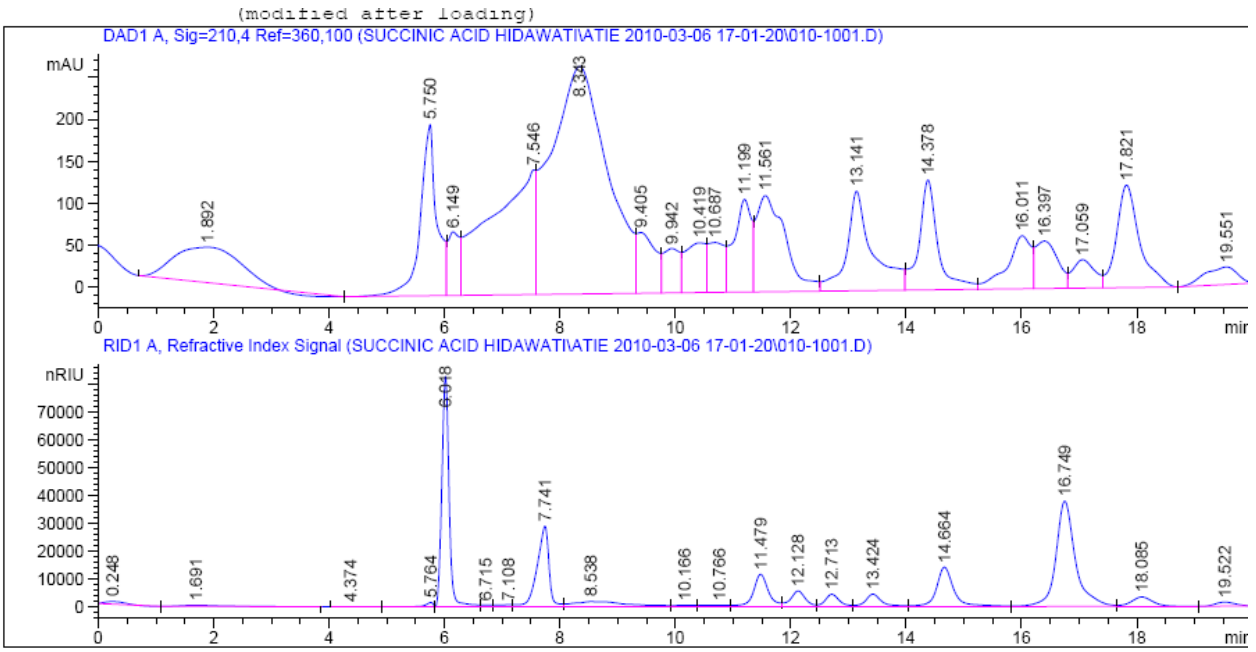
1	0.296	BV	0.4227	2.77477e4	1120.87036	1.0148
2	1.744	VV	0.4738	1.20043e4	382.83765	0.4390
3	3.708	VV	0.1939	366.57565	23.35915	0.0134
4	3.820	VV	0.1113	232.44710	25.35641	8.501e-3
5	4.392	VV	0.6409	4765.37549	92.68369	0.1743
6	5.761	VV	0.1535	1.63281e4	1573.51636	0.5972
7	6.016	VV	0.1311	7.31153e5	8.46052e4	26.7408
8	6.709	VV	0.4163	2.30648e4	700.99683	0.8436
9	7.736	VV	0.2042	3.92667e5	2.80791e4	14.3612
10	8.561	VV	0.7917	9.69558e4	1623.65552	3.5460
11	9.725	VV	0.3325	1.30800e4	548.00677	0.4784
12	10.215	VV	0.2594	1.01083e4	550.95740	0.3697
13	10.760	VV	0.4388	2.37165e4	707.22186	0.8674
14	11.476	VV	0.2827	1.95528e5	1.01664e4	7.1512
15	12.124	VV	0.2919	7.11730e4	3522.27979	2.6030
16	12.698	VV	0.2890	1.12993e4	555.81274	0.4133
17	13.419	VV	0.5598	4.83350e4	1129.45300	1.7678
18	14.663	VV	0.3449	3.01137e5	1.25431e4	11.0136
19	16.751	VV	0.3471	5.99982e5	2.55742e4	21.9434
20	18.097	VV	0.4511	1.17234e5	3750.14038	4.2876
21	19.530	VBA	0.3667	3.73411e4	1616.79004	1.3657

Totals :			2.73422e6	1.78892e5	



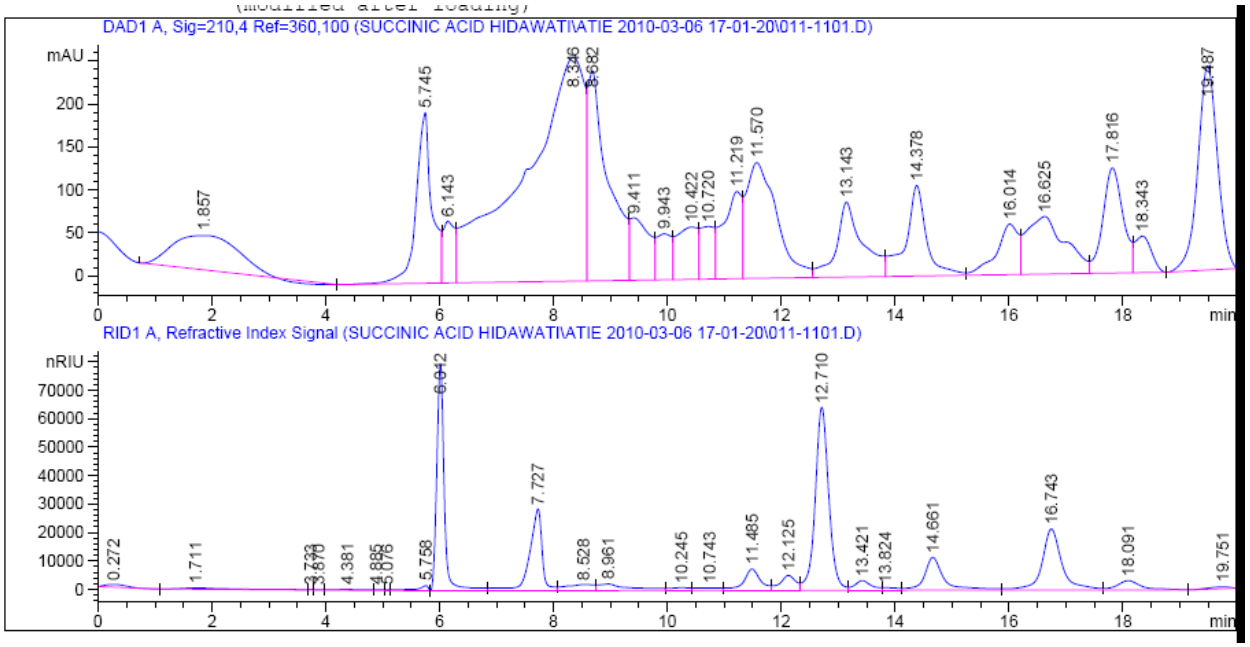
Signal 2: RID1 A, Refractive Index Signal

Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	0.295	BV	0.4337	2.95491e4	1158.33557	1.0347
2	1.724	VV	0.7820	2.91118e4	499.10339	1.0194
3	3.834	VV	0.1059	1002.39545	115.13210	0.0351
4	4.486	VV	0.6137	1.29045e4	250.60120	0.4519
5	5.761	VV	0.2151	2.83300e4	1808.97559	0.9920
6	6.017	VV	0.1324	7.27257e5	8.30311e4	25.4659
7	6.703	VV	0.4277	3.13806e4	926.23657	1.0988
8	7.733	VV	0.2054	4.02618e5	2.82095e4	14.0982
9	8.663	VV	0.8164	1.20234e5	1799.10889	4.2102
10	9.678	VV	0.2709	1.42008e4	756.33380	0.4973
11	10.180	VV	0.3272	1.75632e4	762.16064	0.6150
12	10.762	VV	0.4190	2.77285e4	865.79584	0.9710
13	11.483	VV	0.2932	1.91770e5	9524.25781	6.7151
14	12.125	VV	0.3023	7.82229e4	3708.25610	2.7391
15	12.703	VV	0.2725	1.26780e4	651.24933	0.4439
16	13.425	VV	0.4931	6.32784e4	1697.94202	2.2158
17	14.667	VV	0.3490	3.02332e5	1.23184e4	10.5865
18	16.746	VV	0.3495	6.11221e5	2.58338e4	21.4027
19	18.108	VV	0.4561	1.17789e5	3738.79443	4.1245
20	19.532	VBA	0.3698	3.66406e4	1580.66943	1.2830
Totals :				2.85581e6	1.79236e5	



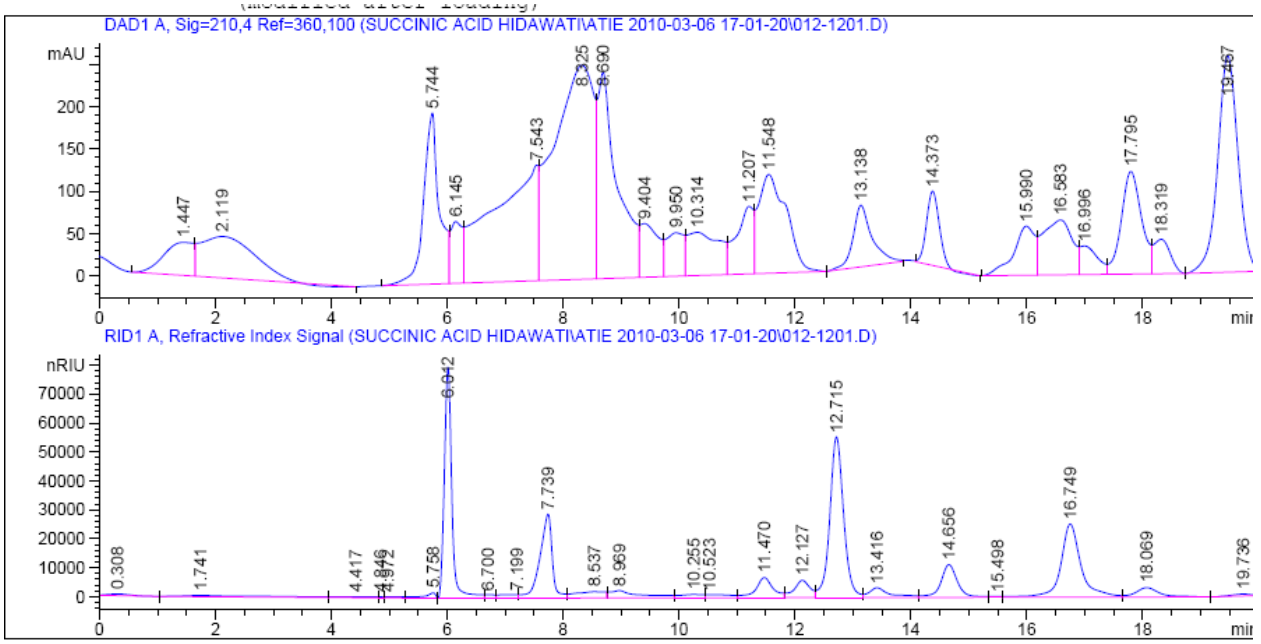
Signal 2: RID1 A, Refractive Index Signal

Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	0.248	BV	0.3801	2.02939e4	929.09363	0.6428
2	1.691	VV	0.6680	2.14661e4	448.91223	0.6799
3	4.374	BV	0.3209	816.53320	31.14940	0.0259
4	5.764	VV	0.1375	1.35947e4	1508.89673	0.4306
5	6.018	VV	0.1320	7.23978e5	8.30261e4	22.9315
6	6.715	VV	0.1818	8455.25000	650.79083	0.2678
7	7.108	VV	0.2517	1.25572e4	674.55731	0.3977
8	7.741	VV	0.2059	4.15667e5	2.90325e4	13.1659
9	8.538	VV	0.8167	1.20464e5	1827.23059	3.8156
10	10.166	VV	0.3518	1.25521e4	522.07489	0.3976
11	10.766	VV	0.4189	1.64873e4	517.97809	0.5222
12	11.479	VV	0.2662	2.09703e5	1.16499e4	6.6422
13	12.128	VV	0.2636	1.00401e5	5647.27734	3.1801
14	12.713	VV	0.2556	7.59799e4	4443.69141	2.4066
15	13.424	VV	0.2864	8.75883e4	4480.39893	2.7743
16	14.664	VV	0.3103	2.98654e5	1.41909e4	9.4597
17	16.749	VV	0.3479	8.94122e5	3.80016e4	28.3206
18	18.085	VV	0.3964	9.01536e4	3416.53931	2.8555
19	19.522	VBA	0.3665	3.42052e4	1458.57312	1.0834
Totals :				3.15714e6	2.02458e5	



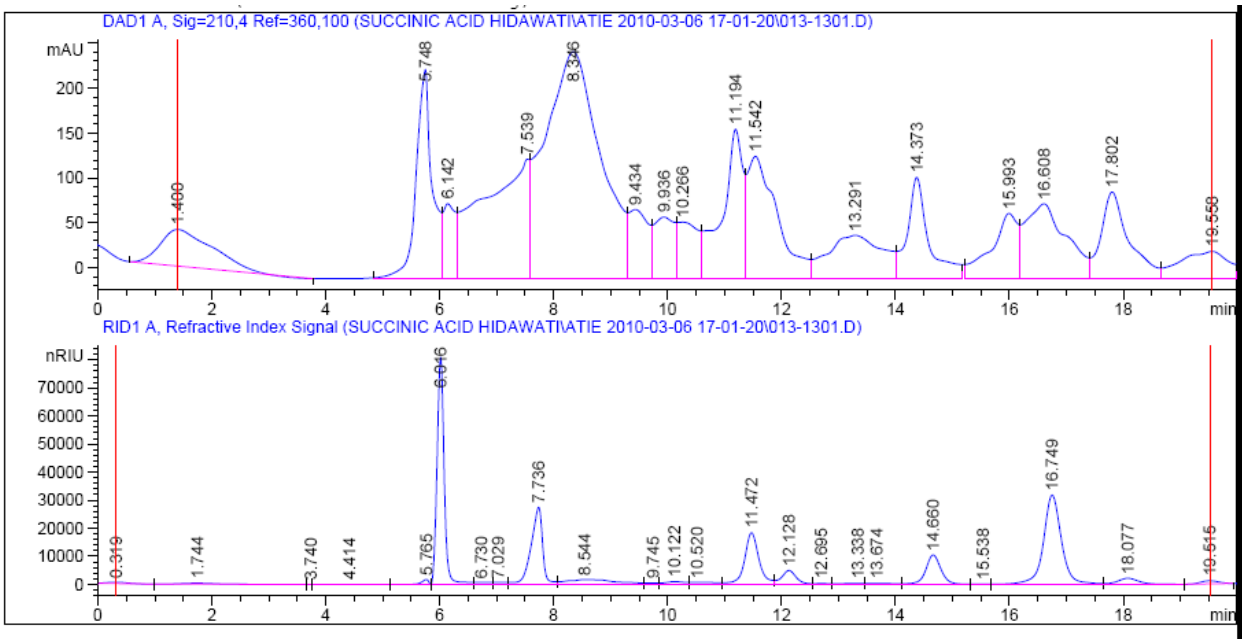
Signal 2: RID1 A, Refractive Index Signal

Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	0.272	BV	0.4006	2.35470e4	982.36810	0.6209
2	1.711	VV	0.8266	3.12785e4	501.75986	0.8247
3	3.733	VV	0.0725	805.04968	147.05412	0.0212
4	3.870	VV	0.1484	2011.91467	165.44554	0.0530
5	4.381	VV	0.5970	1.24278e4	251.97069	0.3277
6	4.885	VV	0.1449	3192.15430	277.63898	0.0842
7	5.076	VV	0.0713	1557.73035	300.31613	0.0411
8	5.758	VV	0.2069	2.63944e4	1764.52136	0.6960
9	6.012	VV	0.1356	7.21700e5	7.98836e4	19.0294
10	7.727	VV	0.2173	4.38989e5	2.87120e4	11.5750
11	8.528	VV	0.4515	7.30814e4	2110.75781	1.9270
12	8.961	VV	0.5025	9.19417e4	2324.62207	2.4243
13	10.245	VV	0.3442	2.49071e4	993.75269	0.6567
14	10.743	VV	0.4064	2.84011e4	912.24933	0.7489
15	11.485	VV	0.2956	1.54102e5	7508.62256	4.0633
16	12.125	VV	0.2747	9.92309e4	5245.95361	2.6165
17	12.710	VV	0.2493	1.06520e6	6.43588e4	28.0865
18	13.421	VV	0.2973	6.93812e4	3387.31763	1.8294
19	13.824	VV	0.2551	1.61424e4	897.95459	0.4256
20	14.661	VV	0.3429	2.74544e5	1.15161e4	7.2390
21	16.743	VV	0.3569	5.21000e5	2.14435e4	13.7374
22	18.091	VV	0.4313	9.61629e4	3253.03955	2.5356
23	19.751	VBA	0.4040	1.65659e4	629.98352	0.4368
Totals :				3.79256e6	2.37569e5	



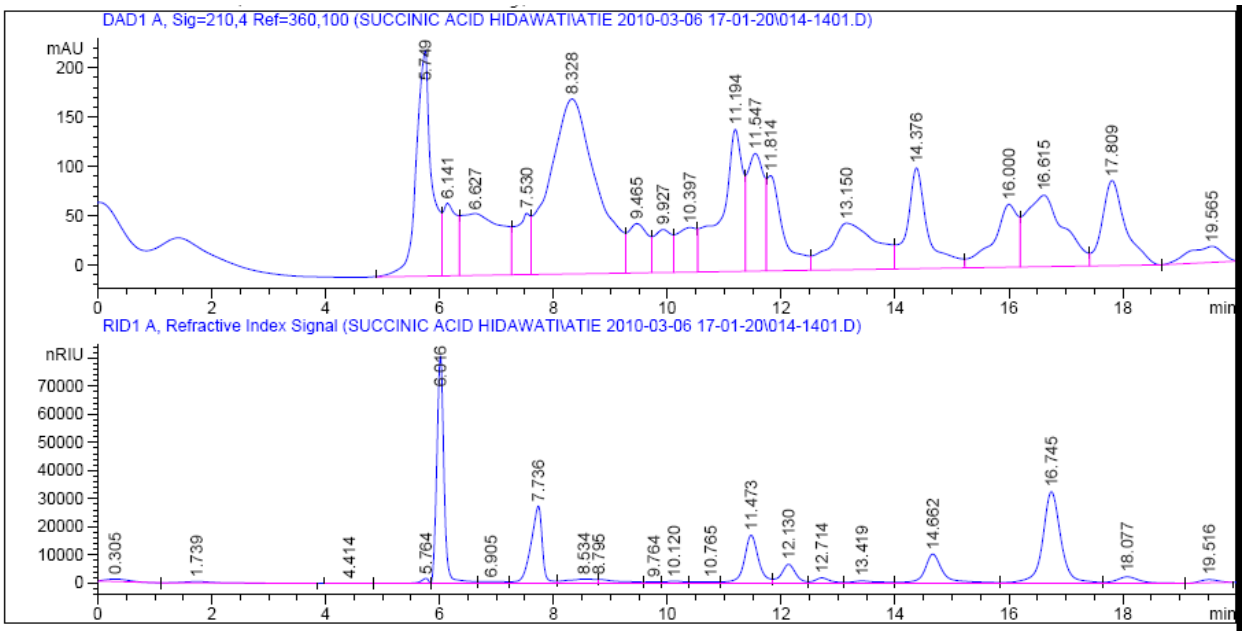
Signal 2: RID1 A, Refractive Index Signal

Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	0.308	BV	0.3866	1.37478e4	542.08893	0.3671
2	1.741	VV	1.3126	4.60196e4	442.54752	1.2288
3	4.417	VV	0.5829	1.76985e4	365.08615	0.4726
4	4.846	VV	0.0723	2199.77148	390.02277	0.0587
5	4.972	VV	0.2773	9068.27832	406.88684	0.2421
6	5.758	VV	0.1979	2.59913e4	1831.77258	0.6940
7	6.012	VV	0.1346	7.14290e5	7.98339e4	19.0732
8	6.700	VV	0.1754	1.39372e4	1136.68689	0.3722
9	7.199	VV	0.2949	2.66968e4	1196.52344	0.7129
10	7.739	VV	0.2110	4.27787e5	2.90038e4	11.4229
11	8.537	VV	0.4889	7.75109e4	2176.76196	2.0697
12	8.969	VV	0.4774	9.44915e4	2538.73413	2.5231
13	10.255	VV	0.3816	3.27756e4	1159.43066	0.8752
14	10.523	VV	0.3908	3.25125e4	1045.85315	0.8682
15	11.470	VV	0.2977	1.42609e5	6949.20166	3.8080
16	12.127	VV	0.2732	1.12766e5	6002.76562	3.0111
17	12.715	VV	0.2496	9.20246e5	5.55084e4	24.5727
18	13.416	VV	0.3470	8.40037e4	3369.40820	2.2431
19	14.656	VV	0.2967	2.25394e5	1.13378e4	6.0185
20	15.498	VV	0.1870	4533.68945	296.56021	0.1211
21	16.749	VV	0.3555	6.11396e5	2.52924e4	16.3257
22	18.069	VV	0.4222	9.32001e4	3259.04102	2.4887
23	19.736	VBA	0.3682	1.61239e4	677.89368	0.4305
Totals :				3.74500e6	2.34764e5	



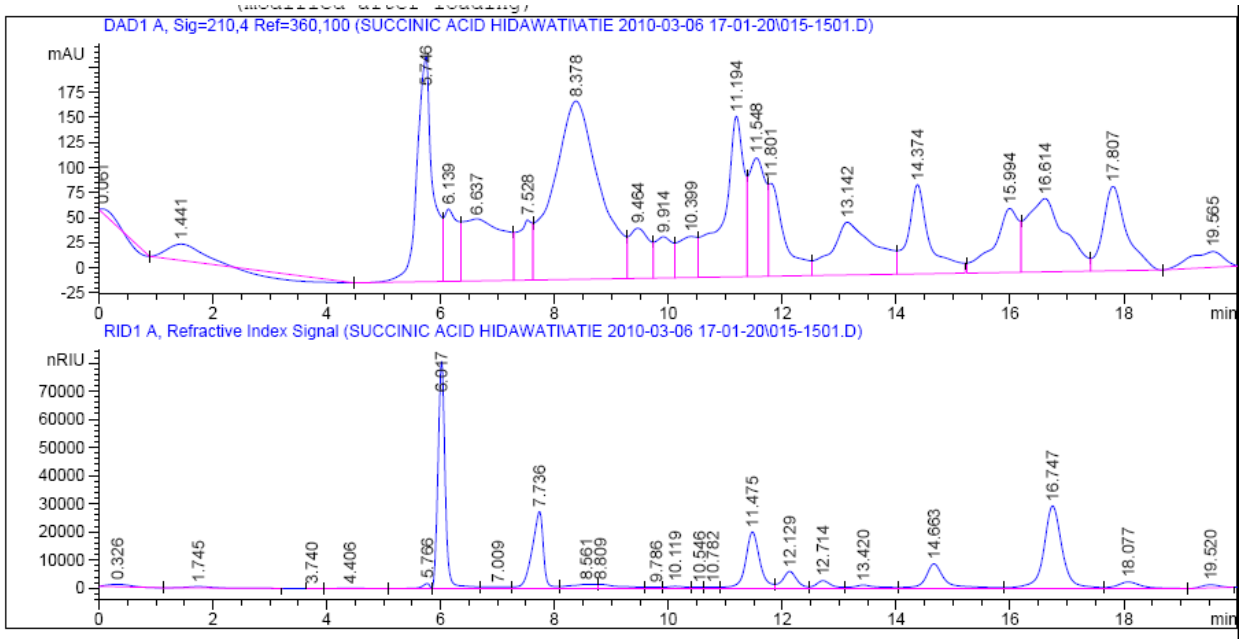
Signal 2: RID1 A, Refractive Index Signal

Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	0.319	BV	0.3782	1.06996e4	427.75833	0.3911
2	1.744	VV	0.5911	1.61699e4	385.31326	0.5911
3	3.740	VV	0.0629	7.36573	1.80019	2.693e-4
4	4.414	VV	0.4135	1866.97424	54.37744	0.0682
5	5.765	VV	0.1305	1.46920e4	1747.05957	0.5371
6	6.016	VV	0.1312	7.01138e5	8.10010e4	25.6304
7	6.730	VV	0.2575	1.44381e4	755.67432	0.5278
8	7.029	VV	0.2289	1.08924e4	684.15533	0.3982
9	7.736	VV	0.2053	3.87816e5	2.75463e4	14.1768
10	8.544	VV	0.7594	1.06197e5	1735.09521	3.8821
11	9.745	VV	0.2180	8196.26660	521.34625	0.2996
12	10.122	VV	0.3368	2.46370e4	1024.08521	0.9006
13	10.520	VV	0.4135	2.23651e4	721.49664	0.8176
14	11.472	VV	0.2613	3.23509e5	1.83956e4	11.8260
15	12.128	VV	0.2679	8.90907e4	4958.42871	3.2568
16	12.695	VV	0.2529	5224.49316	296.70975	0.1910
17	13.338	VV	0.3862	1.01353e4	344.77637	0.3705
18	13.674	VV	0.4497	1.16020e4	349.71027	0.4241
19	14.660	VV	0.2822	1.95052e5	1.04660e4	7.1302
20	15.538	VV	0.2692	2261.82324	108.46830	0.0827
21	16.749	VV	0.3301	6.99618e5	3.17963e4	25.5749
22	18.077	VV	0.3940	5.59438e4	2137.14233	2.0451
23	19.515	VV	0.3514	2.40151e4	1093.07141	0.8779
Totals :				2.73557e6	1.86552e5	



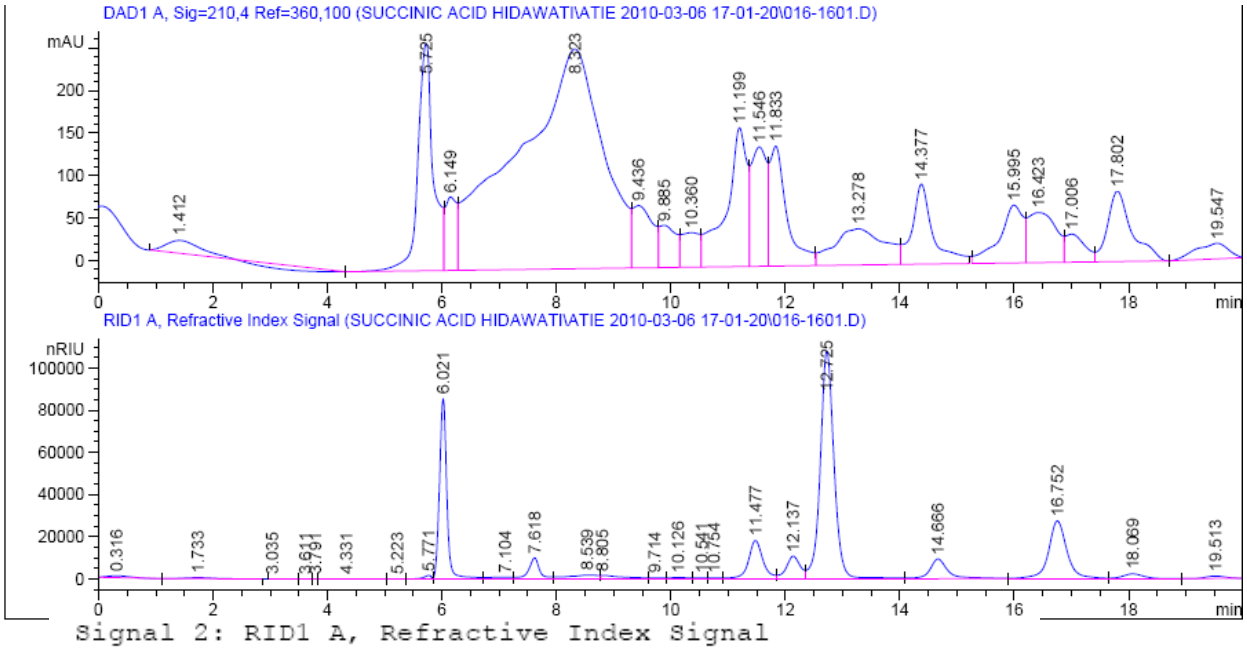
Signal 2: RID1 A, Refractive Index Signal

Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	0.305	BV	0.4355	2.26770e4	852.87219	0.8001
2	1.739	VV	0.4392	1.40193e4	481.26855	0.4946
3	4.414	VV	0.3305	1296.75513	50.38675	0.0458
4	5.764	VV	0.1356	1.56757e4	1772.53650	0.5531
5	6.016	VV	0.1321	7.06048e5	8.08781e4	24.9117
6	6.905	VV	0.4092	2.04354e4	618.61316	0.7210
7	7.736	VV	0.2028	3.80860e5	2.74695e4	13.4380
8	8.534	VV	0.4478	5.21380e4	1528.01575	1.8396
9	8.795	VV	0.3664	3.69871e4	1353.18311	1.3050
10	9.764	VV	0.2530	8188.59961	455.33551	0.2889
11	10.120	VV	0.3252	1.81652e4	787.76862	0.6409
12	10.765	VV	0.3921	1.67173e4	555.49634	0.5898
13	11.473	VV	0.2625	3.02087e5	1.70777e4	10.6586
14	12.130	VV	0.2624	1.19111e5	6739.25635	4.2026
15	12.714	VV	0.2788	3.71675e4	1928.90515	1.3114
16	13.419	VV	0.4605	2.91338e4	849.68866	1.0279
17	14.662	VV	0.3324	2.37881e5	1.03729e4	8.3932
18	16.745	VV	0.3316	7.26539e5	3.25560e4	25.6347
19	18.077	VV	0.4157	6.44963e4	2299.91162	2.2756
20	19.516	VV	0.3486	2.45803e4	1121.58057	0.8673
Totals :				2.83420e6	1.89749e5	



