EXTRACTION OF STEVIOSIDE FROM *STEVIA REBAUDIANA* LEAVES USING CELLULASE

NOR HAYATI BT SHAHIDAN

BACHELOR OF CHEMICAL ENGINEERING (BIOTECHNOLOGY)

UNIVERSITI MALAYSIA PAHANG

UNIVERSITY MALAYSIA PAHANG

CENTRE FOR GRADUATE STUDIES

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Name : Dr Wan Mohd Hafizuddin B. Wan Yusoff

Signature : ……

Institution :

EXTRACTION OF STEVIOSIDE FROM *STEVIA REBAUDIANA* LEAVES USING CELLULASE

NOR HAYATI BT SHAHIDAN

A thesis submitted in fulfillment of the requirement

for the award of the degree of

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UNIVERSITY MALAYSIA PAHANG

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SUPERVISOR'S DECLARATION

I hereby declare that I have checked this thesis and in my opinion, this thesis is adequate in term of scope and quality for the award of the degree of Bachelor in Chemical Engineering in Biotechnology.

Signature : …….

Name of Supervisor : Dr. Wan Mohd Hafizuddin B. Wan Yussof

Date : 5 JULY 2012

STUDENT'S DECLARATION

I hereby declare that the work in this thesis "*Extraction of Stevioside from Stevia Rebaudiana leaves using cellulase*" is my own except for quotations and summaries which have been duly acknowledge. The thesis has not been accepted for any degree and is not concurrently submitted for award of other degree.

Signature : ……

Name : NOR HAYATI BT SHAHIDAN

ID Number : KE08002

Date : 5 JULY 2012

Dedicated especially to my beloved mother, my father, siblings, lectures, friends and to ones who give me support and inspiration that made this work possible.

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ABSTRACT

Stevioside is a diterpene glycoside present in *Stevia Rebaudiana* leaves that has the ability to sweeten at rated between 70 to 350 times than sucrose (0.4% w/v). It has no calorific value. Unlike many low calorie sweeteners, stevioside is stable at high temperature. The objective of this research is to extract stevioside from *stevia rebaudiana* leaves by using cellulase from *Aspergillus Niger*. Acetate buffer and ethanol were used as a medium for enzyme and as a solvent, respectively. In this present study, the enzymatic extraction of stevioside from *stevia rebaudiana* leaves was carried out using cellulase with various parameters that affect the production of stevioside such as concentration of enzyme, incubation time and temperature. Cellulase was observed to give the highest stevioside yield (16230 ± 0.3 µg/ml) at 40^{o}C. This indicated that the maximum temperature for cellulase activity was 40^{o}C. The results signify that the enzymatic extraction method is an alternative to solvent based stevioside extraction, based on its higher efficiency. Thus, it can be concluded that the extraction of stevioside from *Stevia rebaudiana* leaves using cellulase can be maximized under the maximum conditions for the cellulase activity where the used of solvent can be minimized in degrading the cell wall Together with the maximum heat and correct combination of the solvent used, a new and efficient way of extracting high yield of stevioside can be obtained.

ABSTRAK

Stevioside adalah glikosida diterpene yang hadir dalam daun *Stevia rebaudiana* yang dikatakan mempunyai rating tertinggi pemanis antara 70 hingga 350 kali dari sukrosa (0.4% w/v) dan tidak mempunyai nilai kalori. Tidak seperti pemanis berkalori rendah yang lain, stevioside stabil pada suhu yang tinggi. Objektif kajian ini adalah untuk mengestrak stevioside daripada daun *Stevia rebaudiana* dengan menggunakan sellulase daripada *Aspergillus Niger*. Penampan asetat sebagai medium untuk enzim dan etanol sebagai pelarut digunakan dalam pengekstrakan enzim untuk mengekstrak stevioside daripada stevia rebaudiana dengan sellulase menggunakan pelbagai parameter seperti kepekatan enzim, masa pengeraman dan suhu. Sellulase diperhatikan dapat memberikan hasil stevioside tertinggi (16230 ± 0.3 µg/ml) pada suhu 40^{o}C. Ini menunjukkan bahawa suhu maksimum untuk aktiviti sellulase adalah pada suhu 40^{o}C. Keputusan daripada kajian ini menunjukkan bahawa kaedah pengeksrakan enzim adalah alternative bagi pengekstrakan stevioside yang berasaskan pelarut, berdasarkan peningkatan kecekapan yang ditunjuk. Kesimpulannya, pengekstrakan stevioside daripada stevia rebaudiana menggunakan sellulase dikatakan dapat dimaksimumkan di bawah syarat-syarat yang boleh memaksimumkan aktiviti enzim dimana penggunaan pelarut dapat diminimumkan dalam proses penguraian dinding-dinding sel. Bersama-sama dengan haba maksimum dan gabungan pelarut yang betul yang digunakan, cara yang baru dan efisien untuk mengeluarkan hasil stevioside yang tinggi boleh diperolehi.

TABLE OF CONTENT

CHAPTER 2 LITERATURE REVIEW

LIST OF TABLES

LIST OF FIGURE

CHAPTER 1

INTRODUCTION

1.1 Introduction

The increasing number of diabetic people has alarmed the global community. Presently in Malaysia, diabetes is a growing concern with three million out of 28 million of its population diagnosed with disease. In particular, the increasing prevalence is closely linked with obesity, creating significant market opportunities in develop healthier lifestyle people in Malaysia.

Stevia (*Stevia Rebaudiana*) is a herb with incredibility sweetening property which produce sweet taste and has no calorific value. *Stevia rebaudiana* is one of 154 members of the genus stevia and one of only two species that produce sweet steviol glycosides, which has more abundant of rebaudiosides A and steviosides that responsible for the sweet taste. With its ability to sweeten at rated between 70 to 400 times that of sucrose, it is commonly called “sweetleaf” or “sugarleaf” because of the higher sweetness level found in its leaf.

There are five main compounds in the stevia glycosides extract namely stevioside, rebaudioside A, rebaudioside C, dulcoside and steviobioside. With its ability to reduce the

cravings for sweet, it can be part in weight loss program and to treat disease diabetes, and high blood pressure. Currently, most of the stevia glycosides have been extracted from *stevia rebaudiana* leaves using the conventional technique such as maceration or thermal extraction that requiring long processing time and low efficiency.

1.2 Problem Statement

Traditionally, people used maceration or thermal extraction that required long processing time and low efficiency (Vinatoru, 2001). In order to increase the productivity and improve the yield and quality of the extracted stevioside, enzyme extraction has been reported elsewhere, improved the extraction yield for the extraction of plant based bioactive. Enzyme extraction method was also said can minimized the use of solvent and heat (Kaour *et al.*, 2010). This application will be implemented in this present study.

1.3 Research Objective

The objective of this research is to extract stevioside from *Stevia Rebaudiana* leaves by using cellulase.

1.4 Scope of Research

In order to achieve the objectives, the following scopes have been identified:

i. To examine the effect of enzyme concentration on the stevioside yield.
ii. To check the effect of extraction time on the productivity of stevioside.
iii. To investigate the effect of extraction temperature on the stevioside yield.

1.5 Rational and Significant of Research

The increasing number of diabetic people that is growing concern in Malaysia and other disease that closely link to obesity cannot be ignored. This study is significant to people who having both diseases where the uses of sucrose in their lifestyle need to be replacing with the natural sweetener that has no calorific value. This will created new healthier life. Besides, this study is beneficial to the stevia extraction industries in order to choose the type of technique that should be applied from an economical point of view.

CHAPTER 2

LITERATURE REVIEW

2.1 Stevia Rebaudiana

Stevia is the generic term used for food ingredient derived from the herb *stevia rebaudiana* (Carakostas, 2008). Because of high sweetener levels found in its leaves, stevia is commonly called as sweetleaf or sugarleaf (Kansaf, 2004). Stevia has important industrial uses in food and beverages, as well as used as medicine such as for low uric acid treatment, anesthetic and anti-imflammatory (Jayaraman *et al*., 2008). Gardana *et al*., (2003) have been suggested that the extraction of stevia sweetener exert beneficial effect on human health, including antioxidant, carcinogenic and anti-human rotavirus. Before, there were several toxicological studies have been carried out to verify the possible mutagenic and genotoxic effect of srevia extracts on bacterial cells and mammalian species. The extract obtained from stevia leaves contain a complex mixture of compounds, among them is glycosides such as stevioside and rebaudioside A. The residual taste associated with stevia extracts is partially due to the glycoside and some other compound such as terpenes (Guzen *et al*., 2002).

2.2 Glycoside

Glycoside is known as organic compounds which contain a sugar component and no sugar component. Among the other product of hydrolysis, the sugar part is known as glycone and the no sugar part is known as aglycone (Elkin, 1997). The glycone constituent may be comprised of rhamnose, fructose, glucose, xylose and arabinose.

2.3 Steviol glycoside

Steviol glycoside is a more precise term for a group of intensely sweet compound extracted and purified from *S.rebaudiana*. Stevioside and rebaudioside A are the predominant steviol glycosides found in *S.rebaudiana*. Commercial interest in steviol glycoside sweeteners has been high for a long time. Steviol glycosides also has been commercialize as a food ingredient (Carakostas *et al*., 2008). The other glycosides present in lower concentration are steviolbiosides, dulcosides and rebaudioside C (Kirby *et al*., 2002).

