ENZYMATIC EXTRACTION OF STEVIOSIDE FROM *STEVIA REBAUDIANA* LEAVES USING CELLULASE

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ENZYMATIC EXTRACTION OF STEVIOSIDE FROM STEVIA REBAUDIANA LEAVES

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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 terms 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: 28 FEBRUARY 2013

STUDENT'S DECLARATION

I hereby declare that the work in this thesis "Enzymatic 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 another degree.

Signature:……..

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Date: 28 FEBRUARY 2013

Dedicated especially to my beloved mother, my father, siblings, lecturers, friends and to those who gives me support and inspiration that made this work possible.

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ENZYMATIC EXTRACTION OF STEVIOSIDE FROM *STEVIA REBAUDIANA* LEAVES BY USING CELLULASE

ABSTRACT

Stevioside is one of the components known as diterpene glycoside existing in *Stevia Rebaudiana* leaves which have 250 to 300 sweeter than sucrose at the concentration of 0.4% (w/v). It is used as sweetening agents and taste modifier mostly in food industry. Moreover, stevioside has no calorific value and suitable used in therapeutic especially in treating diabetic people. The objective of this research is to extract the stevioside from *Stevia rebaudiana* leaves by using cellulase from *Aspergillus Niger*. The medium for enzyme used is acetate buffer while ethanol is applied as solvent in the enzymatic extraction of stevioside by performing various parameters such as concentration of enzyme, incubation time and temperature. Enzymatic extraction method gives the highest concentration of stevioside (900µg/ml) at 50°C as maximum temperature. The concentration of cellulase at 2% (w/v) gives the highest concentration of stevioside (830µg/ml) and the incubation times of 60 minutes gives the maximum time required to complete the extraction process of stevioside. Therefore, it can be concluded that the extraction of stevioside from Stevia rebaudiana leaves using cellulase is a new efficient way of obtaining high concentration of stevioside and also can minimize the use of solvent and energy consumption in degrading the cell wall.

ENZYMATIC EXTRACTION OF STEVIOSIDE FROM *STEVIA REBAUDIANA* BY USING CELLULASE

ABSTRAK

Stevioside adalah salah satu komponen yang dikenali sebagai glikosida diterpene yang wujud dalam daun Stevia rebaudiana yang juga mengandungi kemanisan di antara 250 hingga 300 lebih dari sucrosa (0.4% w/v). Stevioside adaalah agen pemanis dan pengubahusuai rasa dalam industri. Tambahan pula,stevioside tidak mengandungi nilai kalori dan sesuai digunakan untuk tujuan terapeutik terutama merawat penghidap penyakit diabetes. Objektif utama penyelidikan ini adalah untuk mengekstrak stevioside daripada daun *Stevia rebaudiana* dengan menggunakan sellulase daripada *Aspergillus niger*. Medium untuk enzim yang digunakan adalah buffer asetat manakala etanol digunakan sebagai pelarut dalam pengekstrakan enzim stevioside dengan mengendalikan pelbagai parameter seperti kepekatan enzim, masa pengeraman dan suhu. Kaedah pengekstrakan enzim memberikan kepekatan tertinggi stevioside (900μg/ml) pada 50°C sebagai suhu maksimum. Kepekatan sellulase pada 2% (w/v) memberikan kepekatan tertinggi stevioside (830μg/ml) dan masa pengeraman selama 60 minit memberikan masa maksimum yang diperlukan untuk melengkapkan proses pengekstrakan stevioside. Kesimpulannya, pengekstrakan stevioside daripada daun *Stevia Rebaudiana* dengan menggunakan sellulase adalah salah satu cara yang baru dan cekap untuk mendapatkan kepekatan stevioside yang tinggi dan juga boleh mengurangkan penggunaan pelarut yang banyak dan juga tenaga untuk pemecahan dinding sel.