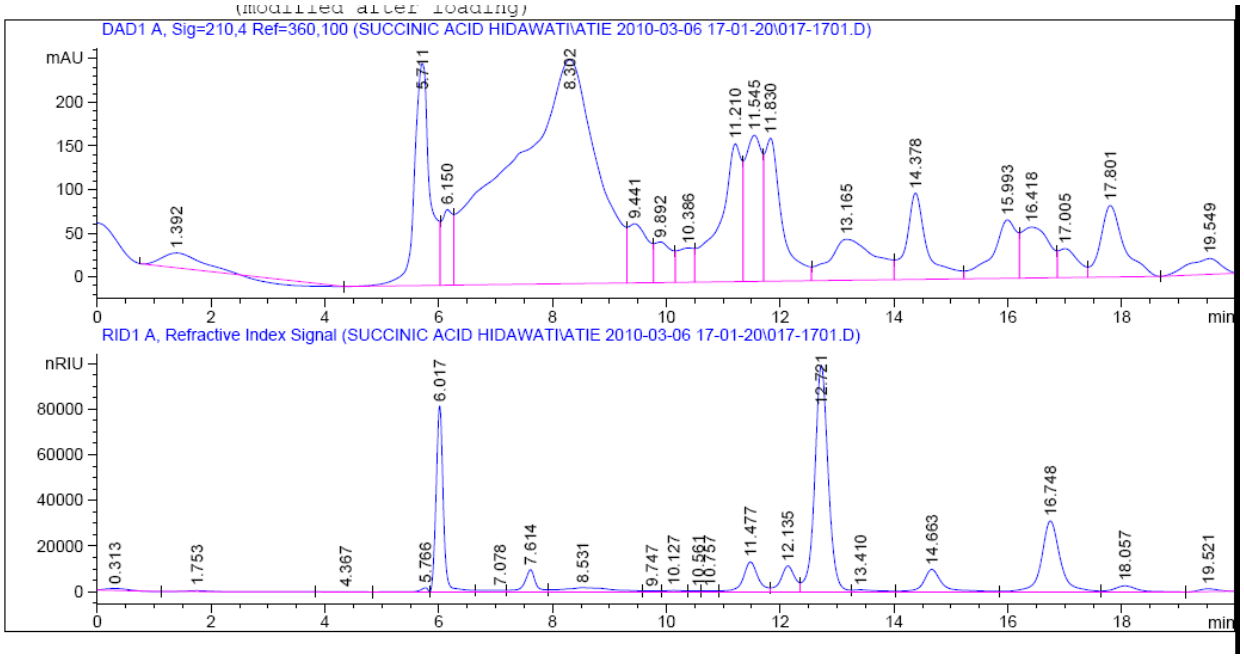
Signal 2: RID1 A, Refractive Index Signal

Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	0.326	BV	0.4668	2.33682e4	869.73962	0.8344
2	1.745	VV	0.4517	1.41470e4	474.23206	0.5052
3	3.740	VV	0.2175	71.91370	4.19032	2.568e-3
4	4.406	VV	0.3724	1224.57556	40.97334	0.0437
5	5.766	VV	0.1302	1.49173e4	1740.28625	0.5327
6	6.017	VV	0.1320	7.03363e5	8.06758e4	25.1155
7	7.009	VV	0.4152	1.98880e4	613.10693	0.7102
8	7.736	VV	0.2023	3.77981e5	2.73407e4	13.4968
9	8.561	VV	0.4177	4.73720e4	1484.30884	1.6915
10	8.809	VV	0.3784	4.10798e4	1438.91272	1.4669
11	9.786	VV	0.2650	8761.26367	474.70715	0.3128
12	10.119	VV	0.3295	1.79007e4	770.15369	0.6392
13	10.546	VV	0.1959	6899.52539	498.91940	0.2464
14	10.782	VV	0.2347	8735.29395	520.50311	0.3119
15	11.475	VV	0.2628	3.51860e5	2.00754e4	12.5641
16	12.129	VV	0.2629	1.07530e5	6067.29297	3.8397
17	12.714	VV	0.2735	5.18018e4	2780.98169	1.8497
18	13.420	VV	0.4255	3.77642e4	1206.85547	1.3485
19	14.663	VV	0.3463	2.11879e5	8780.67090	7.5657
20	16.747	VV	0.3359	6.60685e5	2.93636e4	23.5915
21	18.077	VV	0.4323	6.90722e4	2344.24438	2.4664
22	19.520	VV	0.3479	2.42149e4	1117.40820	0.8647
Totals :				2.80052e6	1.88683e5	



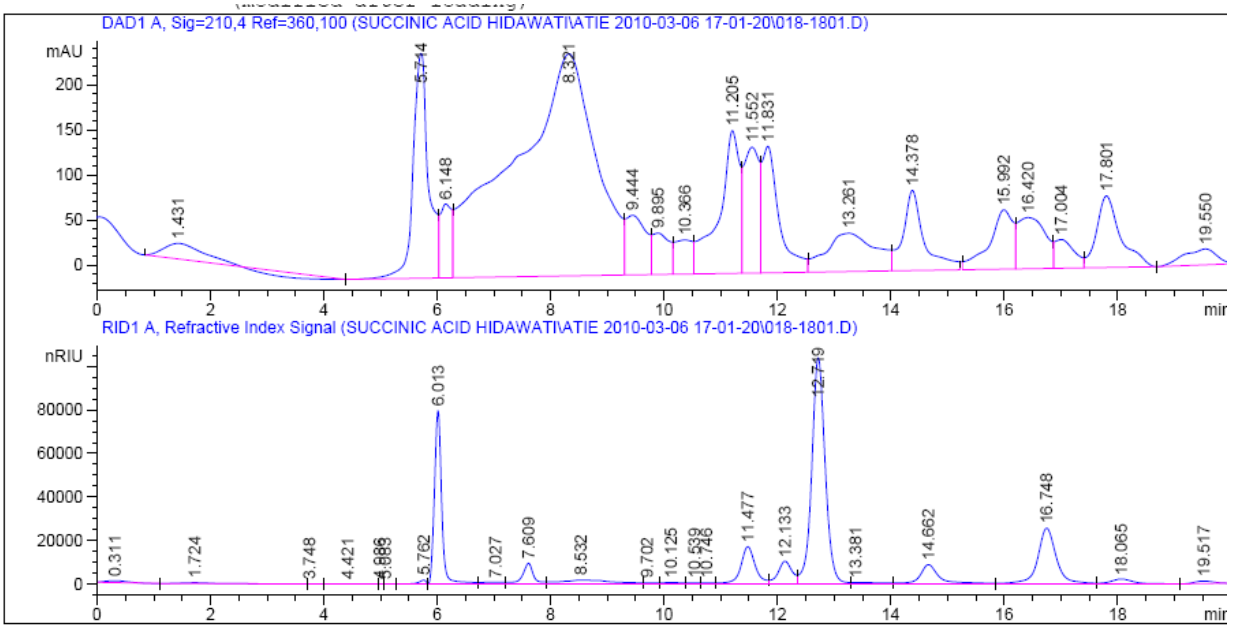
Signal 2: RID1 A, Refractive Index Signal

Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	0.316	BV	0.4697	2.40209e4	879.92883	0.5508
2	1.733	VV	0.4793	1.61720e4	508.20465	0.3708
3	3.035	VV	0.2475	101.23600	4.89940	2.321e-3
4	3.611	VV	0.1271	59.03838	6.27025	1.354e-3
5	3.791	VV	0.0789	38.09484	6.52384	8.735e-4
6	4.331	VV	0.4456	958.44257	25.59212	0.0220
7	5.223	VV	0.1905	869.62268	65.96925	0.0199
8	5.771	VV	0.1356	1.53923e4	1704.67371	0.3529
9	6.021	VV	0.1362	7.61770e5	8.55864e4	17.4674
10	7.104	VV	0.3925	2.41767e4	797.64331	0.5544
11	7.618	VV	0.1915	1.32683e5	1.00001e4	3.0424
12	8.539	VV	0.5224	6.94462e4	1813.37231	1.5924
13	8.805	VV	0.3818	4.72370e4	1638.01880	1.0831
14	9.714	VV	0.2587	8769.60449	505.24078	0.2011
15	10.126	VV	0.3281	1.51801e4	656.45947	0.3481
16	10.541	VV	0.2080	6995.64648	470.55847	0.1604
17	10.754	VV	0.2222	7310.54443	470.76334	0.1676
18	11.477	VV	0.2634	3.19682e5	1.81856e4	7.3303
19	12.137	VV	0.2524	1.82870e5	1.07531e4	4.1932
20	12.725	VV	0.2515	1.81076e6	1.08163e5	41.5206
21	14.666	VV	0.3329	2.13875e5	9383.37891	4.9041
22	16.752	VV	0.3335	6.17020e5	2.74543e4	14.1482
23	18.069	VV	0.3880	5.90256e4	2299.71704	1.3535
24	19.513	VBA	0.3583	2.66978e4	1163.87854	0.6122
Totals :				4.36111e6	2.82544e5	



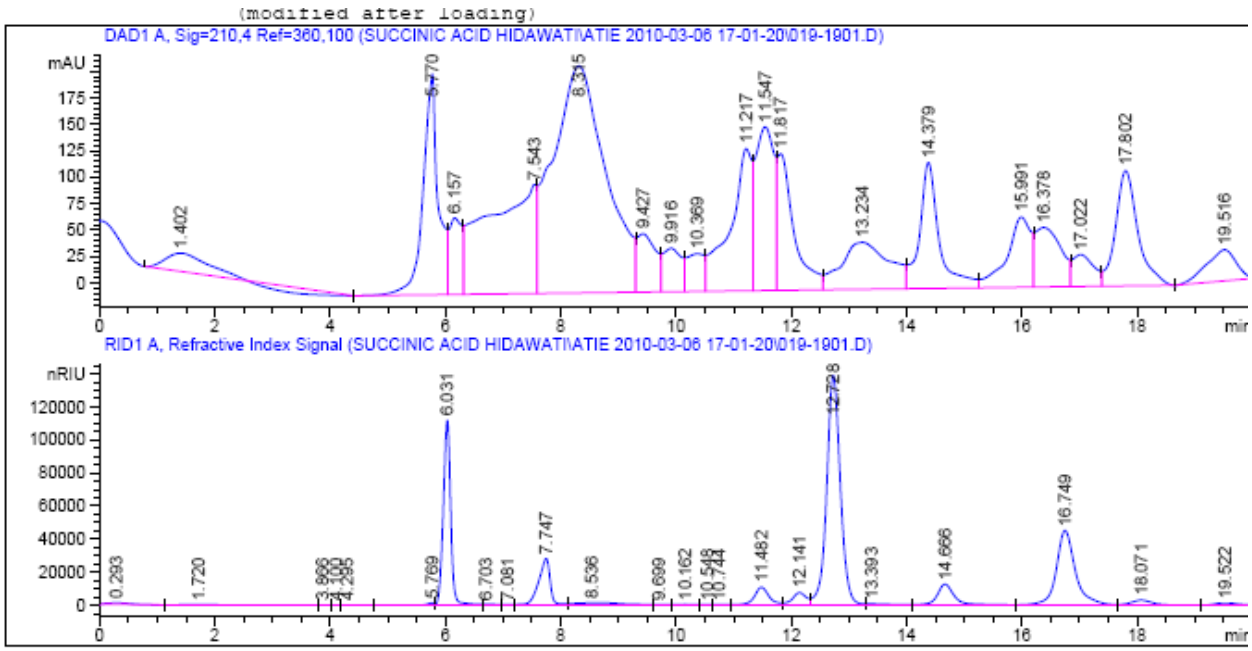
Signal 2: RID1 A, Refractive Index Signal

Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	0.313	BV	0.4294	2.34513e4	893.19775	0.5500
2	1.753	VV	0.4444	1.19041e4	412.81744	0.2792
3	4.367	VV	0.3352	1443.00098	53.32185	0.0338
4	5.766	VV	0.1387	1.73967e4	1871.70422	0.4080
5	6.017	VV	0.1329	7.17159e5	8.15296e4	16.8200
6	7.078	VV	0.3985	2.63504e4	834.88245	0.6180
7	7.614	VV	0.1958	1.33097e5	9763.34668	3.1216
8	8.531	VV	0.8099	1.21246e5	1920.77295	2.8437
9	9.747	VV	0.2749	1.10784e4	557.89752	0.2598
10	10.127	VV	0.3371	1.61505e4	681.06842	0.3788
11	10.561	VV	0.1925	7196.54150	539.10345	0.1688
12	10.757	VV	0.2497	9915.17676	566.05212	0.2325
13	11.477	VV	0.2738	2.43510e5	1.31858e4	5.7112
14	12.135	VV	0.2559	1.97926e5	1.14391e4	4.6421
15	12.721	VV	0.2504	1.63493e6	9.93099e4	38.3451
16	13.410	VV	0.4328	3.17602e4	983.81946	0.7449
17	14.663	VV	0.3425	2.36504e5	9936.81641	5.5469
18	16.748	VV	0.3414	7.15792e5	3.11682e4	16.7880
19	18.057	VV	0.4278	7.89916e4	2716.62329	1.8526
20	19.521	VV	0.3594	2.79200e4	1232.32275	0.6548
Totals :				4.26372e6	2.69596e5	