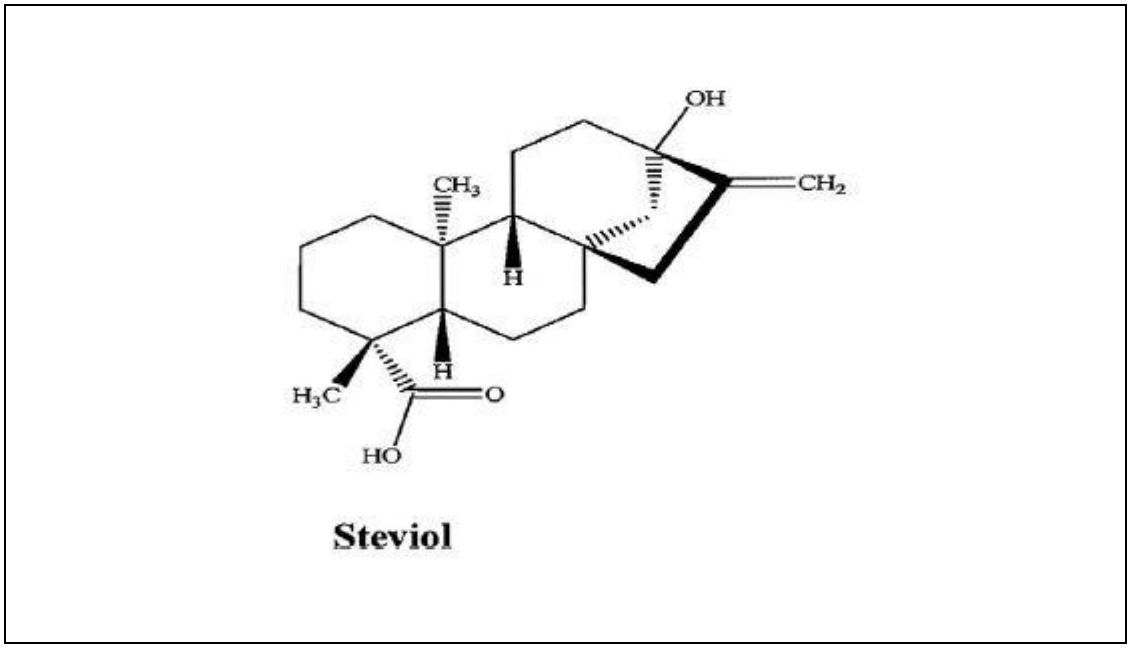

Figure 2.1: Structure of Steviol Glycoside

Compound	Melting point (°C)	Molecular weight	Solubility in water (%)
Stevioside	196 – 198	804	0.13
Rebaudioside A	242 – 244	966	0.80
Rebaudioside B	193 – 195	804	0.10
Rebaudioside C	215 – 217	958	0.21
Rebaudioside D	283 – 286	1128	1.00
Rebaudioside E	205 – 207	966	1.70
Steviolbioside	188 – 192	642	0.03
Dulcoside A	193 – 195	788	0.58

Table 2.1: Physical and solubility data for eight sweet ent-kaurene glycoside from the leaves of *S.rebaudiana*.

2.3.1 Stevioside

Stevioside is a diterpene glycoside present in *S. rebaudiana* bertoni (Geuns, 2003). A simple enzymatic method is describes for the determination of stevioside from *S.rebaudiana* based on the hydrolysis of stevioside with crude hesperidinase. The reaction is followed by monitoring the production of glucose with a glucose oxidase-peroxidase-2 system (Mizukami *et al*., 1982).

According to Kinghorn and Soerjato, (1985), stevioside appear as a white, crystalline and odourless powder. Unlike many low-calorie sweeteners, stevioside is stable at high (100°C) temperatures and over a range of pH values (pH 3-9). It is contain no calories, and does not darken upon cooking (Crammer & Ikan, 1986). The sweetness of these glycosides compared to sucrose is dulcoside A (50–120), rebaudioside A (250–450), rebaudioside B (300–350), rebaudioside C (50–120), rebaudioside D (250–450), rebaudioside E (150–300), steviobioside (100–125), and stevioside (300) (Crammer & Ikan, 1986).

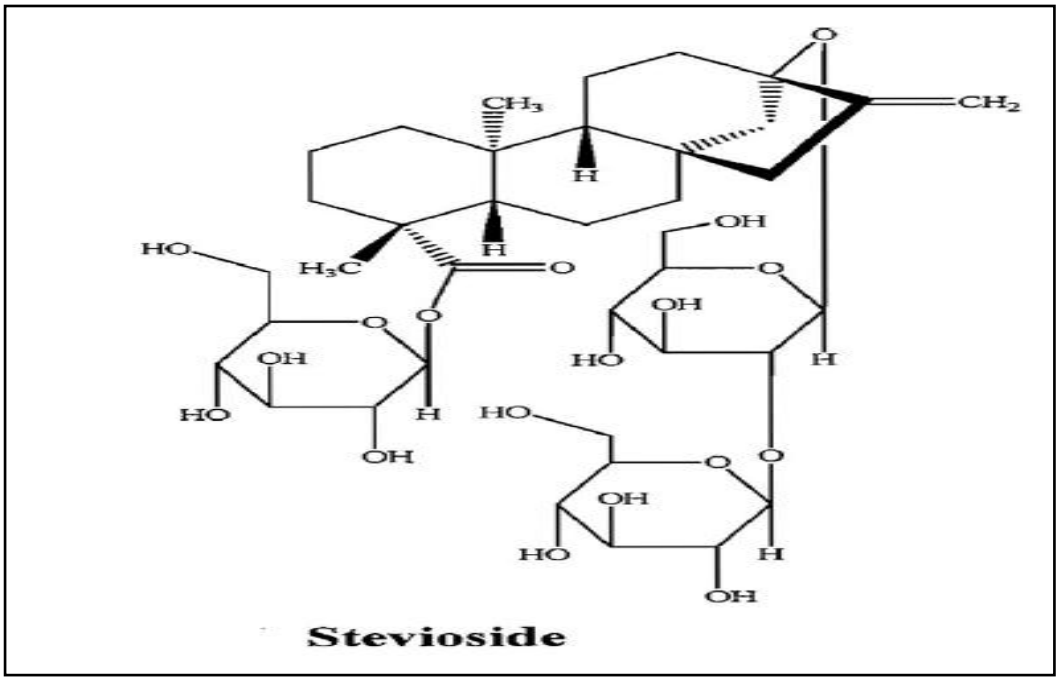

Figure 2.2: Structure of stevioside molecular bonding

Matrix	Physical Properties of Stevioside
Chemical Abstract Name	Kaur-16-en-18-oic acid, 13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]-, β-D-glucopyranosyl ester, (4α)- (9CI)
Other Names	1H-2,10a-Ethanophenanthrene,kaur-16-en-18-oic acid deriv.; Stevioside (6CI, 7CI); α-G-Sweet; Steviosin
Molecular formula	$C_{38}H_{60}O_{18}$
Molecular weight	804.88
Melting point	196°C – 198°C
Solubility	Water, ethanol, dioxane; not in methanol,
Storage temperature	Store at +4°C, in dark place
pKa	12.52 ± 0.70, most acidic
Toxicity (LD_{50})	Not toxic
Polarity	Polar
Optical rotation	- 39.3 ° in water
Wave length maximum	200 nm

Table 2.2: Physical properties of stevioside

2.3.2 Rebaudioside A

Rebaudioside A is also known by the common name rebiana (Prakash *et al*., 2008). Rebaudioside A and stevioside have similar pharmacokinetic and metabolic profiles in rats and human (Roberts and Renwick, 2008; Wheeler *et al*., 2008) and thus studies had been carried out either steviol glycoside are relevant to both. The only different in the structure of stevioside to rebaudioside A was only by a glucose moiety (Prakash *et al*., 2008).

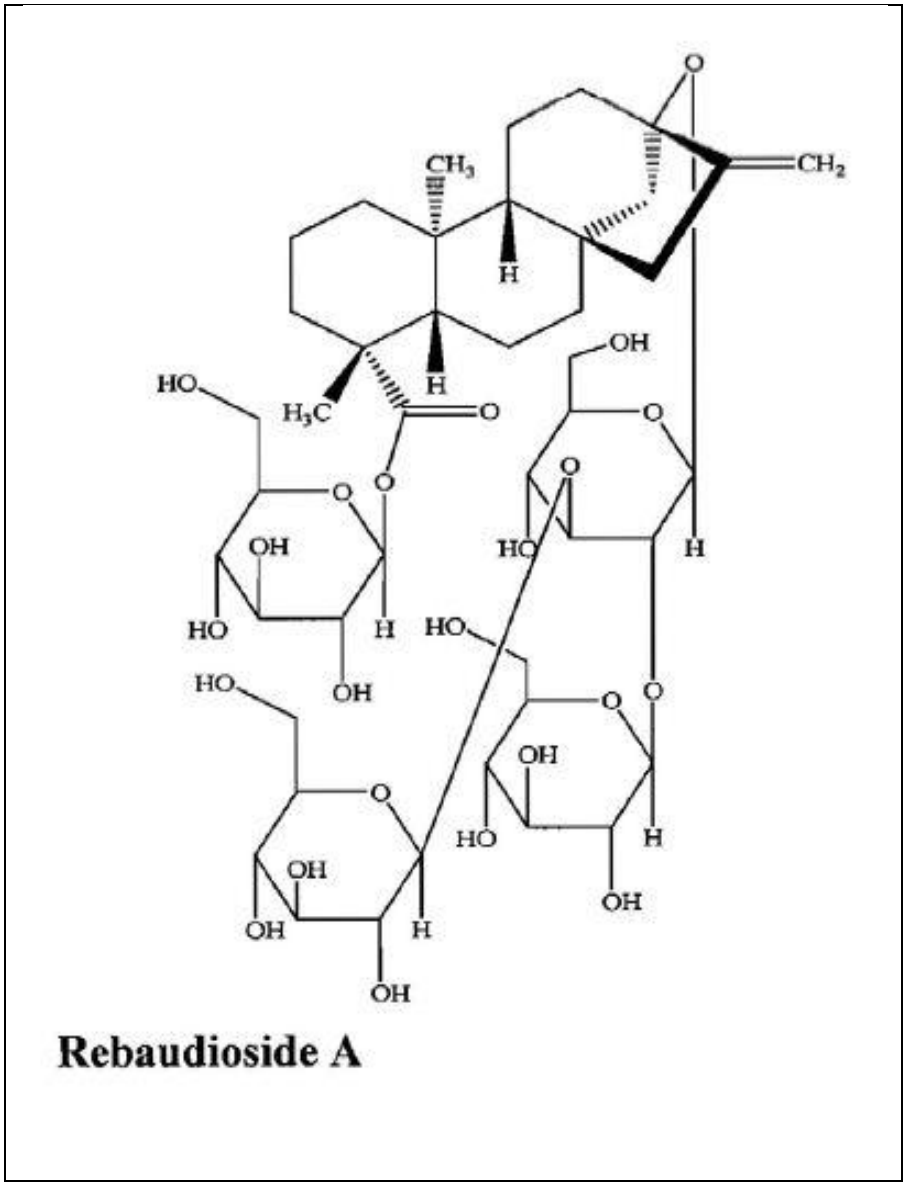

Figure 2.3: Structure of Rebaudioside A

2.4 Extraction process

The most important step in isolating different types of bioactive compound from plants was extraction process. Extraction method ideally, should be quantitative and time saving (Puri *et al*., 2012). Recently, numerous methods have been report for the extraction of bioactive (Puri *et al*., 2012).