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LIST OF SYMBOLS/ ABBREVIATIONS

ºC	Degree Celcius
%	Percentage
w/v	Weight per volume
α	Alpha
β	Beta
mL	Millilitre
nm	Nanometer
M	Molarity
µm	Micrometer
mg	Milligram
g	Gram
µg	Microgram
US	United State

CHAPTER 1

INTRODUCTION

1.1 Background of Study

One of the biggest diseases of world facing today is diabetes. It is approximately 2.6 million of population was diagnosed with diabetes in Malaysia which has been increasing from year to year. Another problem that Malaysia encounters today is obesity which is also called as overweight. This is due to excessive sugar intake in daily life. By performing this study, there will be an opportunity to develop and enlarge the sweetener market by promoting stevioside to these people throughout this country. *Stevia Rebaudiana* is called sugarleaf or sweetleaf because of high level of sweetness found in the leaves. Its ability to sweeten is more than sucrose level which is about 250 to 300 at concentration of 0.4% (w/v).

There are many sweetness components in stevia leaves but the main components are known to be stevioside and Rebaudioside A because of their high producing of sweet taste. The good thing about stevia is that it does not contain caloric value or non-caloric value. It can be part in weight loss management and also to treat diabetic people and people with high blood pressure. At this time, most of stevioside has been extracted by using standard method such as thermal extraction that will take long processing time and producing low efficiency.

1.2 Problem Statement

Basically, people used common method such as thermal extraction which requires solvent and high temperature in order to possess a quality and yield of stevioside. However, this method might take a longer processing time and need high usage of organic solvent which will cause low efficiency of extraction process. Therefore, enzyme extraction method is crucial for green option as to replace this method in order to minimize the usage of organic solvent as well as to reduce the temperature used.

1.3 Research Objective

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

1.4 Scope of Study

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

i. To determine the enzyme concentration on the stevioside yield.

ii. To evaluate the extraction time on the production of stevioside.

iii. To identify the extraction temperature on the production of stevioside.

1.5 Rationale and Significant of Research

The increasing number of diabetic and obesity people in Malaysia is a big concern and should not be taken lightly. This study is important for people who having both diseases and this can help them to create a healthy lifestyle because the stevioside can possibly treat this disease. Hence, the uses of sucrose can be replaced with stevioside as for lowering the sucrose intake.

CHAPTER 2

LITERATURE REVIEW

2.1 Stevia rebaudiana

Stevia rebaudiana (Bertoni) is an herb which can be found in Paraguay and it is classified as one of 154 members of the genus Stevia. This herb is one of only two from genus stevia that producing sweet taste and because of its non-caloric value, it is now proven and has been used in Brazil, Argentina, Paraguay, Korea and Japan. Stevia leaves contain stevioside. Other sweetness components that also present in the leaves are steviolbiodise, rebaudioside A, B, C, D and E as well as dulcoside A (Jan and Geuns, 2003). Table 2.1 shows the physical and solubility of each component from *S.rebaudiana* leaves. The commercialization of *S. rebaudiana* leaves for sweetening and flavouring purposes has been quite fast since first being introduced to Japan. In recent years, about 200 metric tons of purified stevioside and other sweetener products were prepared from about 2,000 metric tons of dried plant leaves for the Japanese market (Kinghorn *et al.* 2002).

Table 2.1 Physical and Solubility Data for Eight Sweet Ent-kaurene Glycoside from Leaves of *S.rebaudiana.*

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

(Source: Kinghorn *et al.,* 2002)

Figure 2.1 A specimen of *Stevia Rebaudiana* leaves.

(sources: Kinghorn *et al*., 2002)

2.2 Stevioside

Stevioside is a diterpene glycoside that present in the leaves of *Stevia rebaudiana* which has 250 to 300 times more sweetener than sucrose at concentration of 0.4% (w/v). Stevioside is appeared as white crystalline and colourless powder. Although Stevioside is potentially sweet, it also has an unpleasant taste which has limit used. It was concluded that stevioside and rebaudioside were not cariogenic under the conditions of the study. Stevioside do not seem to present a potential toxicity risk for humans at the low consumption levels used in sweetening (Mcmurtry, 2009).