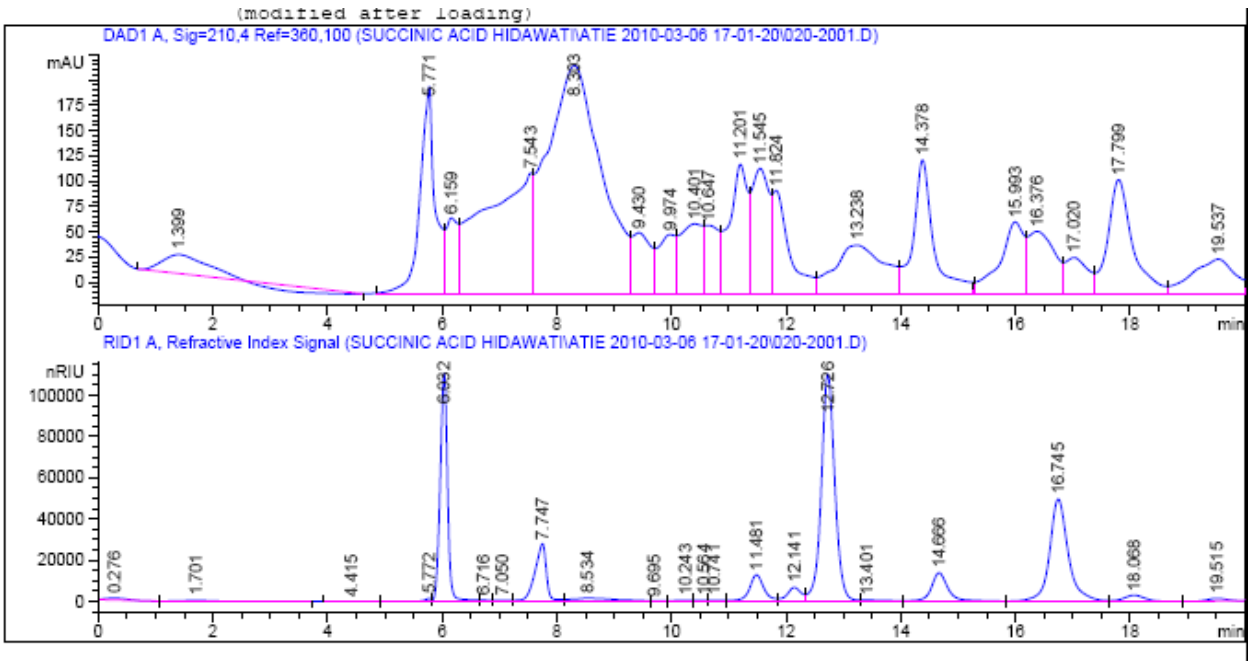
Signal 2: RID1 A, Refractive Index Signal

Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	0.311	BV	0.4340	2.22181e4	845.42120	0.5316
2	1.724	VV	0.4460	1.12705e4	386.49957	0.2696
3	3.748	VV	0.1341	19.71108	1.89779	4.716e-4
4	4.421	VV	0.3595	862.22260	29.58085	0.0206
5	4.986	VV	0.0691	9.60843	1.92418	2.299e-4
6	5.083	VV	0.0682	11.92112	2.33399	2.852e-4
7	5.762	VV	0.1324	1.50598e4	1756.99988	0.3603
8	6.013	VV	0.1360	7.07483e5	7.96979e4	16.9262
9	7.027	VV	0.3595	2.13444e4	771.45447	0.5107
10	7.609	VV	0.1930	1.27732e5	9534.71777	3.0559
11	8.532	VV	0.8264	1.16417e5	1809.09875	2.7852
12	9.702	VV	0.2497	8855.14355	533.88721	0.2119
13	10.125	VV	0.3411	1.58014e4	662.10748	0.3780
14	10.539	VV	0.2186	7590.63818	492.77759	0.1816
15	10.746	VV	0.2172	7301.55615	483.44717	0.1747
16	11.477	VV	0.2650	3.03864e5	1.71491e4	7.2698
17	12.133	VV	0.2537	1.78920e5	1.04543e4	4.2806
18	12.719	VV	0.2490	1.72020e6	1.04090e5	41.1549
19	13.381	VV	0.4606	2.47333e4	687.69897	0.5917
20	14.662	VV	0.3444	2.13231e5	8896.84180	5.1014
21	16.748	VV	0.3405	5.87617e5	2.56668e4	14.0584
22	18.065	VV	0.4124	6.28316e4	2263.54053	1.5032
23	19.517	VV	0.3574	2.64412e4	1165.98926	0.6326
Totals :				4.17981e6	2.67384e5	



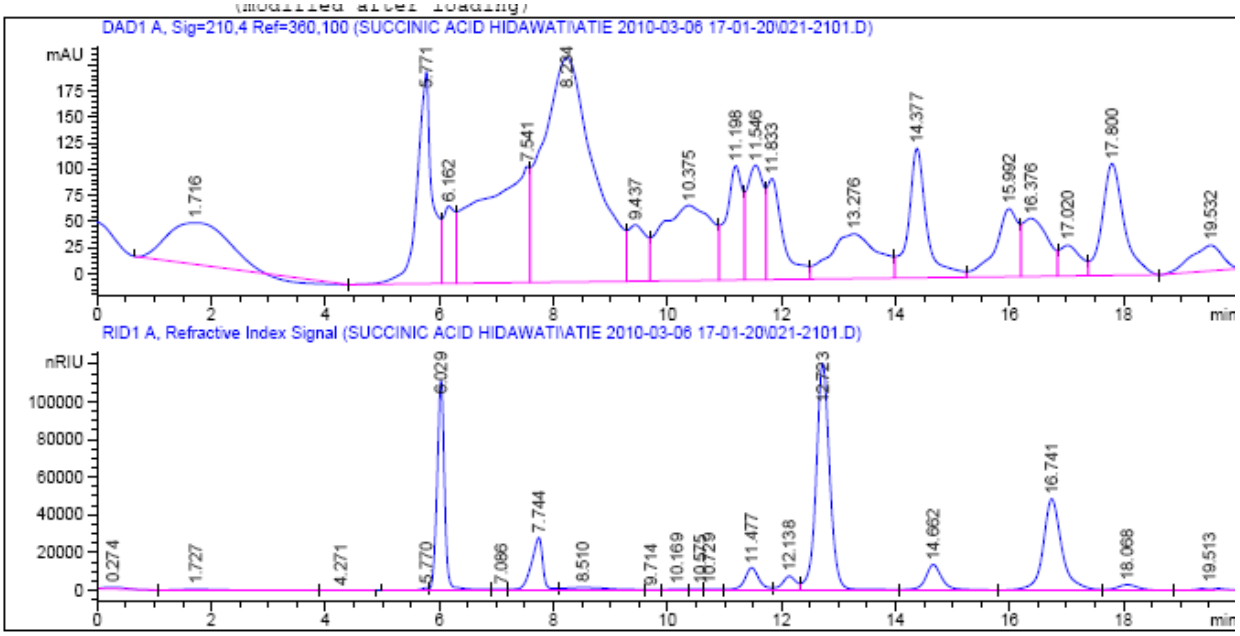
Signal 2: RID1 A, Refractive Index Signal

Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	0.293	BV	0.4192	2.04096e4	810.03534	0.3569
2	1.720	VV	0.4754	1.29119e4	412.39325	0.2258
3	3.866	VV	0.1632	53.25336	4.07668	9.312e-4
4	4.100	VV	0.1203	100.08095	11.83039	1.750e-3
5	4.295	VV	0.2651	518.39777	23.19140	9.065e-3
6	5.769	VV	0.1408	1.39040e4	1467.62183	0.2431
7	6.031	VV	0.1352	1.00692e6	1.11957e5	17.6079
8	6.703	VV	0.2424	1.16093e4	619.49738	0.2030
9	7.081	VV	0.1771	7838.67871	588.29462	0.1371
10	7.747	VV	0.2045	4.01726e5	2.83064e4	7.0249
11	8.536	VV	0.7197	9.54162e4	1661.44055	1.6685
12	9.699	VV	0.2700	8268.01758	446.60361	0.1446
13	10.162	VV	0.3656	1.46265e4	584.01172	0.2558
14	10.548	VV	0.1850	5791.42383	449.25095	0.1013
15	10.744	VV	0.2478	7900.61865	450.46552	0.1382
16	11.482	VV	0.2738	2.01413e5	1.09071e4	3.5221
17	12.141	VV	0.2527	1.32031e5	7670.11133	2.3088
18	12.728	VV	0.2519	2.30731e6	1.39060e5	40.3474
19	13.393	VV	0.4502	2.89344e4	842.86951	0.5060
20	14.666	VV	0.3199	2.78582e5	1.27428e4	4.8715
21	16.749	VV	0.3431	1.04651e6	4.52770e4	18.3000
22	18.071	VV	0.4037	8.32847e4	3083.65894	1.4564
23	19.522	VBA	0.3671	3.25495e4	1395.64136	0.5692
Totals :				5.71861e6	3.68771e5	



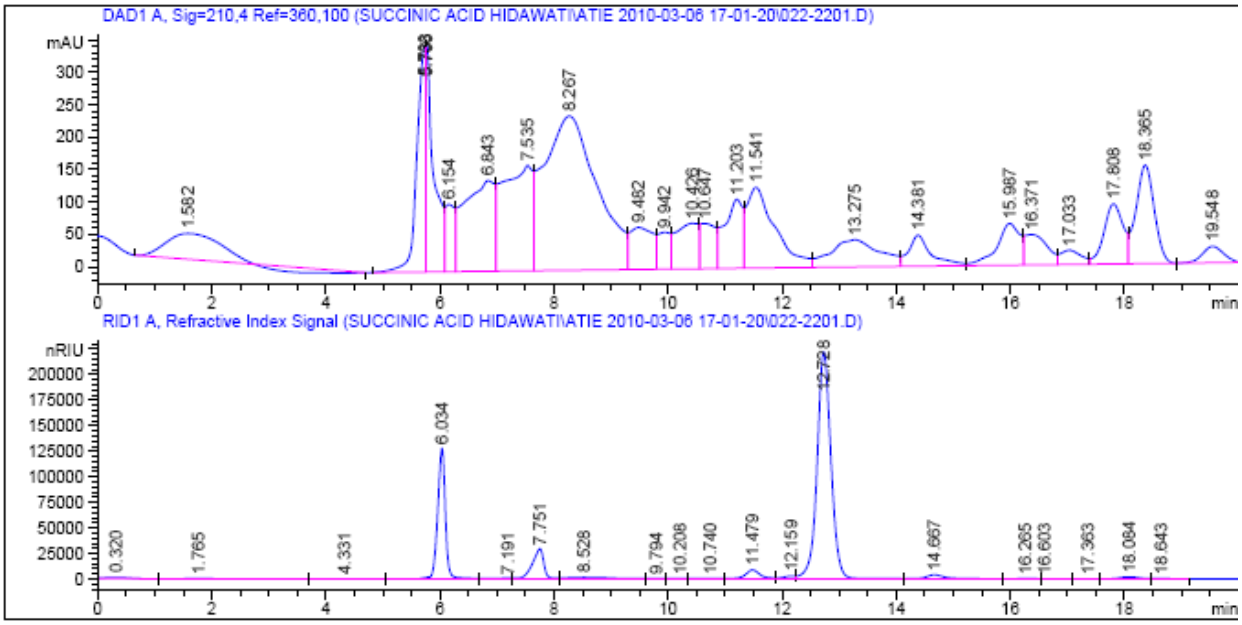
Signal 2: RID1 A, Refractive Index Signal

Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	0.276	BV	0.4098	2.25336e4	924.11322	0.4243
2	1.701	VV	0.4626	1.40045e4	455.23438	0.2637
3	4.415	VV	0.3386	783.04523	27.32987	0.0147
4	5.772	VV	0.1388	1.35807e4	1431.36572	0.2557
5	6.032	VV	0.1348	9.92569e5	1.10792e5	18.6890
6	6.716	VV	0.1812	8599.12305	655.00513	0.1619
7	7.050	VV	0.2604	1.30274e4	648.13629	0.2453
8	7.747	VV	0.2044	3.98884e5	2.81229e4	7.5106
9	8.534	VV	0.7139	9.43018e4	1656.33557	1.7756
10	9.695	VV	0.2521	7353.05908	419.28650	0.1385
11	10.243	VV	0.3352	1.33980e4	573.49426	0.2523
12	10.564	VV	0.2133	9073.51270	606.78857	0.1708
13	10.741	VV	0.2616	1.13475e4	612.09540	0.2137
14	11.481	VV	0.2671	2.34118e5	1.29479e4	4.4082
15	12.141	VV	0.2510	1.16564e5	6827.42041	2.1948
16	12.726	VV	0.2480	1.81413e6	1.10303e5	34.1581
17	13.401	VV	0.4423	2.35363e4	699.35071	0.4432
18	14.666	VV	0.3075	2.91178e5	1.38711e4	5.4826
19	16.745	VV	0.3358	1.12546e6	4.96373e4	21.1911
20	18.068	VV	0.3846	7.65036e4	2992.66064	1.4405
21	19.515	VBA	0.3576	3.00373e4	1312.98914	0.5656
Totals :				5.31098e6	3.45516e5	



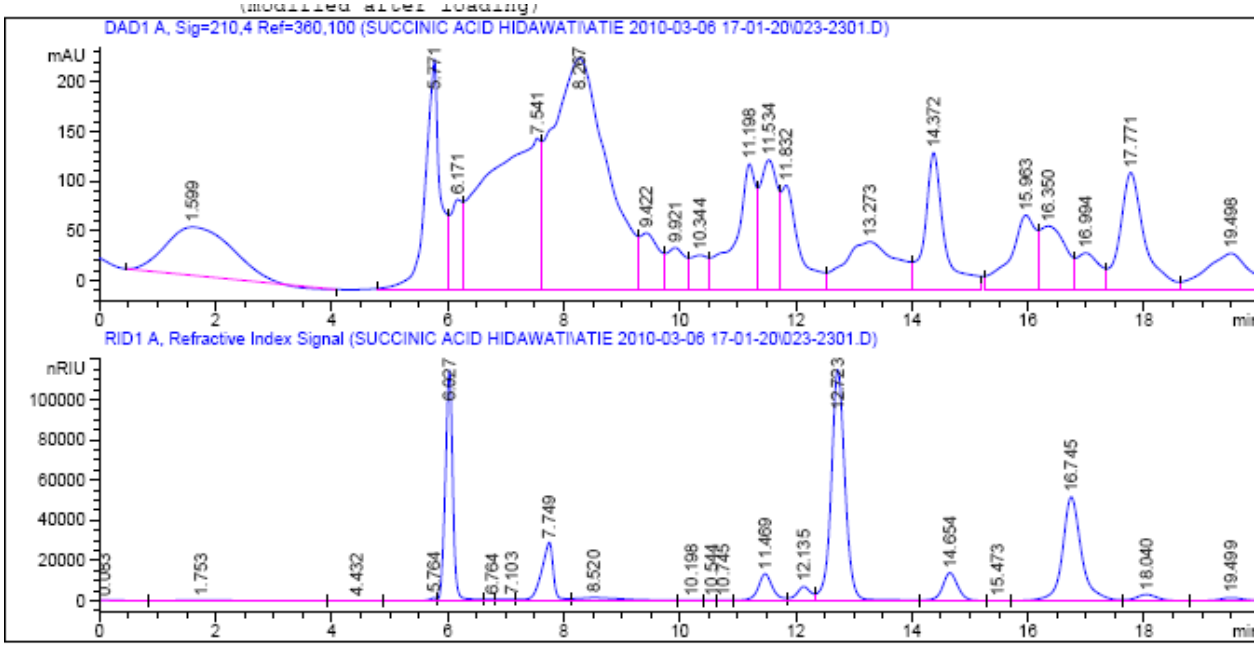
Signal 2: RID1 A, Refractive Index Signal

Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	0.274	BV	0.4036	2.22637e4	926.09399	0.4069
2	1.727	VV	0.5871	2.25561e4	551.77106	0.4122
3	4.271	VV	0.3162	989.30304	38.89694	0.0181
4	5.770	VV	0.1329	1.33288e4	1453.25452	0.2436
5	6.029	VV	0.1391	1.02009e6	1.11491e5	18.6434
6	7.086	VV	0.2122	1.04430e4	632.17340	0.1909
7	7.744	VV	0.2057	3.95910e5	2.80541e4	7.2357
8	8.510	VV	0.6967	9.12719e4	1645.64233	1.6681
9	9.714	VV	0.2612	7474.78369	412.24725	0.1366
10	10.169	VV	0.3530	1.59268e4	649.57916	0.2911
11	10.575	VV	0.2007	8588.99414	610.94183	0.1570
12	10.729	VV	0.2796	1.21445e4	621.97589	0.2220
13	11.477	VV	0.2687	2.16976e5	1.20302e4	3.9655
14	12.138	VV	0.2526	1.28211e5	7530.21338	2.3432
15	12.723	VV	0.2508	2.00735e6	1.20343e5	36.6868
16	14.662	VV	0.3094	2.85327e5	1.36098e4	5.2147
17	16.741	VV	0.3394	1.10606e6	4.85102e4	20.2146
18	18.068	VV	0.3878	7.59954e4	2963.32544	1.3889
19	19.513	VBA	0.3612	3.06769e4	1323.00964	0.5607
Totals :				5.47159e6	3.53398e5	



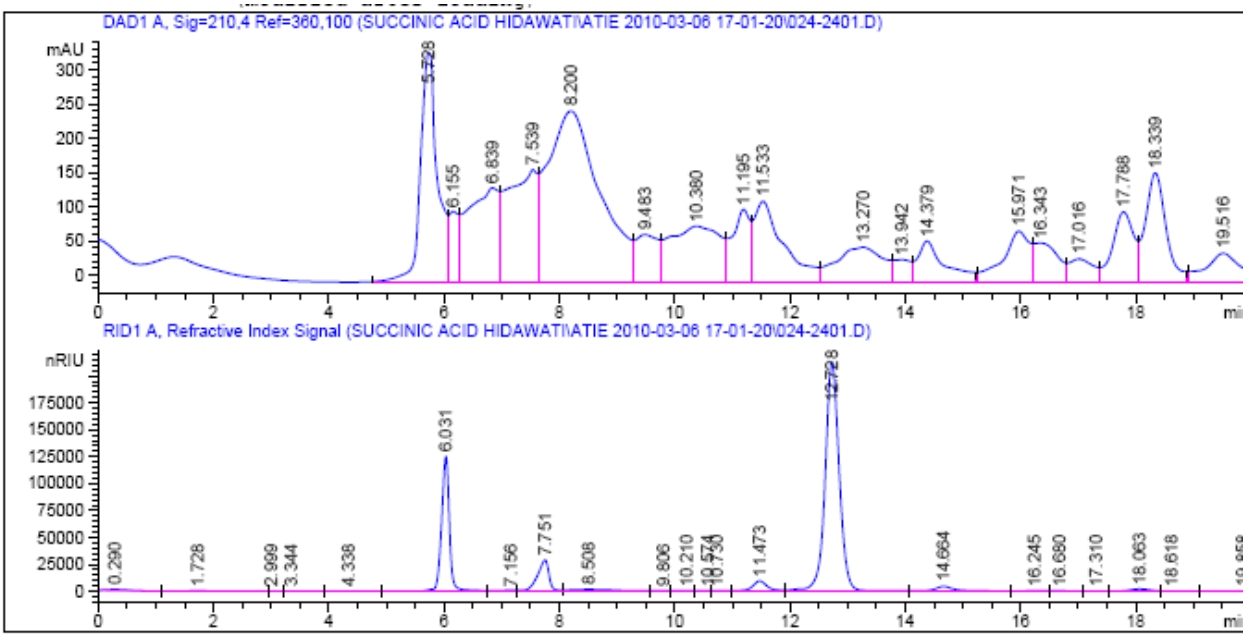
Signal 2: RID1 A, Refractive Index Signal

Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	0.320	BV	0.4528	2.84230e4	1076.23987	0.4667
2	1.765	VV	0.5605	2.11006e4	551.95099	0.3464
3	4.331	VV	0.4289	1205.92151	37.31416	0.0198
4	6.034	VV	0.1429	1.21326e6	1.28123e5	19.9205
5	7.191	VV	0.4155	3.08846e4	967.87732	0.5071
6	7.751	VV	0.2064	4.26564e5	2.97075e4	7.0037
7	8.528	VV	0.7526	1.04744e5	1799.36938	1.7198
8	9.794	VV	0.2958	1.05909e4	493.31152	0.1739
9	10.208	VV	0.3110	1.16814e4	534.81738	0.1918
10	10.740	VV	0.4507	2.35417e4	677.62561	0.3865
11	11.479	VV	0.2824	1.73667e5	8956.50391	2.8514
12	12.159	VV	0.2073	4.28371e4	3006.46118	0.7033
13	12.728	VV	0.2573	3.79071e6	2.22305e5	62.2393
14	14.667	VV	0.3899	1.18508e5	4280.71143	1.9458
15	16.265	VV	0.4038	1.43824e4	511.32617	0.2361
16	16.603	VV	0.3431	8118.38086	354.10052	0.1333
17	17.363	VV	0.3380	3849.30688	154.61528	0.0632
18	18.084	VV	0.3790	5.43951e4	2201.09302	0.8931
19	18.643	VV	0.3057	1.20729e4	595.47638	0.1982
Totals :				6.09054e6	4.06335e5	



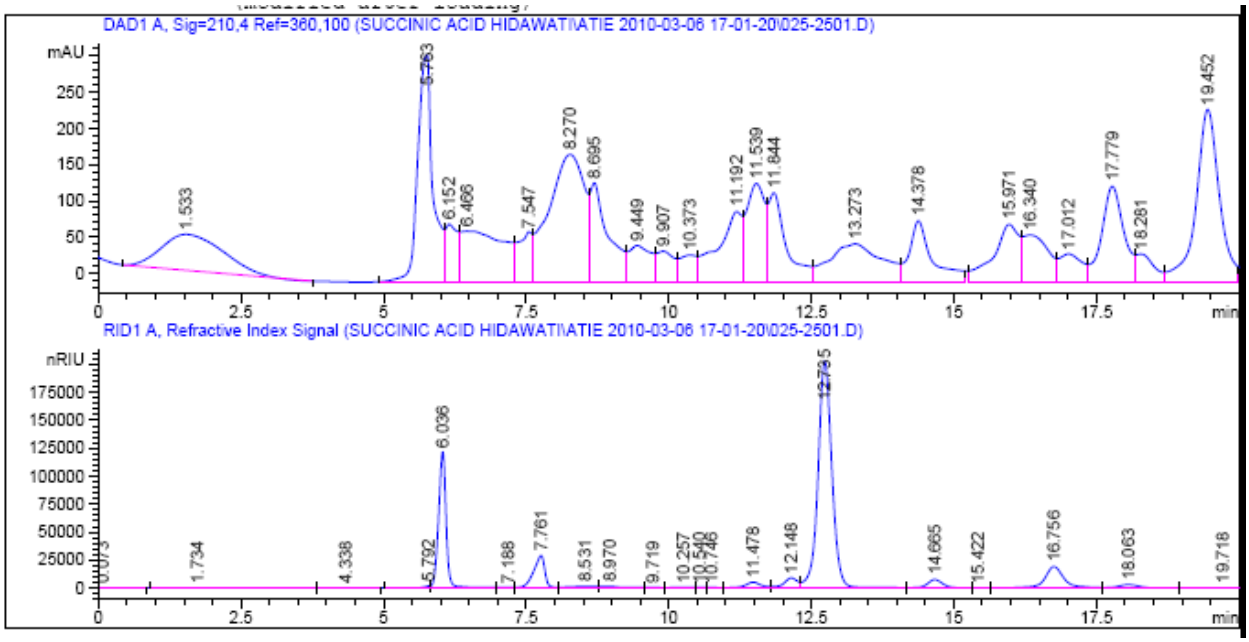
Signal 2: RID1 A, Refractive Index Signal

Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	0.083	BV	0.2992	673.57654	28.75718	0.0124
2	1.753	VV	0.8471	1.91043e4	296.36526	0.3504
3	4.432	VV	0.3399	733.90643	27.45476	0.0135
4	5.764	VV	0.1356	1.37979e4	1497.17224	0.2531
5	6.027	VV	0.1371	1.03056e6	1.14783e5	18.9032
6	6.764	VV	0.1517	7606.69629	717.89795	0.1395
7	7.103	VV	0.2735	1.60339e4	749.11658	0.2941
8	7.749	VV	0.2082	4.22458e5	2.91050e4	7.7490
9	8.520	VV	0.7511	1.02204e5	1700.06128	1.8747
10	10.198	VV	0.3438	1.06193e4	444.08163	0.1948
11	10.544	VV	0.1860	5214.36816	395.94070	0.0956
12	10.745	VV	0.2516	7049.18945	402.95300	0.1293
13	11.469	VV	0.2659	2.38795e5	1.34228e4	4.3801
14	12.135	VV	0.2523	1.20702e5	7101.79297	2.2140
15	12.723	VV	0.2504	1.91275e6	1.14878e5	35.0849
16	14.654	VV	0.2814	2.58246e5	1.39125e4	4.7369
17	15.473	VV	0.2909	2407.94653	107.79530	0.0442
18	16.745	VV	0.3357	1.17018e6	5.16316e4	21.4643
19	18.040	VV	0.3751	7.76897e4	3139.41138	1.4250
20	19.499	VBA	0.3794	3.49450e4	1412.47961	0.6410
Totals :				5.45178e6	3.55754e5	



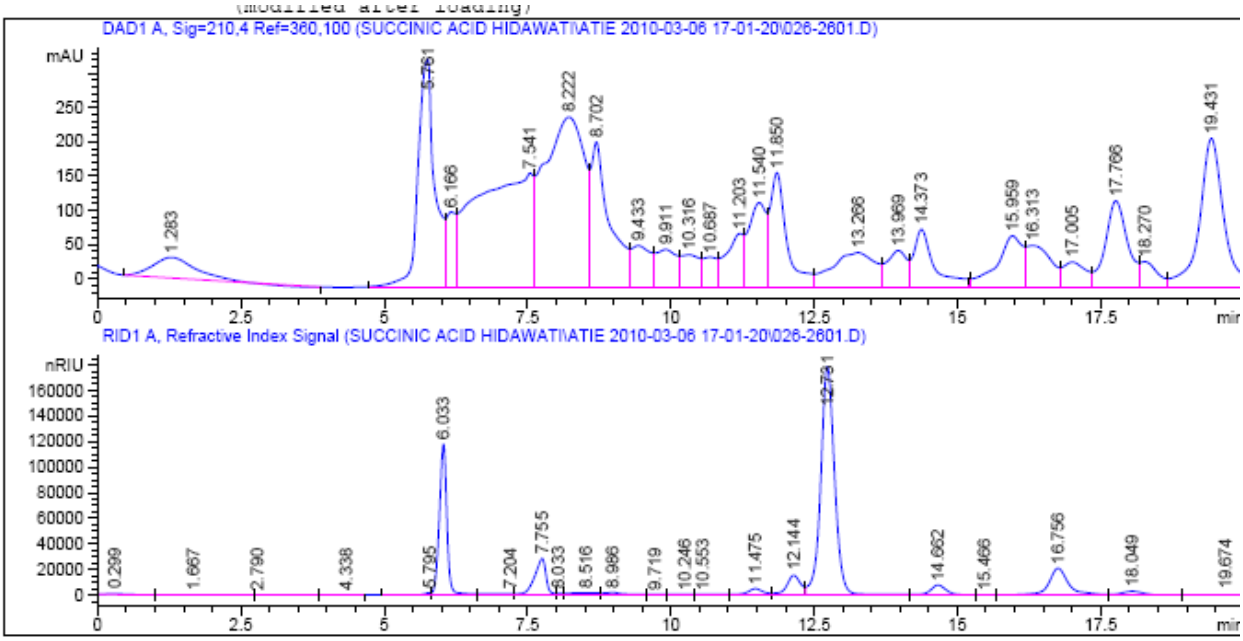
Signal 2: RID1 A, Refractive Index Signal

Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	0.290	BV	0.4117	2.50054e4	1026.16394	0.4263
2	1.728	VV	0.4493	1.16902e4	378.06738	0.1993
3	2.999	VV	0.0413	3.44052	1.83797	5.865e-5
4	3.344	VV	0.1202	54.48816	5.80850	9.289e-4
5	4.338	VV	0.3465	893.20319	31.85692	0.0152
6	6.031	VV	0.1416	1.19479e6	1.25158e5	20.3678
7	7.156	VV	0.3969	2.80924e4	926.67029	0.4789
8	7.751	VV	0.2072	4.13914e5	2.90696e4	7.0561
9	8.508	VV	0.7187	1.04600e5	1829.96289	1.7831
10	9.806	VV	0.2901	1.01655e4	488.91727	0.1733
11	10.210	VV	0.3076	1.26539e4	572.37390	0.2157
12	10.574	VV	0.2431	1.19477e4	689.69531	0.2037
13	10.730	VV	0.2704	1.35866e4	691.10748	0.2316
14	11.473	VV	0.2789	1.81957e5	9529.16699	3.1019
15	12.728	VV	0.2577	3.64233e6	2.13084e5	62.0915
16	14.664	VV	0.3840	1.22450e5	4475.08545	2.0874
17	16.245	VV	0.3942	1.36477e4	503.19342	0.2327
18	16.680	VV	0.3709	9655.64648	381.41931	0.1646
19	17.310	VV	0.3391	3898.09033	155.96716	0.0665
20	18.063	VV	0.3795	5.25331e4	2122.20166	0.8955
21	18.618	VV	0.3099	1.17662e4	570.22852	0.2006
22	19.858	VBA	0.1755	431.43408	50.40704	7.355e-3
Totals :				5.86607e6	3.91742e5	