Intensification technique has been reported lately in order to improve the efficiency and productivity of extraction process. Raman G., (2002) has reported that process intensification has become a very interesting approach, transforming current practices in biochemical engineering and bringing forth new processing technique. Cravotto., (2008) said that intensification is a secure and worthy method of improving either a rather lengthy (time consuming) or and energy intensive (far from normal conditions) process, searching for the increase of at least one of the major parameter governing it: the kinetic, through the partial transfer rates, the interfacial area or the driving force, seen as the distance from the actual state of the process and its equilibrium.

Chemical extraction method for bioactive are widely used due to their well established and easy to perform. Mixtures of a good solvent such as acetone and water have been used for the extraction of antioxidants (Awika *et al*., 2003). In order to increase the productivity, several intensification techniques like ultrasonic waves, supercritical fluids or microwaves were associated with the extraction of plant's compounds to improve the yield and quality of extracted products (Wang, 2006).

Recently, enzyme assisted extraction methods have been reported for the extraction of plant based bioactives such as vanillin and flavorings (Puri *et al*., 2011). Extraction of flavoring using enzyme was significantly increased the product released from plant material and minimizing the uses of solvent and heat (Kaur *et al*., 2010).

2.4.1 Microwave assisted extraction

Microwave assisted extraction is one of the modern technique with the concept of heating the extractant (mostly liquid organic solvents) in contact with the sample with the microwave energy (Pare *et al*., 1994).

Microwave assisted extraction has been proved to be the extraction tool for extraction of phytochemicals from botanicals (Mandal *et al*., 2009). Microwave assisted extraction is the process of heating solvent in contact with a sample with microwave energy (Smith, 2003). Extraction of SDG (SECO diglucoside) using microwave assisted extraction has also been reported recently allowing a gain of time but no marked improvements of yield were obtained (Beejmohun *et al*., 2007)

2.4.2 Enzymatic extraction

Cell wall degradation of polysaccharides is a fundamental step in improving the released of active compound form medicinal raw material (Li *et al*., 2012). Pinelo *et al*., (2008) in their research reported that cell wall degrading enzymes can break down the structural integrity of the cell wall and can increased the solvent accessibility and released the active compounds from intracellular compartment.

Cinar (2004) claimed that enzymatic extraction of carotenoid pigments is found to be new technique. Peptinase and cellulase enzyme were used to disrupt the cell wall of orange peel, sweet potato and carrot, released the carotenoids in the chloroplast and in cell fluids.

The pigments maintained their natural stated which bound with protein (Fennema OW, 1985). The bounded structure also prevents the pigment from oxidation and affects the colour stability. The extraction based on solvent will dissociates the pigments from the proteins and causes water insolubility and ease of oxidation (Bassi *et al*., 1993). Figure 2.4 below shows the chemical structure of some typical orange juice carotenoids.

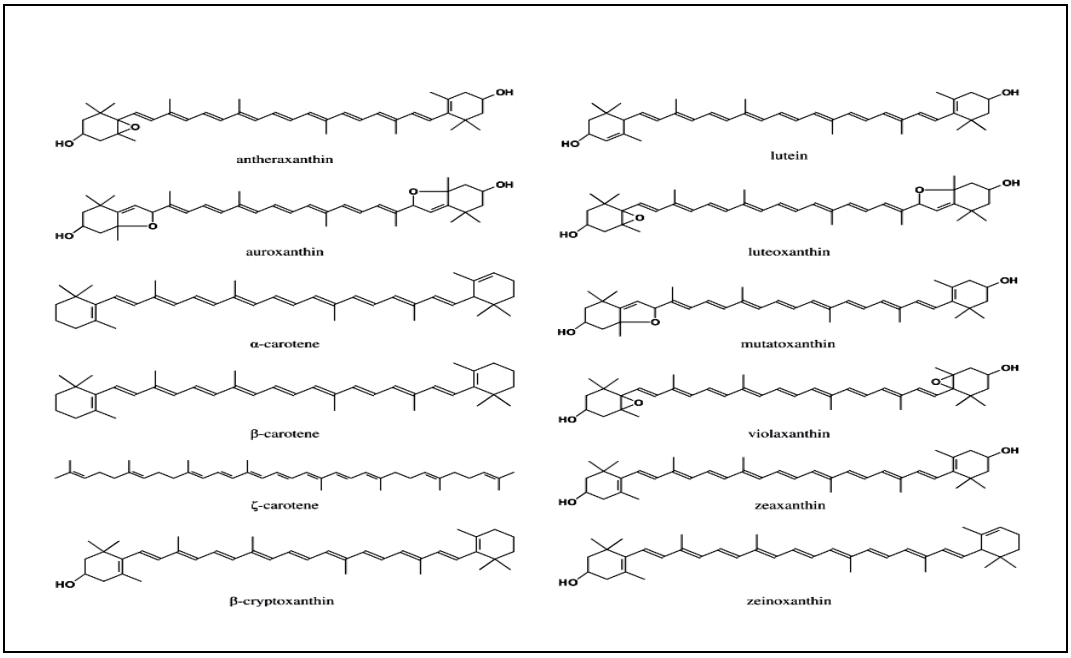

Figure 2.4: Chemical structure of orange juices carotenoids.

Enzyme assisted extraction methods have been reported as a new extraction method for plant based bioactive such as vanillin, phenols, polysaccharide from sterculia, oil from grape seed and flavorings (Puri *et al*., 2011).

The enzyme such as cellulase and pectinase were used to break down the plant cell wall hence rendering intracellular material more accessible for extraction. The used of enzyme in extraction help to minimize the usage of organic solvent thus offers a feasible green option (Puri *et al*., 2012).

2.5 Advantage of Enzymatic Extraction

The application of enzymes for complete extraction of bioactive normally results in a reduction in extraction time, minimizes the usage of solvent and provides increased yield and quality product (Meyer & Sowbhagya, 2010). Decreased the usage of solvent during extraction are particularly important for both regulatory and environmental reasons, providing a greener option than traditionally non enzymatic extraction (Puri, 2011).

2.6 Type of Enzyme

Basically, there are four groups of plant enzymes, and each one of them is responsible for breaking down a certain type of nutrient. Protease is responsible for breaking down a protein, amylase can breaks down the sugar, while lipase works on the fats, and cellulose helps to break down the carbohydrates. Typically, all whole foods contain the necessary enzymes for the body to properly digest that particular food.

The enzymes that can degrade plant cell wall materials include cellulase, hemicellulases, pectinase, chitinase and many ancillary enzymes. Cellulases are part of a large group of glycosyl hydrolases that have been categorized into several families on the basis of their amino acid homology. Hemicellulases are able to degrade hemicelluloses, a class of polysaccharides that can form hydrogen bonds with cellulose fibrils and form a network in plant cell walls (Doi and Kosugi, 2004)

2.6.1 Cellulase

Cellulase is an enzymatic protein that hydrolyzes the cellulose polymer to smaller oligosaccharide, cellobiose and glucose (Criquet, 2002). Cellulase randomly splits cellulose chains into glucose whereas commercial pectinase from aspergillus niger have pectinesterase, polygalacturonase and pectilyase activity (Cinar, 2004). Variety of bacteria and fungi can be used to produce cellulase (Lee *et al*., 2003).

According to Cinar, (2005) cellulase and pectinase enzyme is used to distrupts the cell wall of orange peel, sweet potato and carrot and release caratenoids in the chloroplast in the cell fluids. These pigments remain in their natural state still bound with proteins. The structure with the proteins bound prevents pigment oxidation and also affects the colour stability. A combination of cellulase and pectinase can accelerates the rate of hydrolysis to complete the liquefaction where cellulase can randomly split the cellulose chains into glucose.

2.6.2 Application of Cellulase

Cellulases have been applied successfully in textile and laundry industries because of their ability to modify cellulosic fibres in a controlled and desired manner and improved the quality of fabrics. Cellulase are also increasingly used in household washing powders, since they enhance the detergent performance and allow the removal of small, fuzzy fibrils from fabrics surface and improve the appearance and colour brightness (Bhat, 2000).

The interest in bioconversion of lignocellulosic biomass using cellulase and other enzymes became famous in order to find an alternatives source for renewable the energy (Sukumaran *et al*., 2005).