Table 2.2 Physical Properties of Stevioside

Matrix	Physical Properties of Stevioside
Chemical Abstract Name	Kaur-16-en-18-oic acid, 13-[(2-O-β-D-glucopyranolsyl-β-D-glucopyranoyl)oxy]-, B-D-glucopyranosyl ester, (4α)- (9CI)
Other Names	Ethanophenanthrene, Kaur-16-en-18-oic acid derive.; Stevioside (6CI, 7CI); α-G-Sweet; Steviosin
Molecular Formula	$C_{38}H_{60}O_{18}$
Molecular Weight	804.88
Melting Point	196-198℃

(sources: Kinghorn *et al*., 2002)

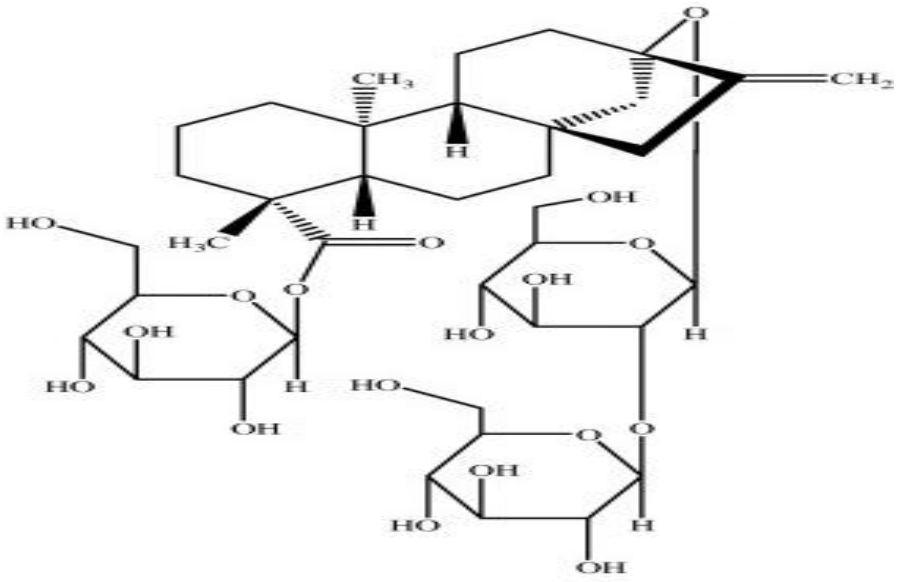

Figure 2.2 Molecular Structures of Steviol and Stevioside in *Stevia Rebaudiana* Leaves.

(Source: Ursula, 2012)

2.2.1 Applications of Stevioside

A refined extract of at least 60 percent of stevioside from the leaves of *S.rebaudiana* and as well as pure stevioside that is free from steviol and isosteviol is approved in Brazil. This stevioside is used for the sweetening of chewing gum, dietetic foods and beverages, medicines, oral hygiene products, and soft drinks. Furthermore, *S. rebaudiana* products are also used as dietary supplements in the United States (US) and other countries such as Italy in Western Europe (Jan & Geuns, 2003)

2.3 Extraction Process

Extraction process is very crucial because of its short time consuming apart from getting the main components of bioactive compounds in plants. The isolations of different types of compounds can proceed by performing the analysis of the product gained using a few equipments such as spectrophotometer, High performance liquid chromatography (HPLC) or gas chromatography (GC) etc. Numerous methods have been used for the bioactive compounds especially the chemical extraction method whereby the chemical used can be obtained easily.

For examples, the mixture of good solvent for antioxidants extraction is acetone and water has been used in order to get the extracts. The high productivity of the extract can be applied by performing the intensification techniques such as ultrasonic waves, supercritical fluids or microwaves were associated with the extraction of plants to improve the yield and quality of extracted products (Wang, 2006). Recently enzyme extraction methods have been reported that the usage of enzyme can increase the product released from plants and therefore, the enzymatic extraction is used as to replace the chemical extraction in order to minimize the use of chemical such as chemical and heat.

2.3.1 Enzymatic Extraction

Selected enzymes can degrade the cell wall by breaking down the structural integrity of the cell wall and increased the solvent accessibility and released the bioactive compounds from intracellular compartments (Pinelo et al., 2008). Examples of enzymes are peptinase, cellulase and xylanase which can disrupt the cell wall of any plants materials. In conventional method, the addition of the enzyme solution to the enzyme extractor affects the carbohydrates compositions, the gallic acid concentration, the acid stability and the cold water solubility and yield. The extractor is preferably temperature controlled as to maximize the effects of the enzymes.