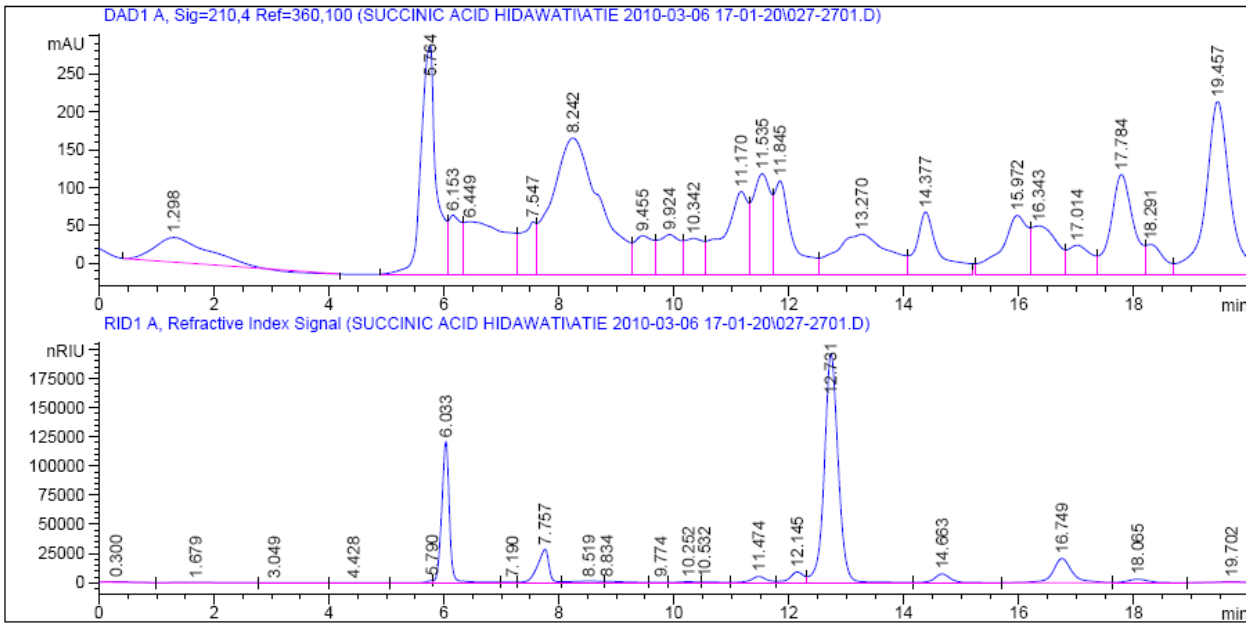
Signal 2: RID1 A, Refractive Index Signal

Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	0.073	BV	0.2480	350.04068	23.52835	5.749e-3
2	1.734	VV	0.7680	1.71098e4	296.61954	0.2810
3	4.338	VV	0.3935	1199.66797	37.63203	0.0197
4	5.792	VV	0.1120	1.34033e4	1771.54626	0.2201
5	6.036	VV	0.1410	1.13552e6	1.22008e5	18.6486
6	7.188	VV	0.2383	1.12863e4	660.40729	0.1854
7	7.761	VV	0.2013	4.04550e5	2.90606e4	6.6439
8	8.531	VV	0.4388	5.02650e4	1515.46033	0.8255
9	8.970	VV	0.3708	3.97973e4	1417.17249	0.6536
10	9.719	VV	0.2971	8894.52734	415.70749	0.1461
11	10.257	VV	0.3272	1.69561e4	718.30804	0.2785
12	10.540	VV	0.1545	4640.84766	428.23331	0.0762
13	10.746	VV	0.2435	6957.98096	409.79343	0.1143
14	11.478	VV	0.2863	1.04407e5	5291.64648	1.7147
15	12.148	VV	0.2491	1.53877e5	9004.00586	2.5271
16	12.735	VV	0.2555	3.43615e6	2.03283e5	56.4319
17	14.665	VV	0.2867	1.43322e5	7535.35742	2.3538
18	15.422	VV	0.2292	1684.75037	92.74756	0.0277
19	16.756	VV	0.3454	4.46530e5	1.91523e4	7.3334
20	18.063	VV	0.3838	7.47779e4	2954.83765	1.2281
21	19.718	VBA	0.4105	1.73460e4	664.26233	0.2849
Totals :				6.08903e6	4.06741e5	

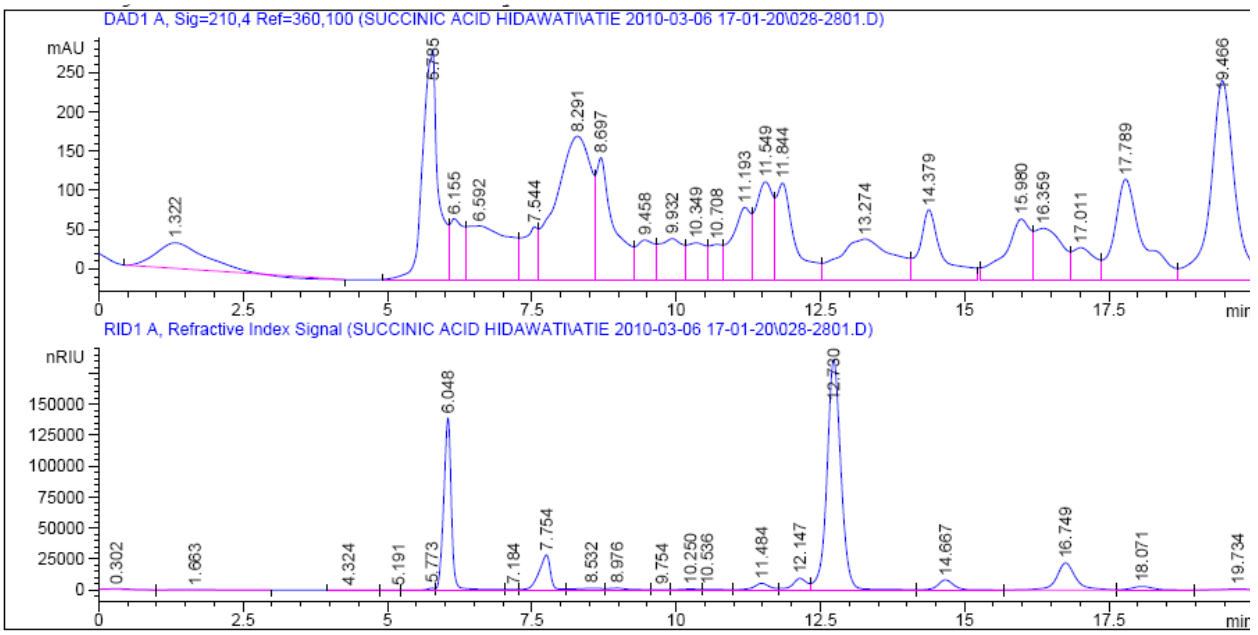


Signal 2: RID1 A, Refractive Index Signal

Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	0.299	BV	0.4038	1.15810e4	470.56198	0.1988
2	1.667	VV	0.5387	7712.92480	197.07079	0.1324
3	2.790	VV	1.6338	292.40891	2.09920	5.019e-3
4	4.338	VV	0.2816	638.70233	26.87808	0.0110
5	5.795	VV	0.1108	1.33310e4	1786.13220	0.2288
6	6.033	VV	0.1379	1.08909e6	1.18041e5	18.6940
7	7.204	VV	0.4469	3.44737e4	980.55005	0.5917
8	7.755	VV	0.2037	4.03119e5	2.85369e4	6.9194
9	8.033	VV	0.1101	9989.97266	1285.77576	0.1715
10	8.516	VV	0.4614	5.98508e4	1809.98328	1.0273
11	8.986	VV	0.3359	4.99816e4	1992.00330	0.8579
12	9.719	VV	0.3027	9438.10059	454.66879	0.1620
13	10.246	VV	0.3427	1.67940e4	673.64014	0.2883
14	10.553	VV	0.3778	1.75645e4	608.55902	0.3015
15	11.475	VV	0.2820	9.70627e4	5014.64941	1.6661
16	12.144	VV	0.2514	2.60630e5	1.54054e4	4.4736
17	12.731	VV	0.2550	3.01010e6	1.78591e5	51.6675
18	14.662	VV	0.2885	1.49527e5	7797.48389	2.5666
19	15.466	VV	0.2539	1975.16309	96.52477	0.0339
20	16.756	VV	0.3494	4.92872e5	2.08347e4	8.4600
21	18.049	VV	0.3819	7.27478e4	2893.32690	1.2487
22	19.674	VBA	0.4339	1.71241e4	642.85992	0.2939
Totals :				5.82590e6	3.88142e5	

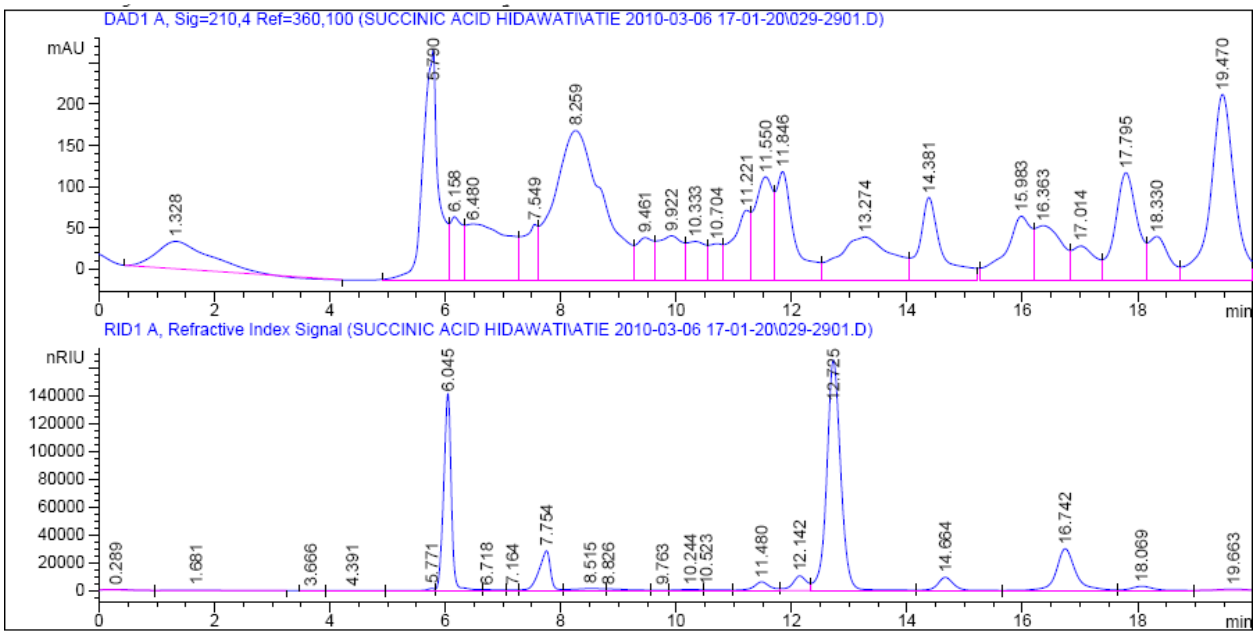


Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	0.300	BV	0.4074	1.46723e4	588.66479	0.2444
2	1.679	VV	0.5387	7978.47803	197.29665	0.1329
3	3.049	VV	0.1511	196.91425	18.35467	3.280e-3
4	4.428	VV	0.3089	903.89520	35.12605	0.0151
5	5.790	VV	0.1117	1.40299e4	1884.04797	0.2337
6	6.033	VV	0.1390	1.12991e6	1.21197e5	18.8185
7	7.190	VV	0.2310	1.07041e4	642.94269	0.1783
8	7.757	VV	0.2028	3.98980e5	2.87712e4	6.6450
9	8.519	VV	0.4618	5.40684e4	1555.13647	0.9005
10	8.834	VV	0.4045	3.35425e4	1174.23340	0.5586
11	9.774	VV	0.2651	8322.40820	425.21500	0.1386
12	10.252	VV	0.3558	2.09974e4	805.81287	0.3497
13	10.532	VV	0.3557	1.48134e4	531.05249	0.2467
14	11.474	VV	0.2863	1.06021e5	5373.95557	1.7658
15	12.145	VV	0.2507	1.57762e5	9254.96973	2.6275
16	12.731	VV	0.2553	3.31122e6	1.96080e5	55.1480
17	14.663	VV	0.2940	1.47648e5	7516.06250	2.4591
18	16.749	VV	0.3442	4.82193e5	2.07789e4	8.0309
19	18.065	VV	0.3794	7.42902e4	2957.74927	1.2373
20	19.702	VBA	0.4360	1.59934e4	608.97272	0.2664
Totals :				6.00425e6	4.00397e5	

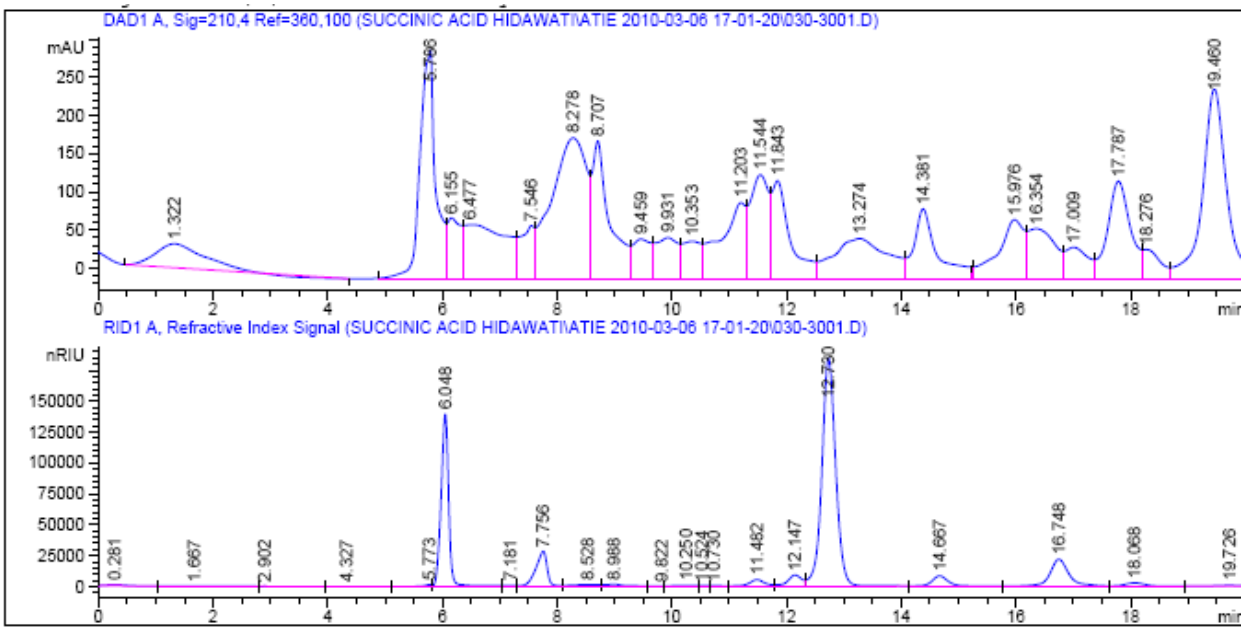


Signal 2: RID1 A, Refractive Index Signal

Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	0.302	BV	0.4084	1.59506e4	637.88196	0.2653
2	1.663	VV	0.5583	8256.02930	206.96159	0.1373
3	4.324	VV	0.2976	810.58105	32.97061	0.0135
4	5.191	VV	0.1236	48.99901	5.06773	8.149e-4
5	5.773	VV	0.1275	1.32944e4	1559.44519	0.2211
6	6.048	VV	0.1378	1.28231e6	1.39089e5	21.3248
7	7.184	VV	0.2094	9454.00000	630.76392	0.1572
8	7.754	VV	0.2007	3.94071e5	2.84118e4	6.5534
9	8.532	VV	0.4440	4.79139e4	1499.89001	0.7968
10	8.976	VV	0.3500	4.06595e4	1546.25903	0.6762
11	9.754	VV	0.2637	8278.27441	413.62051	0.1377
12	10.250	VV	0.3543	1.92959e4	749.72784	0.3209
13	10.536	VV	0.3604	1.47649e4	525.23053	0.2455
14	11.484	VV	0.2867	1.06826e5	5456.80811	1.7765
15	12.147	VV	0.2528	1.60787e5	9434.58496	2.6739
16	12.730	VV	0.2530	3.13664e6	1.85917e5	52.1625
17	14.667	VV	0.2936	1.60683e5	8193.33984	2.6722
18	16.749	VV	0.3445	5.06401e5	2.17990e4	8.4215
19	18.071	VV	0.3853	7.23902e4	2846.48999	1.2039
20	19.734	VBA	0.3768	1.43781e4	595.27411	0.2391
Totals :				6.01320e6	4.09551e5	