2.6.2.1 Food and Animal Feed

In food industry, cellulase is mostly used in extraction and clarification process of fruit juices, production of fruit purees and extraction of olive oil (Galante *et al*., 1998). Other than that, cellulase was also used in caretonoid extraction to produced food coloring agents (Kvietok *et al*., 1995). In animal feed, Bedford *et al*., (2003) reported about the use of Trichorderma cellulase in feed additive to improve the feed conversion ratio and increasing the digestibility of a cereal-based feed.

2.6.2.2 Pulp and Paper Industry

Cellulase and hemicellulase have been employed in pulp and paper industry for biomechanical pulping for modification of the coarse mechanical pulp and hand sheet strength and for improving drainage and run ability of paper mills (Prasad *et al*., 1992). Besides, cellulase was also employed in removing the inks, coating and toner from paper (Yang *et al*., 2004). Hsu *et al*., and Sharyo *et al*., (2002) concluded that both this enzyme mostly used in paper industry including the soft paper such as sanitary paper and paper tower where the cellulase are used to removed the adhered paper.

2.6.2.3 Biofuel

A wide application of cellulase with its potential in converting the cellulosic materials to glucose and other fermentable can be used as microbial substrates for the production of single protein and variety of fermentation product such as ethanol (Sukamaran *et al*., 2005).

The production of bioethanol from lignocellulosic residue is a multi step process involving pre treatment to remove lignin and hemicellulase. Treatment of cellulase at temperature 50^{o}C will hydrolyze the cellulosic residue and generate the fermentable sugar

that will be used to produce alcohol from hydrolyzed cellulosic material (Sudha *et al*., 1997).

2.7 Plant cell wall

There are two type of cells wall can be distinguished which is primary cell wall and secondary cell wall. For the primary cell walls, the walls are deposited during cell growth. In order to avoid the rupture of cells, it needs to be stable and extensible to allow the cell expansion. Primary cell walls mainly consist of polysaccharides that can be classified as cellulose, hemicelluloses and pectin. Secondary cell walls are deposited after the end the cell growth (Rieter, 2002).

2.7.1 Cellulose

In plant, cellulose consists of two parts which are crystalline structure and amorphous structure. The cellulose strains form are bundled together and were called cellulose fibrils or cellulose bundles. These cellulose fibrils are mostly independent and weakly bound through hydrogen bonding (Perez *et al*., 2005). Cellulose is a major component of plant cell wall and the most abundant carbohydrate polymer in nature (Doi et al., 2004). Different cellulose synthase perform cellulose synthesis in the primary cell wall and secondary cell wall (Malcom *et al*., 2007). Cellulose is the most abundant polymer in the biosphere with estimation of synthesis rate of 1010 tonnes per years (Juwaed *et al*., 2011). Although abundant, cellulose is particularly difficult polymer to degrade as it is insoluble and present as hydrogen bonded crystalline (Sanddler *et al*., 1999). Beguin et al., (1994) concluded that Cellulose is more susceptible to enzymatic degradation in its amorphous form.

The hydrolysis of solid cellulose employed simultaneous synergetic action of several different isozymes of cellulase (Belgium and Albert, 1994). The hydrolysis of insoluble cellulose is not linear with the enzyme dosage and reaction time (Wu *et al*., 2006).

Cellulose is the only renewable carbon source that is available in large quantity (Ryu *et al*., 1980). Cellulose is found principally in plant residue such as leaves and wood and cannot be direct assimilated by cellulolytic microorganism due to its molecular size and water insolubility (Criquet *et al*., 2002).

2.7.2 Hemicellulose

Hemicellulose is a complex carbohydrate structure that consists of different polymer like pentose, hexoses and sugar acid (Saha, 2003). Hemicellulose has lower molecular weight than cellulose and branches with short lateral chains that consist of different sugar (Fenger *et al*., 1984). This hemicellulose also serves as a connection between lignin and the cellulose fibers and gives the whole cellulose-hemicelluloses-lignin network more rigidity (Perez *et al*., 2005).

2.8 Carbohydrate Analysis

Carbohydrates may be added to food products or occur naturally to provide nutrient and to improve the texture and overall quality of food product. It was also known as one of the most important ingredients in food and raw material (Cui *et al*., Taylor & Francis, 2005). Carbohydrates are quite heterogeneous, differing in primary structure, degree of polymerization and charged. Due to the heterogeneity of this group of compounds, it makes the analyzing the total carbohydrates content of a sample quite complex (Taylor & Francis, 2005).

Before analyze any class of carbohydrate such as monosaccharide, the sample prepared must remove the substance that can interfere with analysis. There are two type of chemical reaction method that has been applied to analyze neutral sugars which are phenol-sulfuric reaction method and anthrone reaction method (Miller *et al*., 1998).

2.8.1 Application of Phenol Reaction Method

The phenol reaction method is used widely in order to determine the total concentration of carbohydrates in a sample solution. This method is also appropriate for both reducing and non reducing sugars. This method is advantageous due to the low cost of the reagent and readily available (Gilles *et al*., 1956). The absorption curve resulting from the analysis, are characteristic for different type of sugars. Figure 2.5 below shows example absorbance curve of phenol reaction absorbance maxima for hexose and pentose.

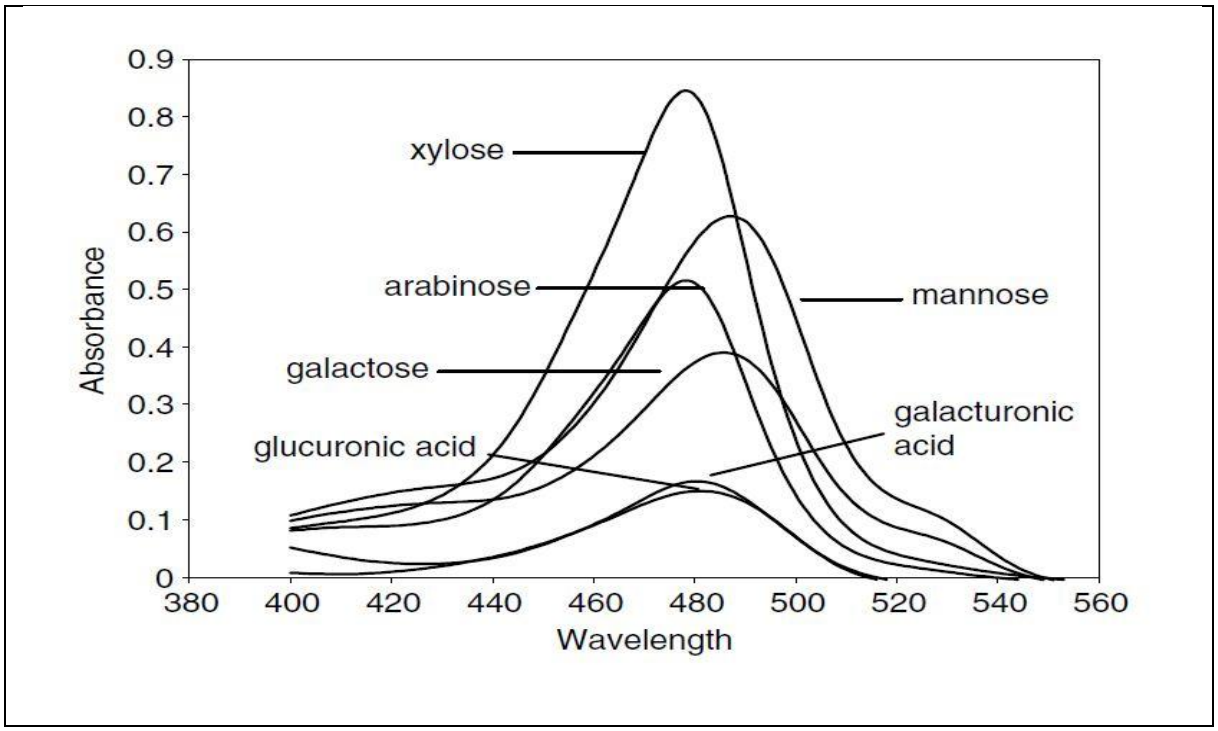

Figure 2.5: Phenol Reaction Absorbance Maxima for Hexose and Pentose

2.8.2 Application of Anthrone Reaction Method

The anthrone reaction method is the most applicable reaction method to a solution containing one type of hexose. This is because a reaction of anthrone and sugar with similar structure will result in a different rate and quantities of color development. However, this reaction method can also be used for quantitative analysis of oligo and polysaccharide with only one type is present in the solution (Edward *et al*., 2003)

2.9 Analysis of Stevioside

Anthrone method was used in this present study to determine the concentration of rebaudioside A and stevioside in solvent. It was reported by (Ludwig *et al*., 1956), an investigation was made of the suitability of the anthrone method for the estimation of carbohydrate in food and oral rinsings where this method was found offers a quick and relative accurate technique for determination of this type.

Anthrone method is based on the condensation of furaldeyde derivatives, generated by carbohydrate in the presence of a strong acid, with a reagent, anthrone to produced colored compounds. The reaction of carbohydrates in a strongly acidic environment with anthrone results in a blue-green color and the absorbance is read at 628nm (Cui *et al*., 2004).

2.10 Principle of Spectrophotometry

A spectrophotometer is the combination of two devices namely spectrometer and a photometer. The spectrometer is used in producing light of any selected wavelength while photometer is used in measuring the intensity of light. These two devices both are placed at either site of the cuvette that filled with a liquid. The spectrometer will reach the photometer that measures its intensity by producing the desired wavelength and passes through the tubes. The amounts of absorption of light at the appropriate wavelength represent the amounts of concentration in the solution (Kirsten, 2008).

Spectrophotometric methods have become the most frequently used and important methods of quantitative analysis. This method is applicable to many industrial and clinical problems involving the quantitative determination of compounds that are colored or react to form a colored product. Table 2.1 below shows pairs of complementary colors and the corresponding wavelength ranges.