2.3.2 The Advantages of Enzyme Extraction

This method has impressive effects with characteristics of high catalytic efficiency, high specificity, mild reactive conditions and preserving the original efficacy of active compounds to the maximum method. Enzymatic extraction has many advantages, such as shorter time, less solvent, higher extraction rate and better products with lower cost (Meyer & Sowbhagya, 2010).

2.4 Cellulase Enzyme

Fungi are the major production of cellulase although bacteria and actinomycetes also can produce a yield of cellulase. Commercial crude enzyme cellulase from this Fungal like *Aspergillus niger* and *Trichoderma* which are known to be efficient cellulase producers are now can be used in agriculture. This species attack cellulose and produced large amount of cell free cellulase yet still able to hydrolyze cellulose into soluble sugar by fermentation such as glucose (Milala *et al.*, 2009). Cellulose is a linear polymer of anhydroglucose units linked together by b-1,4-glycosidic bonds and found as a major component of plant biomass. The main component of cellulase enzyme is endo-β-D-glucase and catalyzes the hydrolysis of cellulose by tearing apart the sugar residues within the molecules. It is also can convert cellulose into glucose and can be used in industrial scale (Sohail *et al*., 2009).

2.4.1 Preparation of Cellulase

Sohail and co-workers (2009) stated that the commercial cellulase preparation from *Trichoderma reesei* is popular as it contains of both exo-glucanase and endo-glucanase but a low level of β-glucosidase. Hence, *Aspergillus niger* is the most common fungal that widely used in industry as it possesses all three essential components of cellulase system and produced relatively large quantities of endoglucanase and β-glucosidase but low levels of exoglucanase. The production of cellulase was conducted in two methods which are solid state fermentation and submerged fermentation.

A comparison between both methods shown that submerged method has shearing forces deactivated the enzymes, so the enzyme's activity will be decreased. Meanwhile, the production of cellulase from solid state fermentation shown the higher activity of enzyme was produced when agricultural waste was used as the carbon source.

2.4.2 Application of Cellulase in Industry

The role of celullase in pulp and paper processing is by smoothing the fibers because paper is composed of natural polymers such as cellulose, hemicelluloses, and lignin and therefore, microbial enzymes and organisms might be useful in its processing (Kirk and Jefrries, 1996). Celullase is also used to modify the surface properties of cellulosic fibers and fabric in order to achieve a desired surface effect (Kotchoni *et al.,* 2003). Celullase has been used in degrading the environmental waste such as plant waste especially in lignocellulosics.

2.5 Analysis of Stevioside using Anthrone Method

The anthrone reaction method is widely used for determine the total concentration of carbohydrates in a sample solution. The anthrone–sulfuric method is most relevant to solutions containing one type of hexose because even sugars with similar structures result in different rates and quantities of colour development. The anthrone method has been modified for use with a micro-plate, thus permitting the analysis of many samples within a short period of time and reducing the quantity of reagent needed (Edward *et al*., 2003).

Anthrone method was used in this study in order to determine the concentration of rebaudioside A and stevioside in solvent. This method is based on the condensation of furaldehyde derivatives which is generated by carbohydrate in the presence of a strong acid and with a reagent, the anthrone reagent can produce colored compounds. The reaction of carbohydrates in a strong acidic with anthrone resulted in a blue-green colour and the absorbance is read at 628nm (Cui *et al*., 2004)

CHAPTER 3

METHODOLOGY

3.0 Introduction

This chapter explained about the material used and also described the procedures on how to extract the stevioside from *Stevia Rebaudiana*. The flow of the experimental work in Figure 3.1 below has shown the process of enzymatic extraction of stevioside.