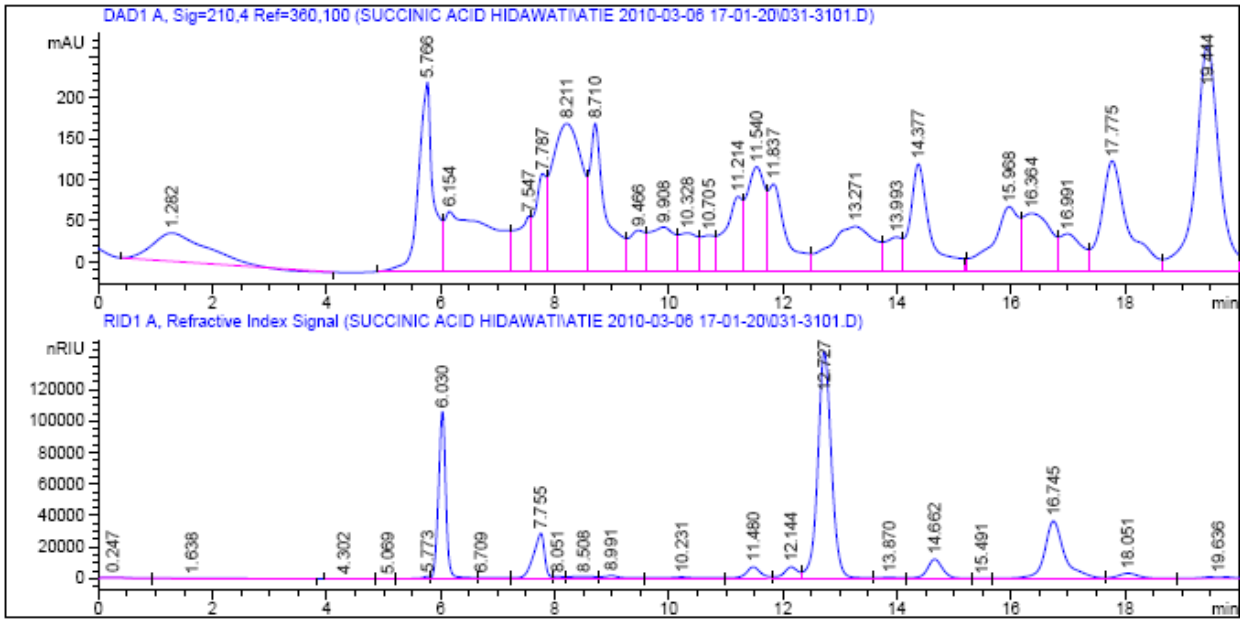


Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	0.289	BV	0.4040	1.17611e4	477.45203	0.1981
2	1.681	VV	0.5829	9658.73730	235.15472	0.1627
3	3.666	VV	0.1928	153.10745	9.93021	2.579e-3
4	4.391	VV	0.3489	1238.78857	44.74978	0.0209
5	5.771	VV	0.1300	1.39167e4	1592.58215	0.2344
6	6.045	VV	0.1373	1.29829e6	1.41527e5	21.8659
7	6.718	VV	0.2911	1.39739e4	619.83264	0.2353
8	7.164	VV	0.1767	7077.51318	563.94000	0.1192
9	7.754	VV	0.2004	3.95751e5	2.85791e4	6.6653
10	8.515	VV	0.4529	5.24216e4	1524.93042	0.8829
11	8.826	VV	0.3884	3.19401e4	1167.08215	0.5379
12	9.763	VV	0.2399	6907.67480	388.42593	0.1163
13	10.244	VV	0.3709	2.10677e4	780.86353	0.3548
14	10.523	VV	0.3463	1.49643e4	544.87280	0.2520
15	11.480	VV	0.2787	1.18943e5	6235.11084	2.0033
16	12.142	VV	0.2506	1.79009e5	1.05091e4	3.0149
17	12.725	VV	0.2551	2.78851e6	1.65325e5	46.9644
18	14.664	VV	0.2882	1.80773e5	9442.30566	3.0446
19	16.742	VV	0.3431	6.92739e5	2.99705e4	11.6672
20	18.069	VV	0.3886	7.73170e4	3005.89087	1.3022
21	19.663	VBA	0.4934	2.10912e4	718.97394	0.3552
Totals :				5.93750e6	4.03263e5	

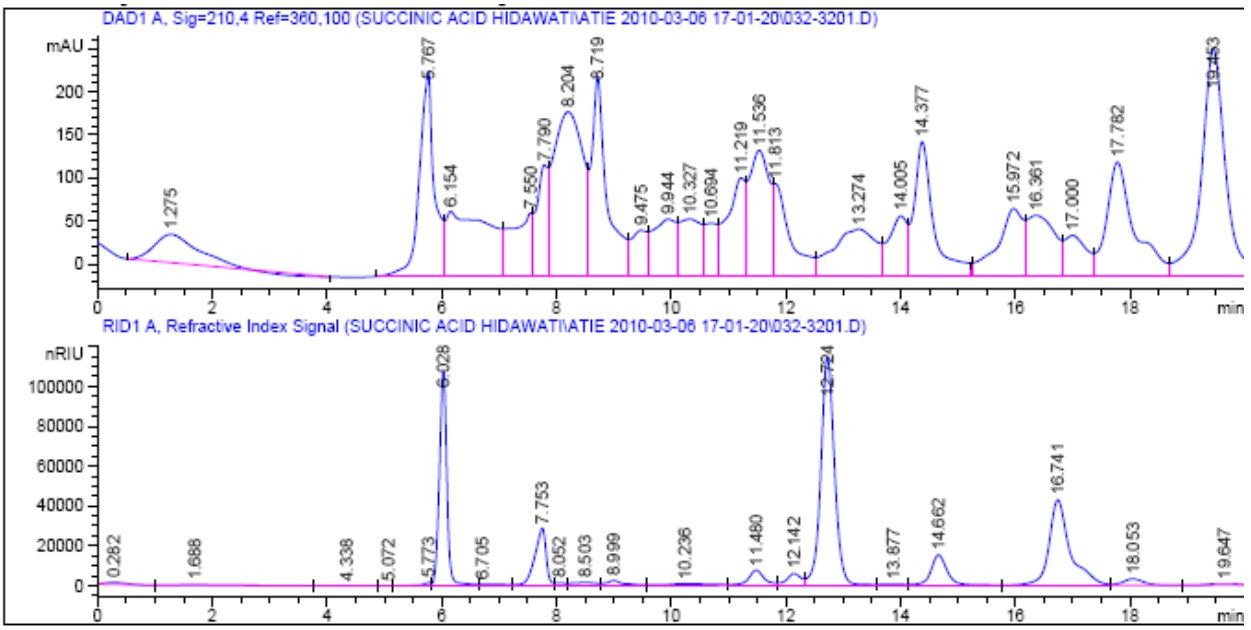


Signal 2: RID1 A, Refractive Index Signal

Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	0.281	BV	0.3956	1.85403e4	769.30731	0.3059
2	1.667	VV	0.5576	9142.77734	232.79634	0.1508
3	2.902	VV	0.6200	460.12061	8.78100	7.592e-3
4	4.327	VV	0.3814	869.75421	29.07526	0.0144
5	5.773	VV	0.1278	1.33052e4	1524.18799	0.2195
6	6.048	VV	0.1381	1.29130e6	1.39700e5	21.3051
7	7.181	VV	0.2089	9608.10156	651.04889	0.1585
8	7.756	VV	0.2015	3.97430e5	2.85200e4	6.5572
9	8.528	VV	0.4487	4.96299e4	1534.17944	0.8188
10	8.988	VV	0.3295	4.58066e4	1866.43994	0.7558
11	9.822	VV	0.2124	7266.33398	416.14023	0.1199
12	10.250	VV	0.3746	2.12327e4	772.67590	0.3503
13	10.524	VV	0.1617	6292.91455	532.08527	0.1038
14	10.730	VV	0.2359	8286.44727	501.75458	0.1367
15	11.482	VV	0.2874	1.09857e5	5541.46729	1.8125
16	12.147	VV	0.2536	1.58723e5	9279.34961	2.6188
17	12.730	VV	0.2557	3.13079e6	1.85021e5	51.6550
18	14.667	VV	0.3104	1.80052e5	8551.27441	2.9707
19	16.748	VV	0.3470	5.15494e5	2.19843e4	8.5051
20	18.068	VV	0.3853	7.23563e4	2844.90674	1.1938
21	19.726	VBA	0.3802	1.45226e4	607.92249	0.2396
Totals :				6.06096e6	4.10889e5	

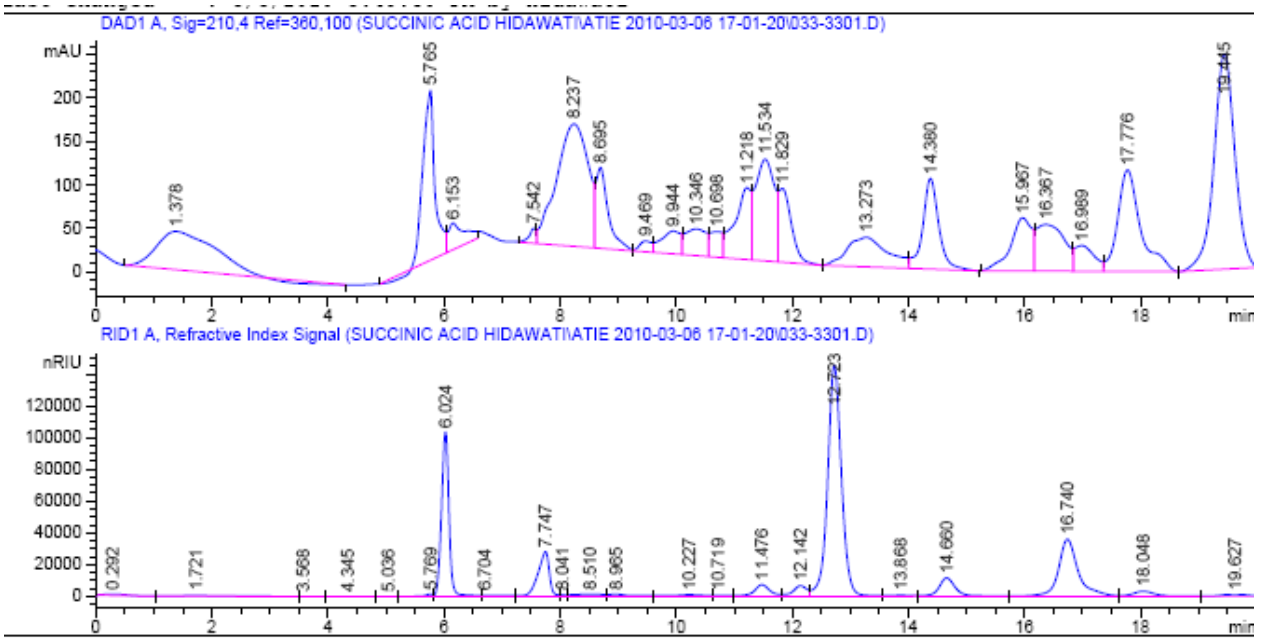


Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	0.247	BV	0.3923	9418.96680	408.24307	0.1719
2	1.638	VV	0.5992	1.09446e4	243.88499	0.1998
3	4.302	VV	0.3061	732.73523	28.52755	0.0134
4	5.069	VV	0.1322	64.01626	6.38021	1.169e-3
5	5.773	VV	0.1325	1.36766e4	1527.49084	0.2497
6	6.030	VV	0.1375	9.55857e5	1.06119e5	17.4496
7	6.709	VV	0.4064	1.94083e4	598.50098	0.3543
8	7.755	VV	0.1994	3.96025e5	2.87867e4	7.2296
9	8.051	VV	0.1686	1.58911e4	1361.08142	0.2901
10	8.508	VV	0.4016	4.62268e4	1541.93689	0.8439
11	8.991	VV	0.3151	4.51838e4	1938.98254	0.8249
12	10.231	VV	0.6400	4.49935e4	948.23364	0.8214
13	11.480	VV	0.2767	1.39390e5	7372.17773	2.5446
14	12.144	VV	0.2512	1.26303e5	7391.13770	2.3057
15	12.727	VV	0.2532	2.40892e6	1.44237e5	43.9759
16	13.870	VV	0.3742	1.91830e4	723.31653	0.3502
17	14.662	VV	0.2857	2.31273e5	1.22158e4	4.2220
18	15.491	VV	0.2629	2411.54297	114.54693	0.0440
19	16.745	VV	0.3551	8.80769e5	3.64803e4	16.0789
20	18.051	VV	0.3819	8.28060e4	3269.82495	1.5117
21	19.636	VBA	0.4780	2.83315e4	991.87103	0.5172
Totals :				5.47781e6	3.56306e5	



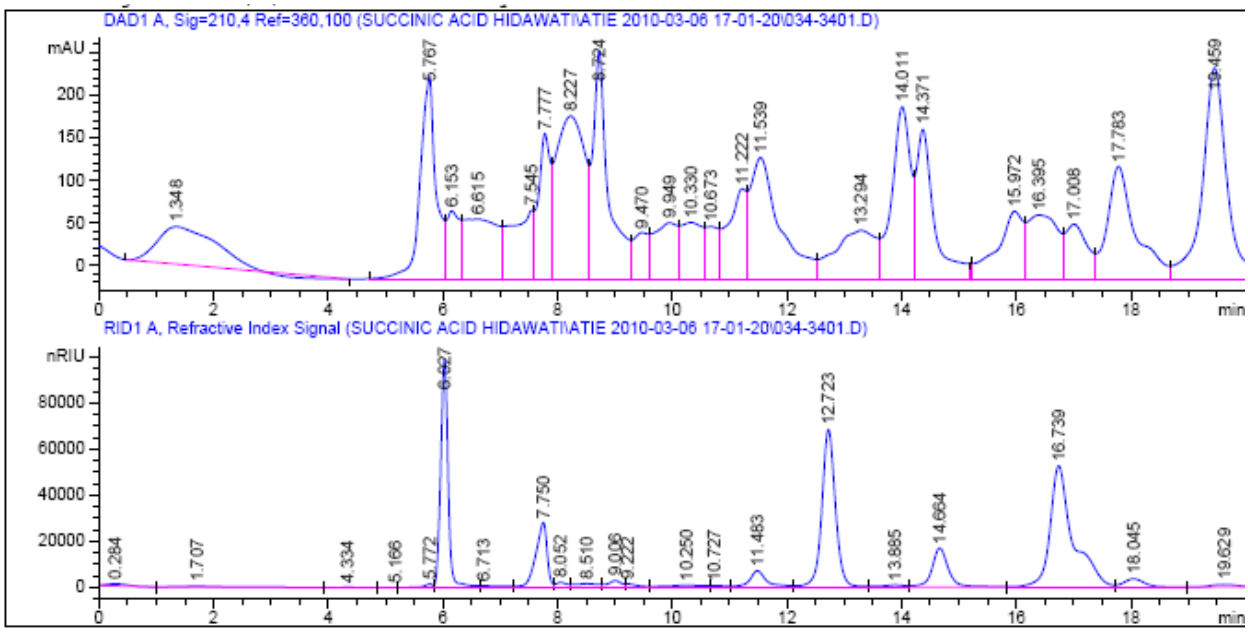
Signal 2: RID1 A, Refractive Index Signal

Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	0.282	BV	0.4092	2.20323e4	891.65326	0.4152
2	1.688	VV	0.5199	1.00137e4	285.10831	0.1887
3	4.338	VV	0.4086	1383.76294	40.58613	0.0261
4	5.072	VV	0.1361	87.44312	8.14290	1.648e-3
5	5.773	VV	0.1303	1.27478e4	1454.81897	0.2402
6	6.028	VV	0.1377	9.73838e5	1.07902e5	18.3520
7	6.705	VV	0.4052	1.96829e4	608.97925	0.3709
8	7.753	VV	0.1999	3.98075e5	2.88346e4	7.5017
9	8.052	VV	0.1706	1.68198e4	1420.03564	0.3170
10	8.503	VV	0.3975	4.70702e4	1578.71362	0.8870
11	8.999	VV	0.2977	5.45839e4	2502.36694	1.0286
12	10.236	VV	0.7364	5.29207e4	943.54425	0.9973
13	11.480	VV	0.2816	1.48497e5	7684.87451	2.7984
14	12.142	VV	0.2531	1.03762e5	6015.62695	1.9554
15	12.724	VV	0.2498	1.90628e6	1.14865e5	35.9239
16	13.877	VV	0.3845	1.82614e4	666.32770	0.3441
17	14.662	VV	0.3004	3.09887e5	1.53462e4	5.8398
18	16.741	VV	0.3701	1.10104e6	4.29856e4	20.7490
19	18.053	VV	0.3849	8.35590e4	3289.81982	1.5747
20	19.647	VBA	0.4781	2.59032e4	906.46887	0.4881
Totals :				5.30644e6	3.38231e5	



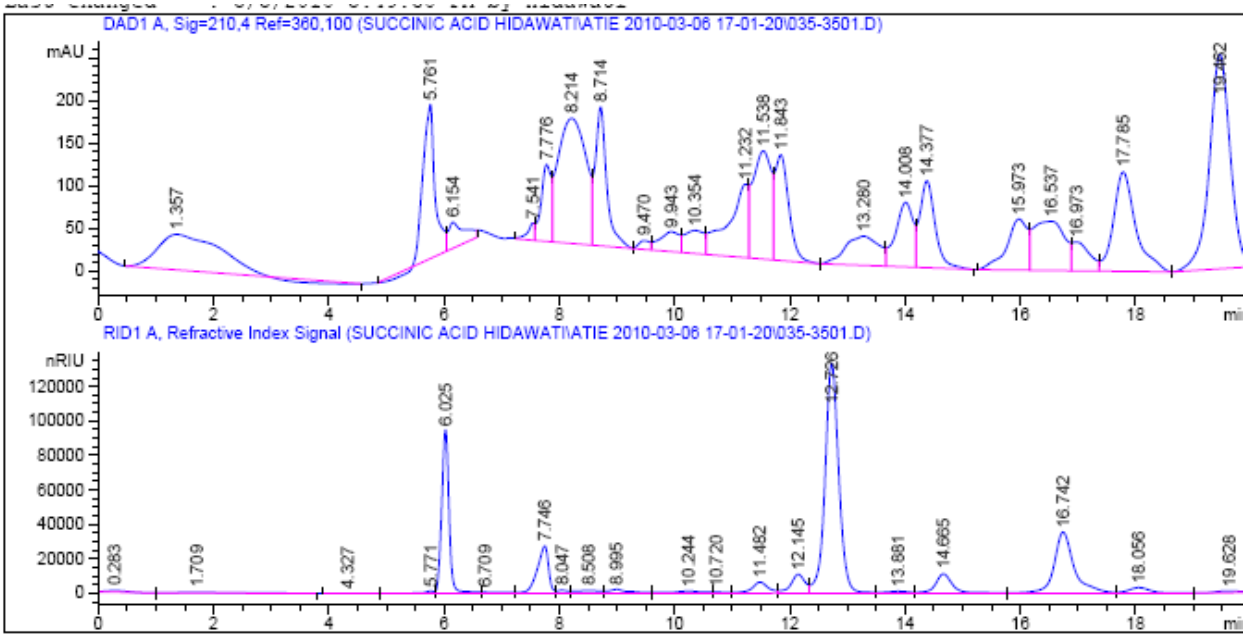
Signal 2: RID1 A, Refractive Index Signal

Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	0.292	BV	0.4115	2.21879e4	897.67621	0.4089
2	1.721	VV	0.6248	1.58356e4	345.94870	0.2919
3	3.568	VV	0.1107	31.20984	3.81144	5.752e-4
4	4.345	VV	0.2999	816.30432	32.69604	0.0150
5	5.036	VV	0.1681	148.87071	13.00628	2.744e-3
6	5.769	VV	0.1325	1.34904e4	1507.66260	0.2486
7	6.024	VV	0.1345	9.25254e5	1.03560e5	17.0529
8	6.704	VV	0.4064	1.93295e4	596.04181	0.3563
9	7.747	VV	0.1991	3.86350e5	2.81407e4	7.1206
10	8.041	VV	0.1069	7003.96924	934.43073	0.1291
11	8.510	VV	0.4317	5.09414e4	1564.50305	0.9389
12	8.965	VV	0.3715	3.90501e4	1387.76501	0.7197
13	10.227	VV	0.5479	3.99550e4	1005.45459	0.7364
14	10.719	VV	0.2602	1.21064e4	644.51471	0.2231
15	11.476	VV	0.2840	1.43497e5	7419.45898	2.6447
16	12.142	VV	0.2515	1.14198e5	6672.04980	2.1047
17	12.723	VV	0.2500	2.41740e6	1.45515e5	44.5540
18	13.868	VV	0.3980	2.00910e4	694.43127	0.3703
19	14.660	VV	0.3015	2.35455e5	1.16059e4	4.3396
20	16.740	VV	0.3479	8.52364e5	3.59519e4	15.7095
21	18.048	VV	0.3889	8.17906e4	3177.07202	1.5074
22	19.627	VBA	0.4646	2.84861e4	1025.09412	0.5250
Totals :				5.42579e6	3.52695e5	



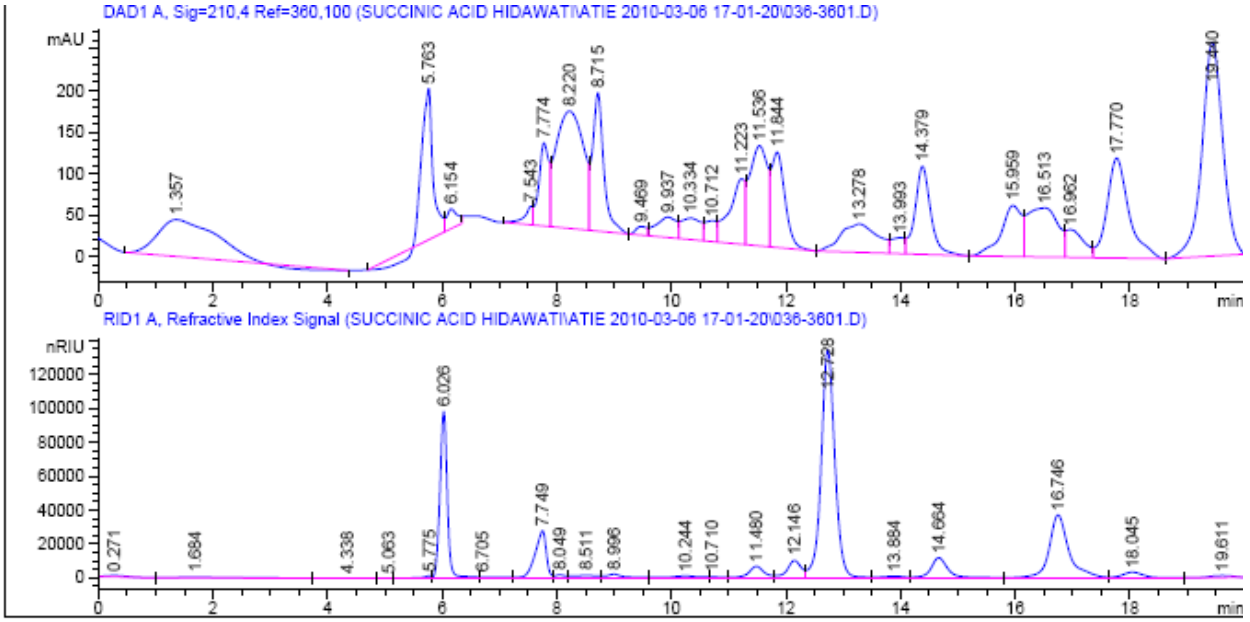
Signal 2: RID1 A, Refractive Index Signal

Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	0.284	BV	0.4073	2.15264e4	870.49902	0.4468
2	1.707	VV	0.6258	1.72893e4	386.29355	0.3589
3	4.334	VV	0.3198	1234.17322	49.36218	0.0256
4	5.166	VV	0.1598	167.02391	12.70993	3.467e-3
5	5.772	VV	0.1395	1.41551e4	1512.13879	0.2938
6	6.027	VV	0.1369	8.90562e5	9.93812e4	18.4857
7	6.713	VV	0.4201	2.20317e4	663.02637	0.4573
8	7.750	VV	0.2012	3.88204e5	2.82725e4	8.0581
9	8.052	VV	0.1884	2.76761e4	2129.40796	0.5745
10	8.510	VV	0.3837	4.68952e4	1616.95520	0.9734
11	9.006	VV	0.2351	4.91366e4	2954.37524	1.0199
12	9.222	VV	0.2125	2.19835e4	1477.17358	0.4563
13	10.250	VV	0.5656	4.26144e4	1019.15204	0.8846
14	10.727	VV	0.2685	1.35008e4	705.88007	0.2802
15	11.483	VV	0.3068	1.53498e5	7207.29492	3.1862
16	12.723	VV	0.2495	1.13599e6	6.85371e4	23.5801
17	13.885	VV	0.3883	2.87035e4	1028.34253	0.5958
18	14.664	VV	0.3038	3.48389e5	1.70033e4	7.2316
19	16.739	VV	0.3951	1.47417e6	5.27666e4	30.5999
20	18.045	VV	0.3866	9.35140e4	3660.85889	1.9411
21	19.629	VBA	0.4766	2.63253e4	925.36145	0.5464
Totals :				4.81757e6	2.92179e5	



Signal 2: RID1 A, Refractive Index Signal

Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	0.283	BV	0.4088	2.09618e4	862.58215	0.4015
2	1.709	VV	0.5766	2.02412e4	494.80688	0.3877
3	4.327	VV	0.3758	1204.37317	38.04594	0.0231
4	5.771	VV	0.1378	1.29205e4	1373.98523	0.2475
5	6.025	VV	0.1337	8.41127e5	9.48515e4	16.1116
6	6.709	VV	0.4023	2.03052e4	636.63531	0.3889
7	7.746	VV	0.1977	3.74168e5	2.74794e4	7.1671
8	8.047	VV	0.1774	2.11205e4	1699.08521	0.4046
9	8.508	VV	0.4042	4.97517e4	1627.25134	0.9530
10	8.995	VV	0.3272	5.55024e4	2262.49097	1.0631
11	10.244	VV	0.4746	4.52561e4	1281.57324	0.8669
12	10.720	VV	0.2509	1.12217e4	630.46790	0.2149
13	11.482	VV	0.2855	1.27340e5	6478.25977	2.4392
14	12.145	VV	0.2553	1.88878e5	1.09463e4	3.6179
15	12.726	VV	0.2495	2.21002e6	1.33349e5	42.3325
16	13.881	VV	0.3842	3.02149e4	1103.51392	0.5788
17	14.665	VV	0.3032	2.29916e5	1.11499e4	4.4040
18	16.742	VV	0.3509	8.49750e5	3.54572e4	16.2768
19	18.056	VV	0.3843	8.14483e4	3212.51782	1.5601
20	19.628	VBA	0.4771	2.92786e4	1041.33667	0.5608
Totals :				5.22063e6	3.35976e5	



Signal 2: RID1 A, Refractive Index Signal

Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	0.271	BV	0.3995	1.97746e4	828.40546	0.3716
2	1.684	VV	0.6135	1.78189e4	414.81149	0.3348
3	4.338	VV	0.3883	1193.51660	37.30985	0.0224
4	5.063	VV	0.1475	171.61346	15.12532	3.225e-3
5	5.775	VV	0.1314	1.24026e4	1371.75269	0.2330
6	6.026	VV	0.1343	8.74540e5	9.80642e4	16.4326
7	6.705	VV	0.3852	1.87689e4	613.07275	0.3527
8	7.749	VV	0.1979	3.79707e5	2.78516e4	7.1347
9	8.049	VV	0.1804	2.27239e4	1817.87585	0.4270
10	8.511	VV	0.3976	4.76884e4	1589.19360	0.8961
11	8.996	VV	0.3073	5.15047e4	2275.26880	0.9678
12	10.244	VV	0.5125	4.58282e4	1254.83801	0.8611
13	10.710	VV	0.2505	1.11599e4	634.77155	0.2097
14	11.480	VV	0.2801	1.31444e5	6847.75342	2.4698
15	12.146	VV	0.2545	1.77150e5	1.03091e4	3.3287
16	12.728	VV	0.2517	2.22859e6	1.34477e5	41.8751
17	13.884	VV	0.3835	2.91754e4	1060.92603	0.5482
18	14.664	VV	0.3101	2.49997e5	1.18888e4	4.6974
19	16.746	VV	0.3499	8.86561e5	3.71188e4	16.6585
20	18.045	VV	0.3820	8.37029e4	3327.39087	1.5728
21	19.611	VBA	0.4622	3.20842e4	1163.07507	0.6029
Totals :				5.32199e6	3.42961e5	