Wavelength (nm)	Color Absorbed	Color Observed
400	Violet	Yellow-Green
435	Blue	Yellow
495	Green	Purple
560	Yellow	Blue
650	Orange	Greenish blue
800	Red	Bluish green

Table 2.3: Pairs of Complementary Colors and Corresponding Wavelength Ranges

2.11 Summary

Extraction of phytochemicals is importance to the pharmaceutical and the dietary supplement industries. It was also known as the starting step in qualitative and quantitative analysis of medicinal plant constituent, but until today very substantial amount of work has been done to improve the efficiency of this crucial step (Smith, 2003). Salvador *et al*., (2004) in his research employed ultrasound wave to extract active compound such as saponins, steroids and triterpenoids from *Chresta spp*. and concluded that this method was three times faster than with the conventional extraction methods. After that, microwave assisted extraction, in recent times has been proven to be promising ideal extraction tool for the extraction of phytochemicals from botanicals (Mandal *et al*., 2007). But, Beejmohun *et al*., (2007) reported that extraction using microwave assisted extraction may allowing a gain of time but no marked improvement of yield were obtained. Recently, enzyme assisted extraction methods have been reported for the extraction of plant based bioactives such as vanillin and flavorings. This extraction using enzyme was said to be significantly increased the product released from plant material and minimizing the uses of solvent and heat (Kaur *et al*., 2010; Puri *et al*., 2012; Li *et al*., 2012).

CHAPTER 3

METHODOLOGY

3.0 Introduction

This chapter describes the material used in this present study and the methodology applied during this research. The behavior of cellulase in the extraction of stevioside was evaluated in this present study. The flow of the experimental work is shown in Figure 3.1 below.

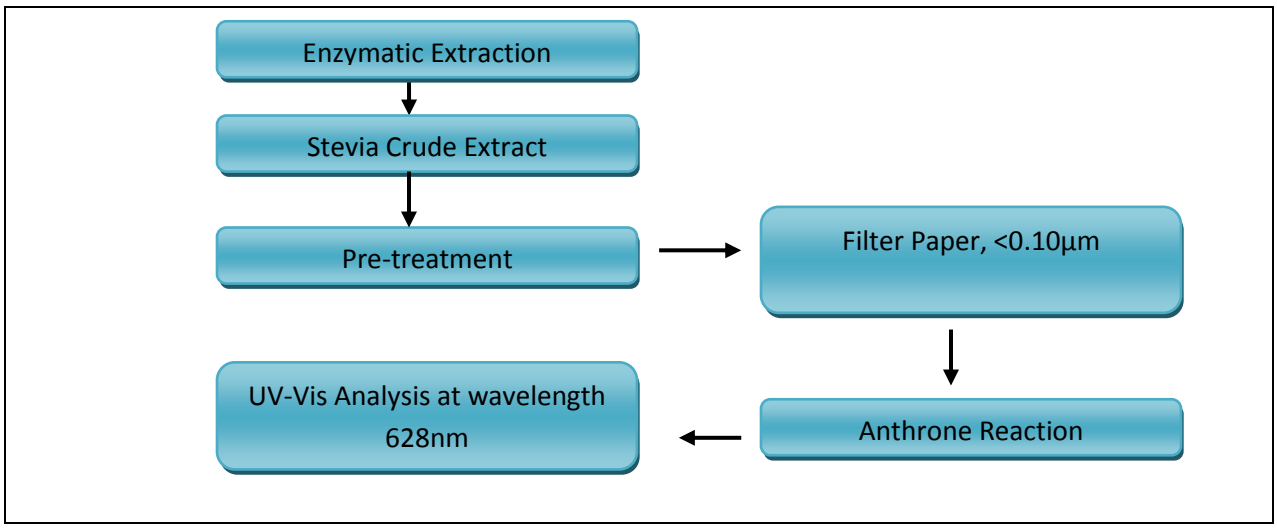

Figure 3.1: Experimental work on the extraction of stevioside using cellulase.

3.1 Material Used

3.1.1 Plant and chemicals

The raw material used in this study was *Stevia rebaudiana* leaves, water and ethanol. *Stevia rebaudiana* leaves were purchased from local market. The ethanol was purchased from Sigma Aldrich (Malaysia). For the analysis, anthrone method was used to determine the concentration of stevioside in solvent, where the anthrone reagent, sulphuric acid and hydrochloric acid were also purchased from Sigma Aldrich (Malaysia). Besides, stevioside (~95%) purity, used as a standard in this study was also purchased from Sigma Aldrich (Malaysia).

3.1.2 Enzymes

Food grade cellulase form *Aspergillus Niger* used in this study was purchased from Sigma Aldrich (Malaysia). This enzyme is water soluble and was found to be highly efficient in the degradation of plant cell wall. Cellulase is an enzymatic protein that hydrolyzes the cellulose polymer to smaller oligosaccharide, cellobiose and glucose.

3.2 Experimental procedure

3.2.1 Stevia leaves preparation

The green *stevia rebaudiana* leaves were selected, whereas the dried brown, yellow were separated. The green leaves were washed with deionized water and dried in oven at 60^{o}C for about 48 hours. The leaves were then grinded into smaller pieces to increase their surface area. The leaves then were stored in clean beaker waiting to be used.

3.2.2 Acetate Buffer Preparation

27.2g of 0.2M sodium acetate was mixed with 800ml of deionized water. The solution was bringing to pH 5 with a few drop of glacial acetic acid.

3.2.3 Enzymatic Extraction

Four different concentration of cellulase enzyme (0.5%, 1%, 2% and 4% w/v) were used in this experiment in order to measure the effect of enzyme extraction on the yield of stevioside. These experiments were performed at different extraction temperature (27, 40, 50 and 60 ^{o}C) and extraction time (30, 60 and 120 min).

Dried *stevia rebaudiana* leaf (10g) was placed in 500 ml conical flask with different enzyme concentration in 10ml of acetate buffer at pH 5.0. The mixtures were shaken at 150 rpm after the addition of 20 ml of ethanol 95%. This step was then repeated for each time (30, 60 and 120 min) and temperature (27, 40, 50 and 60 ^{o}C). After that, the resultant stevia crude extract was filtered with Whatmann filter paper pore size < 10 µm. The filtrate solution was then collected and this step was repeated for the other parameter.

3.2.4 Stevioside Standard Solution

A stevioside standard solution was prepared with four different concentrations of 100, 200, 400 and 800µg by dissolved 3.2 mg of stevioside hydrate in 4ml of distilled water. After that, the solution was diluted with 4 mL of distilled water. Finally, the solution was store at 4°C to be used.

3.3 Analysis

3.3.1 Anthrone Reaction Method

Anthrone (ACROS ORGANICS) and sulfuric acid (96%) were both purchased from Sigma Aldrich (Malaysia). The analysis of stevioside was conducted using UV-Vis spectrophotometer. Anthrone solution was prepared by dissolving 0.1g anthrone in 76% sulfuric acid, and makes it to a volume of 100ml.

An amount of 1 ml stevioside sample from different concentration was mixed with 5 ml of anthrone solution in a close cap test tube. The tubes then were immersed in water bath at boiling point for 12 minutes. After that, it was cooled at room temperature for 20 minutes. The sample was analyzed using UV-Vis spectrophotometer at wavelength of 628 nm.

3.3.2 Standard Solution for Calibration Graph Preparation

A standard solution of stevioside with a concentration of 100, 200, 400, and 800µg/ml were prepared according to section 3.2.4. After that, the sample were analysed using the anthrone reaction method. A calibration graph is plotted according to the four different concentrations of standard stevioside.

CHAPTER 4

RESULT AND DISCUSSION

4.0 Introduction

This chapter present the result achieved from this present study on the effect of processing parameters such as the temperature, incubation time and enzyme concentration that influence the effectiveness of the extraction process. For the sample analysis, anthrone reaction method was used in determining the total concentration of stevioside in the sample. The absorbance of the solution was read and the data was collected at 628 nm by using UV-Vis spectrophotometer. The result of this study is present in term of the effect of the parameter involved in the scope of study, which are the temperature, incubation time and concentration of cellulase used. The higher temperature used in the extraction process have make the cellulase denatured since the maximum temperature for cellulase activity is between 37^{o}C to 55^{o}C, as report by Criquet (2002) and Gautam *et al.*,(2011). The faster the reaction rate of the enzyme solution in degrading the plant cell wall increased the amount of production of stevioside released, given its condition of degrading is at its maximum condition.

4.1 Standard Curve of Stevioside

A standard curve of stevioside sample was prepared as references for the absorbance values obtained from the spectrophotometer. The standard solution of stevioside was done successfully by diluting 3.2 mg of stevioside hydrate in 4 ml of deionized water. Anthrone reaction was selected as suitable method for carbohydrate analysis and used in this study. The result of the reaction of stevioside standard solution with anthrone solution has been measured by absorbance at wavelength 628 nm through the spectrophotometer. The readings of absorbance obtained through spectrophotometer have been tabulated in Table 4.1 below and the standard curve of stevioside was as in Figure 4.1 below.