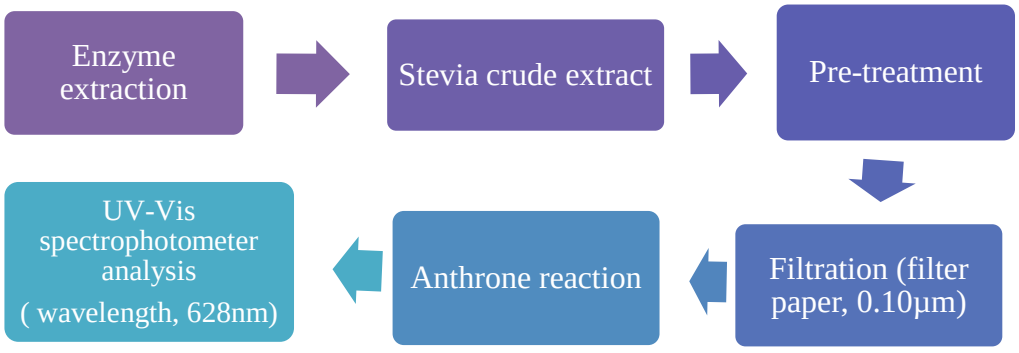

Figure 3.1 Experimental Work on The Extraction Process of Stevia Leaves using Cellulase

3.1 Material Used

3.1.1 Plant and Chemical Reagents

Stevia rebaudiana leaves were purchased from local market**.** Anthrone method was used for analysis of concentration of stevioside. The enzyme used for this study was Cellulase from *Aspergillus Niger* which was purchased from Sigma Aldrich (Malaysia) Ethanol was purchased from Sigma Aldrich (Malaysia).The solvent for this method was the anthrone reagent and sulphuric acid which were also purchased from Sigma Aldrich (Malaysia). Stevioside standard solution was used with the purity of 95% was also purchased from Sigma Aldrich (Malaysia).

Figure 3.2 Anthrone reagent

3.2 Experimental Procedure

3.2.1 Stevia Leaves Preparation

The yellow and brown leaves of *stevia rebaudiana* were removed while the green *stevia rebaudiana* leaves were selected. The leaves were washed with deionized water in 3 times and dried in an oven at 60℃ for 48 hours. After 2 days, the dried leaves were grinded into small pieces as to increase the surface area. The leaves then were placed into the plastic bag and stored in cold storage below 10℃.

Figure 3.3 Dried *Stevia rebaudiana* leaves

3.2.2 Acetate Buffer Preparation

16g of 0.2M sodium acetate was mixed with 800 ml of deionized water. Then, a few drops of glacial acetic acid into the solution until exceed pH 5.

3.2.3 Enzymatic Extraction

This experiment was conducted in order to measure the effect of enzyme concentration on the yield of stevioside by using four different concentration of cellulase enzyme (0.5%, 1%, 2% and 4%). Different extraction temperature was performed at 27, 40, 50 and 60℃ and extraction time 30, 60 and 120 minutes.

5g dried *Stevia rebaudiana* leaves was weighed and placed in 250 ml conical flask with different enzyme concentration and 5ml of acetate buffer at pH 5 was added to each flask. The mixtures were shaken at constant speed of 150 rpm after the addition of 10ml of ethanol 95% by setting the incubator shaker at the temperature of 27℃ for 30 minutes. The steps were repeated with different times for 60, 120 minutes and temperature 40, 50 and 60℃. Then, the stevia crude extract was filtered with Whatmann filter paper pore size <10μm. the filtrate solution was collected and the steps were repeated for other parameter.

Figure 3.4 Preparation of stevia solutions at different concentration of enzymes

3.2.4 Stevioside Standard Solution

Four different concentrations (100, 200, 400 and 800µg) of stevioside standard solution were prepared by dissolving 3.2 mg of stevioside hydrate in 4 ml of distilled water. Then, the solutions were diluted with 4 ml of distilled water and stored at 4℃ to be used.

3.3 Analysis

3.3.1 Anthrone Reaction Method

Anthrone reagent (Acros Organics) and sulphuric acid with the purity of 96% were purchased from Sigma Aldrich (Malaysia). UV-Vis Spectrophotometer was used to analyze the stevioside concentrations. The preparation of anthrone solution is carried out by adding 01g of anthrone reagent into 76% of sulphuric acid at a volume of 100ml.