Concentration, µg/ml	Absorbance, 628 nm
800	2.602
400	1.278
200	0.781
100	0.375
50	0.206

Table 4.1: Absorbance value of Standard Stevioside

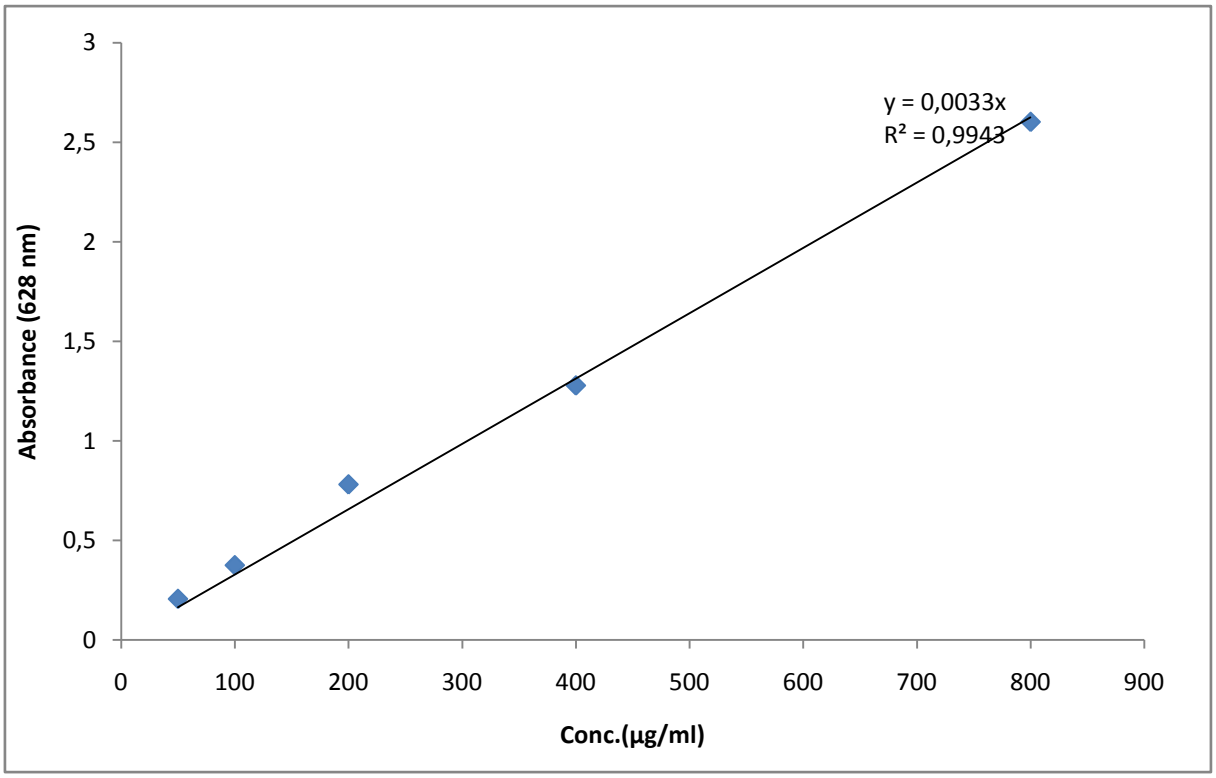

Figure 4.1: Standard curve of Stevioside

A standard curve of stevioside was generated as a reference for this study. The equation of y = mx + c in Figure 4.1 above was determined in order to show the accuracy of the result. Since R^2 value is equal to 0.994 which is near to 1, it can be concluded that this result is accepted and can be used as reference in this present study.

4.2 Enzymatic Extraction of Stevioside

4.2.1 Effect of Temperature

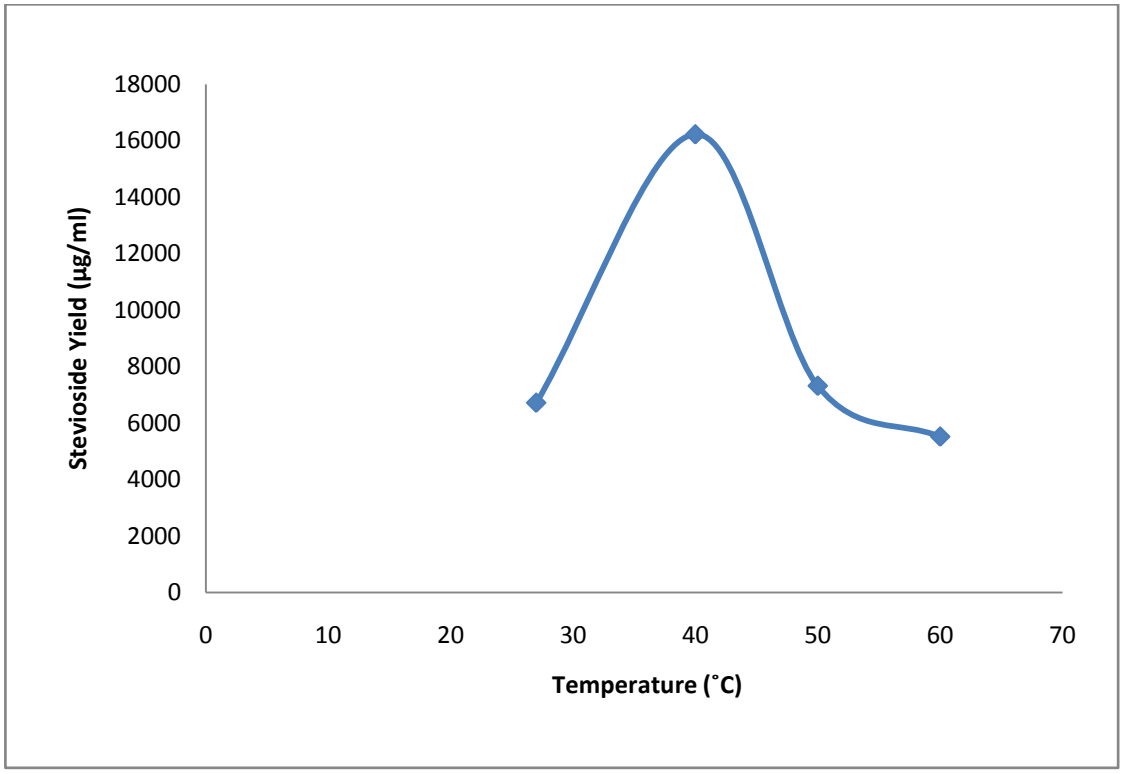

Figure 4.2: Effect of temperature on enzymatic extraction

The stevioside yield was investigated at fixed concentration of enzyme, (2% w/v) and incubation time 60 minutes. Since enzymes have maximum temperature for its activity, variation of temperature (27, 40, 50 and 60^{o}C) was investigated to achieve highest yield of stevioside. The graph from Figure 4.2 shows that the stevioside yield was increased to maximum yield (16230 µg/ml) at temperature 40^{o}C and significantly decreased by 38% when reached 50^{o}C. Since temperature is one of the main factors for the cellulase activity, the yield of stevioside slowly decreases from 7320 µg/ml to 5520 µg by 14% when the temperature reached 60^{o}C.

The maximum temperature for cellulase activity appeared to be 40^{o}C since the yield of stevioside increased by 41% from 6720 µg/ml at room temperature. The enzyme activities at maximum temperature (40^{o}C) have facilitated the degradation of the cell wall of polysaccharides thus maximize the stevioside yield. As it stated by Li *et al.,* (2012) that the reaction rates of enzyme solution gradually accelerated with the increase of temperature up to maximum temperature for enzyme activity and decreased after that due to the enzyme denaturation at temperature above the maximum temperature.

Thus, it was suggested here that the improvement of the stevioside yield become decreased at higher temperature than maximum temperature for the cellulase activity due to the cellulase denaturation. It was known that endogeneous enzymes have good activity at warm temperature but at high temperature its activity became inactivated (Puri *et al.*, 2012). Due to the inactivated enzyme activity at high temperature, the yield of stevioside after 40^{o}C became decreased.

From Figure 4.2, the stevioside yield varied with the changing temperature and highest yield of stevioside occurred at a temperature of 40^{o}C. Therefore, temperature 40^{o}C was considered to be the best choices for cellulase incubation for this study. This is the same as previous experiment, as it states that a temperature of 40^{o}C is the best options for enzyme incubation since the highest yield appeared at a temperature of 40^{o}C (Renouard *et al.*, 2010; Li *et al.*, 2012).

Previously, conventional extraction method for stevioside from *S.rebaudiana* leaves was employed using thermal degradation, pressurizes hot water extraction, supercritical fluid extraction and microwave assisted extraction (Kovylyaeva *et al*., 2007; Pol *et al*., 2007; Puri *et al*., 2011). This conventional extraction method was report required high temperature and high energy when the extract is subject to thermal degradation through overheating (Alupului *et al*., 2009). Thus, by using enzymatic extraction in producing stevioside, less energy was required since the maximum temperature used in the extraction process is not high than the conventional extraction method. Conventionally, solvent

extraction method was frequently used in extracting plant bioactive. Then, enzymatic extraction became an alternative method in recovery purification product since it can minimize the used of solvent in assisted the extraction process. Smith *et al*., (2006) has claimed that the highest recovery was achieved using enzymatic extraction.

4.2.2 Effect of Incubation Time

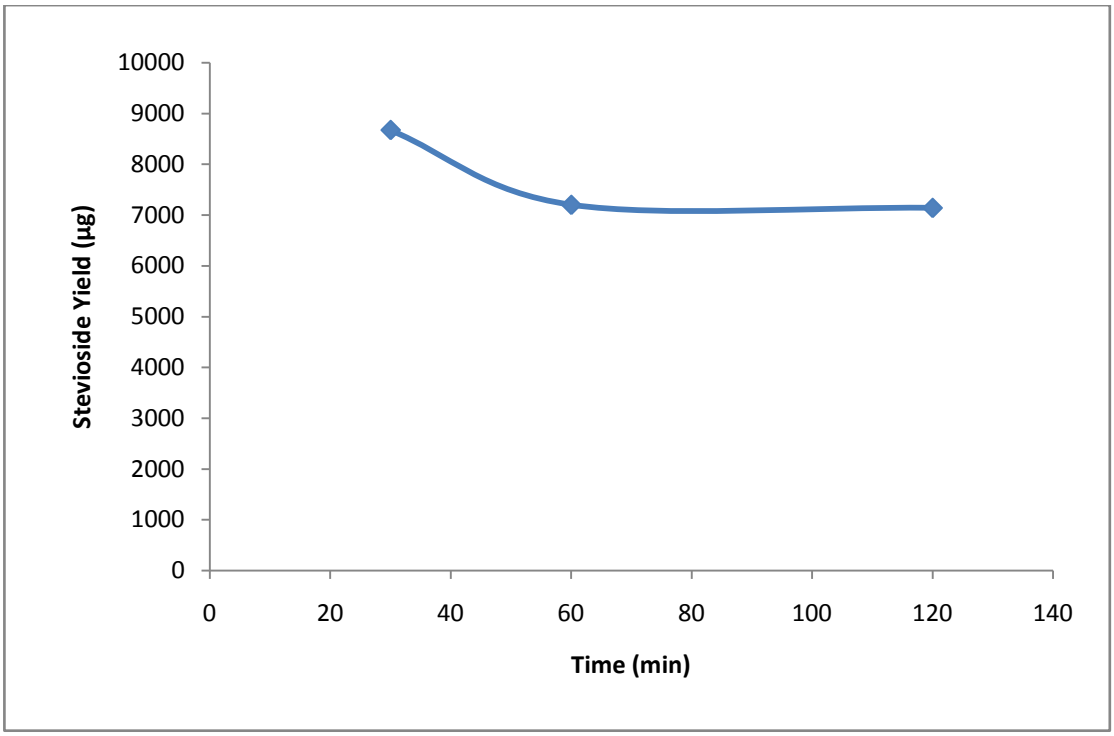

Figure 4.3: Effect of incubation time on enzymatic extraction

Figure 4.3 above shows the result of the effect of the enzyme incubation time on the stevioside yield. The stevioside yield was evaluated at fixed concentration of enzyme (2% w/v) and temperature 50^{o}C. The variation of incubation time (30, 60 and 120 minutes) was studied to achieve highest yield of stevioside. An incubation time of 30 minutes was found to be the maximum time for the enzyme activity due to the maximum production of stevioside that shown as in Figure 4.3.

Extending the duration of incubation up to 60 minutes resulted in slightly decreased in the production of stevioside and the yield of stevioside became slowly decreased and constant without any further conversion occurred when longer the incubation time. Since the maximum temperature for cellulase activity was 40^{o}C, at high temperature the enzyme's activity became inactivated and thus not helping in degrading the cell wall to release the stevioside and increased the production. But rationally, the yield of stevioside released should be increased consistent with the duration of incubation when the maximum temperature for the cellulase activity in degrading the cell wall is used. The yield will only decreased when extending the duration of incubation with the maximum temperature of cellulase used because of the activity of the cellulase reduced due to deactivation. When cellulase is inactivate, the reaction rates were also slowly decreased. In the previous experiment, it can be observed that by extending the incubation time with the maximum temperature of the enzyme used, it has increased the extraction yield and the yield began to decrease with longer incubation (Renouard *et al.*, 2010).

The yield of stevioside was increased initially due to the high cellulase temperature used that over than maximum temperature for the cellulase activity in a small scale of extraction. But rationally, with shorter incubation time it will only led to partially released of the active compound from herbal medicine. This result proved that by longer the incubation time, it failed to improve the extraction efficiency in a small scale of extraction when high temperature was used.

Based on previously researcher Smith *et al*., (2006) it was reported that the used of enzymatic extraction was more effective and can improve the time consuming for the extraction process. Comparing the enzymatic extraction method with aqueous organic extraction method, the used of enzymatic method can save and reduced the incubation time (3 hours) for 50% from 6 hours with aqueous extraction method (Smith *et al*., 2006).

4.2.3 Effect of Cellulase Concentration

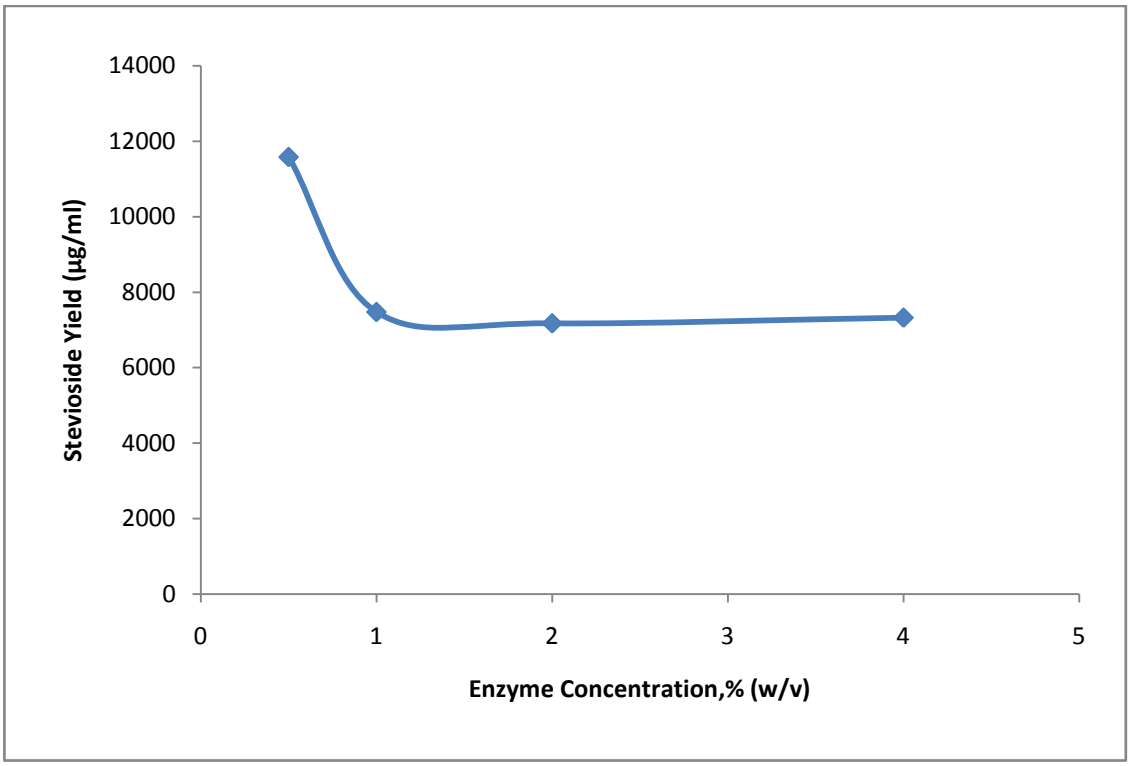

Figure 4.4: Effect of enzyme concentration on enzymatic extraction

The effect of using different cellulase concentration (0.5, 1.0, 2.0 and 4.0% w/v) on stevioside yield is shown in the Figure 4.4 above. The incubation time is fixed at 60 minutes and reaction temperature of 50^{o}C. The highest peak of stevioside yield (11580µg/ml) in the Figure 4.4 shows that the maximum concentration of cellulase was at 0.5 % w/v.

The yield of stevioside as shown in the Figure 4.4 became decreased when high concentration of cellulase was used. The yield became decreased consistently with higher concentration of cellulase probably because of the end product inhibition that occurred due to high concentration of cellulase that speeds up the hydrolysis process. Generally, the reaction rate of enzyme increased gradually with the increase of the concentration until the saturated concentration that suitable in maximizing the product. This is same as observed in

the previous experiment using same method, that the yields start to decrease with higher concentration of enzymes (Lee *et al.*, 2012). The main reasons in the use of cellulase to maximize the production, was its ability to distrupt the cell wall, thus make the extraction became more effective.Because of that, the maximum cellulase concentrations (0.5% w/v) that maximized the production were selected as a suitable concentration for a small scale (10g) extraction of stevioside from *stevia rebaudiana* leaves.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

In this present study, extraction of stevioside from *Stevia rebaudiana* leaves using cellulase was successfully done. The parameter that affects the cellulase in releasing the stevioside such as the temperature, incubation time and cellulase concentration was investigated. The maximum temperature for cellulase activity was found to be at 40^{o}C. The highest yield of stevioside obtained in this present study was 16230µg/ml, under the maximum conditions for cellulase activity.

From the experimental work done to extract stevioside from *Stevia Rebaudiana* leaves using cellulase, the result shows that the yield of stevioside can be maximized under the maximum conditions for the temperature, incubation time and cellulase concentration . It was found that the maximum incubation time for the extraction of stevioside using cellulase was 30 minutes. Since only small extraction scale was used in this present study, the maximum cellulase concentrations that suitable used in achieving the highest yield of stevioside were 0.5 % w/v. Thus, it can be concluded that the extraction of stevioside from *Stevia rebaudiana* leaves using cellulase can be maximized under the maximum conditions for the cellulase activity where the used of solvent can be minimized in degrading the cell wall. From the previous experiments, it was state that extraction using cellulase was proven

to be highly efficient in the degradation of plant cell wall (Li *et al.*, 2012). Therefore, with less solvent used, enzymatic extraction can also be an alternative for the greener option. Together with the maximum heat and correct combination of the solvent used, a new and efficient way of extracting high yield of stevioside can be obtained.

5.2 RECOMMENDATION

To improve this research project accuracy, several recommendations has been proposed during the study. Firstly, the experiment should be run not higher than 40^{o}C. Since, the maximum temperature for cellulase was 40^{o}C it is suggested that the cellulase will be denatured above this temperature.

Secondly, in order to obtain the desired levels of stevioside concentration under the optimized condition, it is highly recommended to do the optimization and resonance surface methodology (RSM). This will provide an optimum set of independent variable that can be used for further study.