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

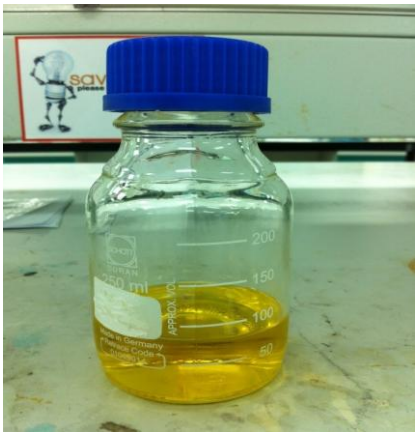

Figure 3.5 Anthrone reagent solutions

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. Next, the samples were analyzed using anthrone reaction method. A calibration graph is plotted according to the four different concentrations of standard stevioside.

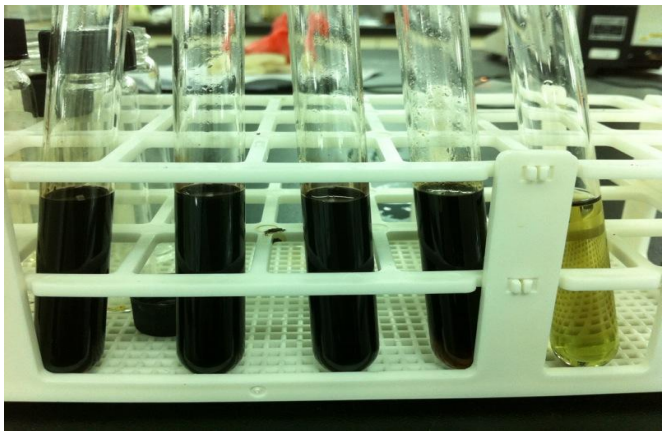

Figure 3.6 Samples stevioside yield to be analyzed using spectrophotometer

CHAPTER 4

RESULT AND DISCUSSION

4.0 Introduction

This chapter explained on the result achieved after performing the experiment based on the effect of parameters which influence the effective of the extraction process for instance the concentration of enzyme, temperature and incubation time. Meanwhile, the anthrone reaction method was used as for the sample analysis in order to determine the concentration of stevioside in the samples. The UV-Vis spectrophotometer was used at the wavelength of 628nm for the collections of data by reading the absorbance from the solution. The result of this study is presented in terms of the effect of the parameter involved in the scope of study, in which temperature, incubation time and concentration of cellulase are used. The maximum temperature used in the extraction process is from 37 to 50℃ as reported by Puri *et al*., (2012) and Gautman *et al*., (2011) since the high temperature involved make the cellulase become denatured.

4.1 Standard Curve of Stevioside

A standard curve of stevioside sample was prepared as references for the absorbance values obtained from spectrophotometer. The standard solution of stevioside was done effectively by diluting 3.2mg of stevioside hydrate in 4 ml of deionized water. Anthrone reaction was selected a 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 628nm 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 in figure 4.1.