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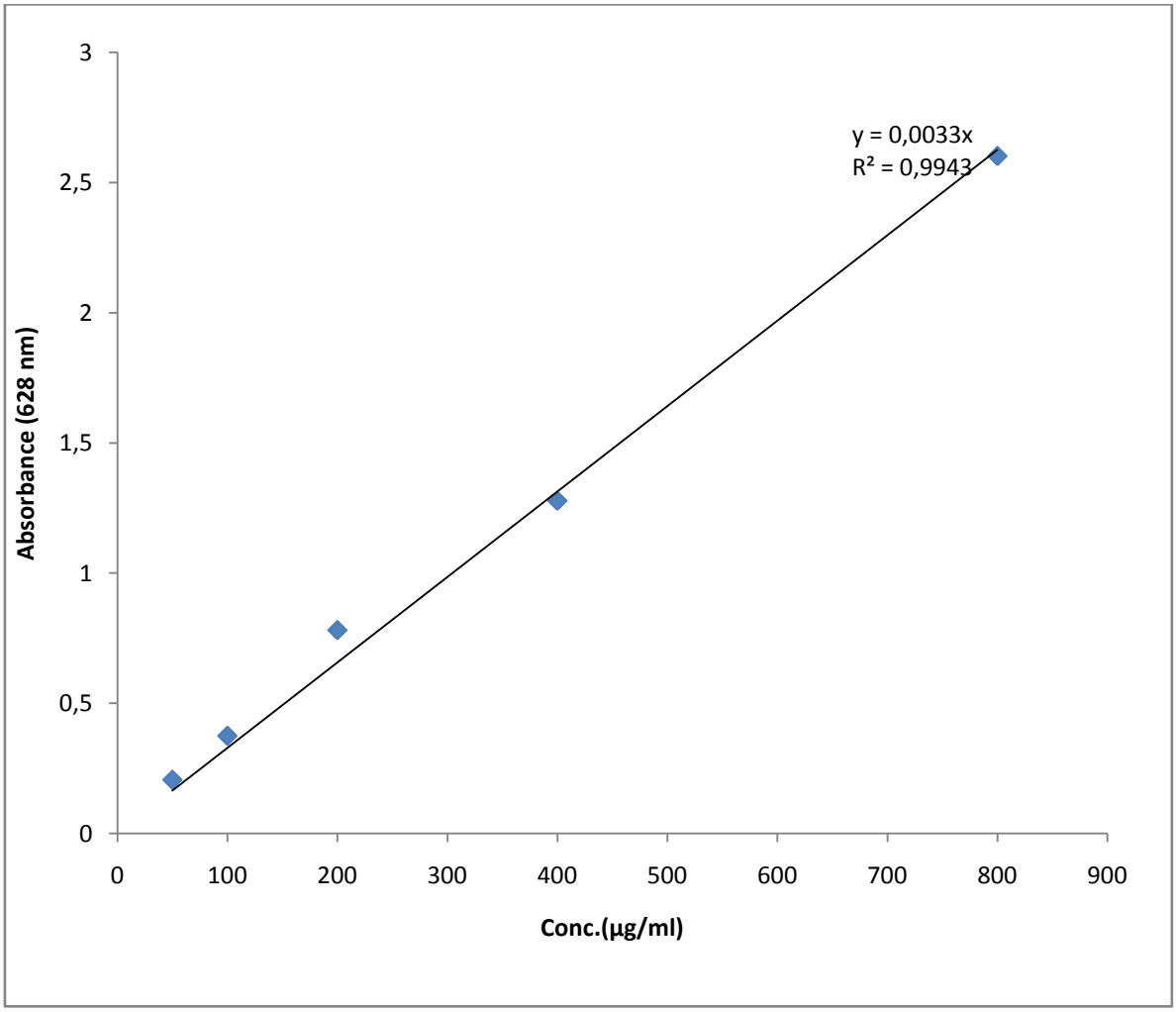
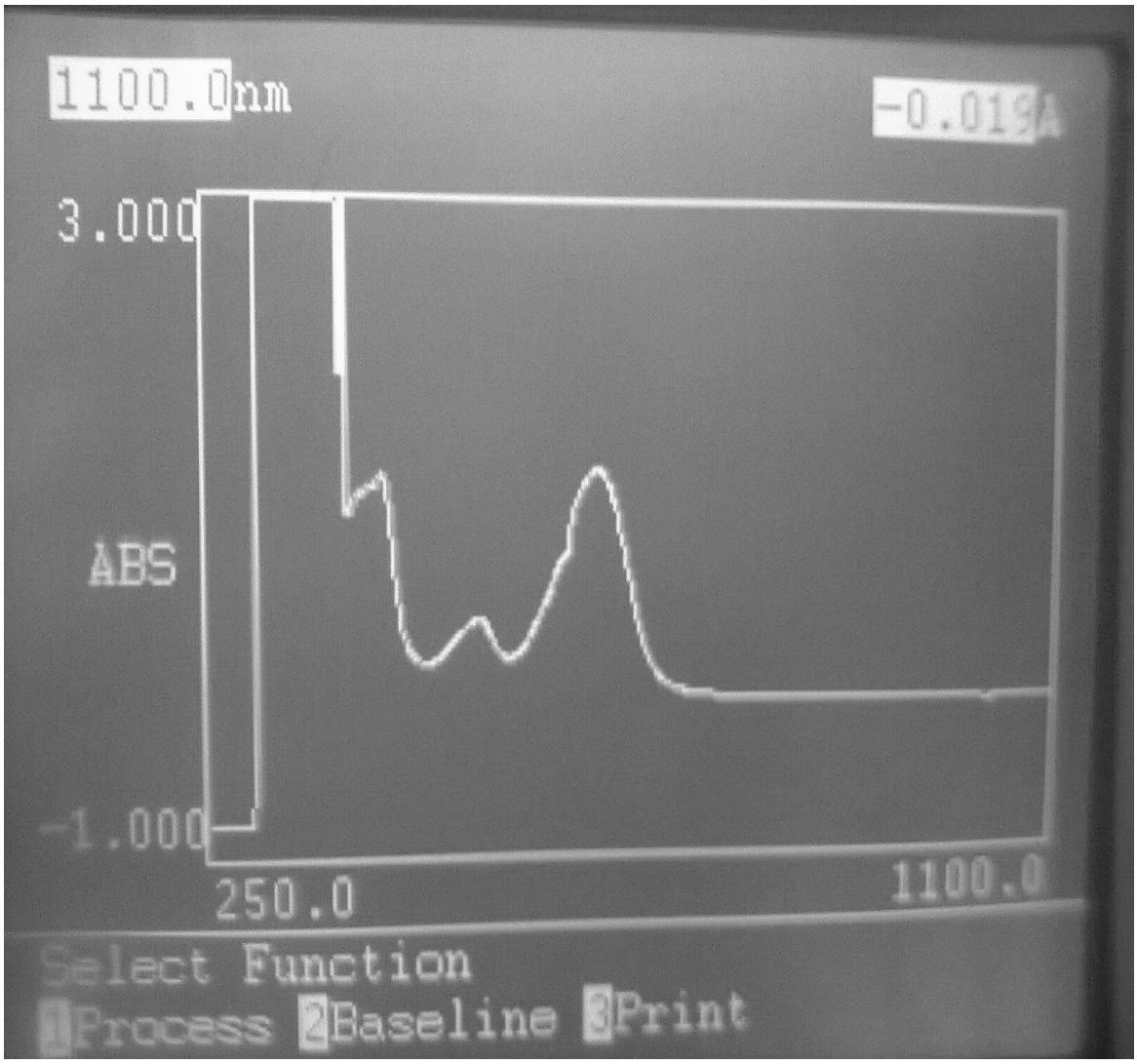
APPENDIX

A-1 Standard Calibration Curve



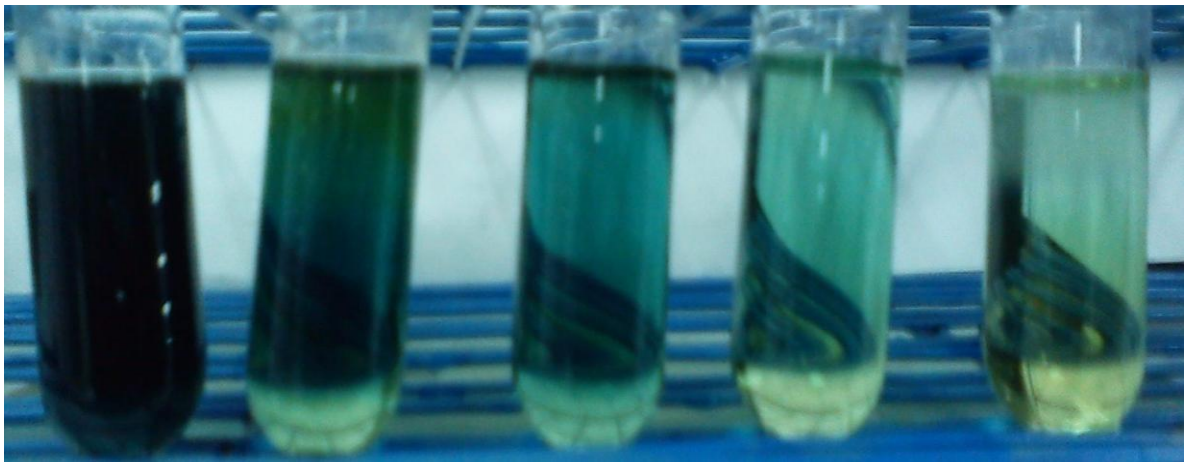
A-2 Wavelengths Scanning of Standard Stevioside



A-3 Range of Wavelengths Scanning

A-4 Anthrone Reagent Used for Analysis

A-4.1 Color Yield in Anthrone Reaction



A-5 Enzyme used

A-6 Stevioside Hydrate

A-7 Incubator Shaker

A-8 UV-Vis Spectrophotometer