Table 4.1 Absorbance value of Standard Stevioside

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

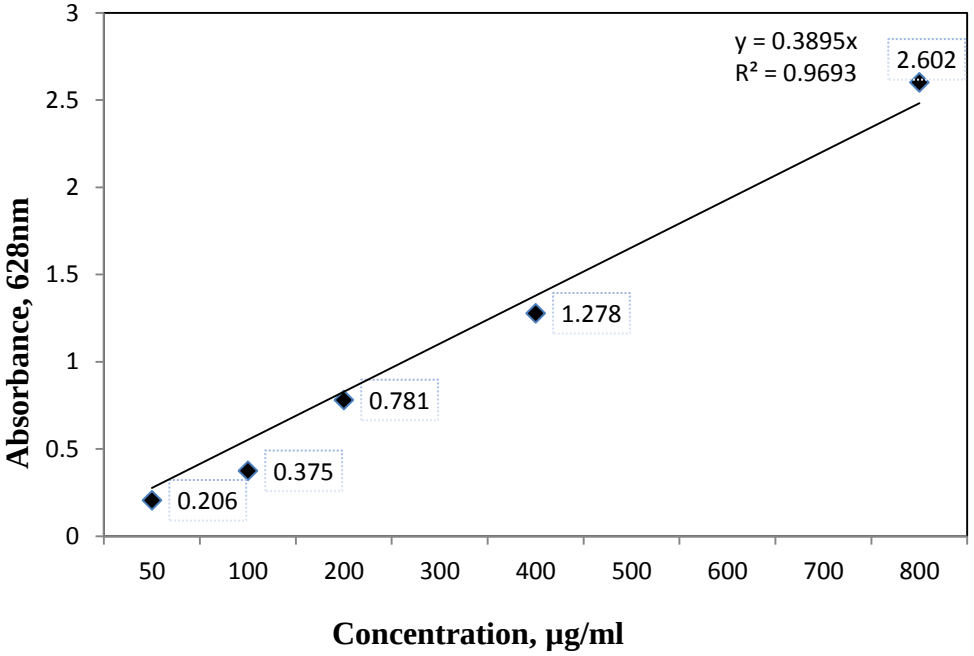

Figure 4.1 Correlation between absorbance (628 nm) and concentration of stevioside Hydrate (50-800 µg/ml)

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 0.9693 which is near to 1, it can be concluded that this result is acceptable and can be used as reference in this study.

4.2 Enzymatic Extraction of Stevioside

4.2.1 Effect of Concentration

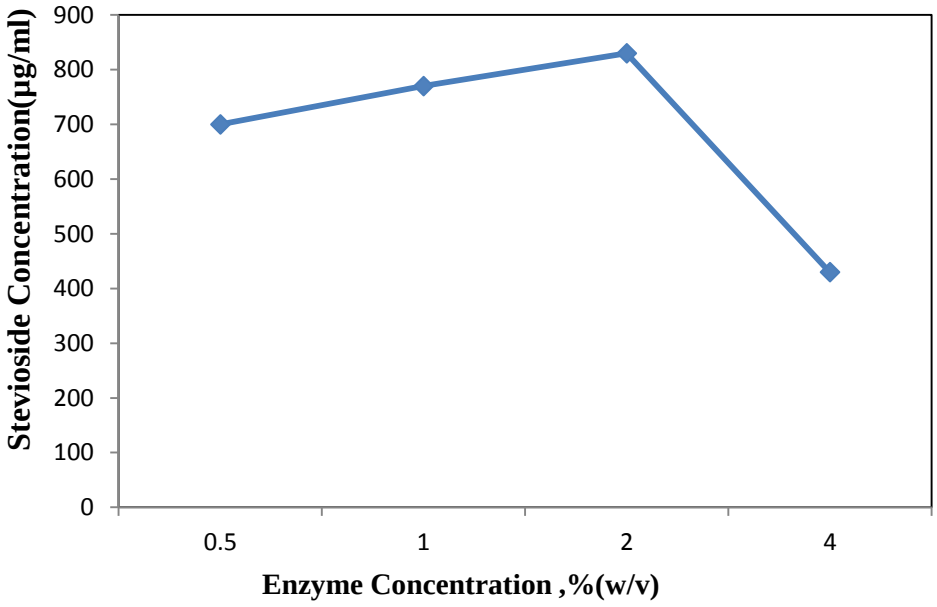

Figure 4.2 Correlation between stevioside soncentration (µg/ml) and enzyme concentration (0.5- 4 % w/v)

The figure 4.2 above has shown the effect of using different cellulase concentration which is at 0.5, 1.0, 2.0 and 4% weight per volume (w/v) on stevioside yield. The incubation time and temperature reaction were fixed at 60 minutes and 50°C. The highest peak of stevioside yield is 830µg/ml in figure 4.2 proved that the maximum concentration of cellulase being used was at 2%.

In general, the rate of enzyme increased steadily with increasing concentration until the yield of stevioside decreases when 4% of cellulase used because of the end product inhibition occurred due to high concentration of cellulase was utilized. In addition, there were no further extraction yield was gain at 4% enzyme concentration. The use of enzyme helps in cell disruption of the cell walls and as a result the extraction is more efficient. However, when the cells are disrupted, the material released from the cells such as sugars which are known to form a complex with enzymes will inhibit the product when high concentration of enzymes is applied (Puri *et al.*, 2012).

Cellulase is a good activity in degrading the cell wall polysaccharides for examples pectic polysaccharide, xyloglucans and heteroxylans. Hence, the treatment with cellulase will lead to loss of integrity and disintegration of the cell wall, improving solvent access to stevioside.

4.2.2 Effect on Temperature

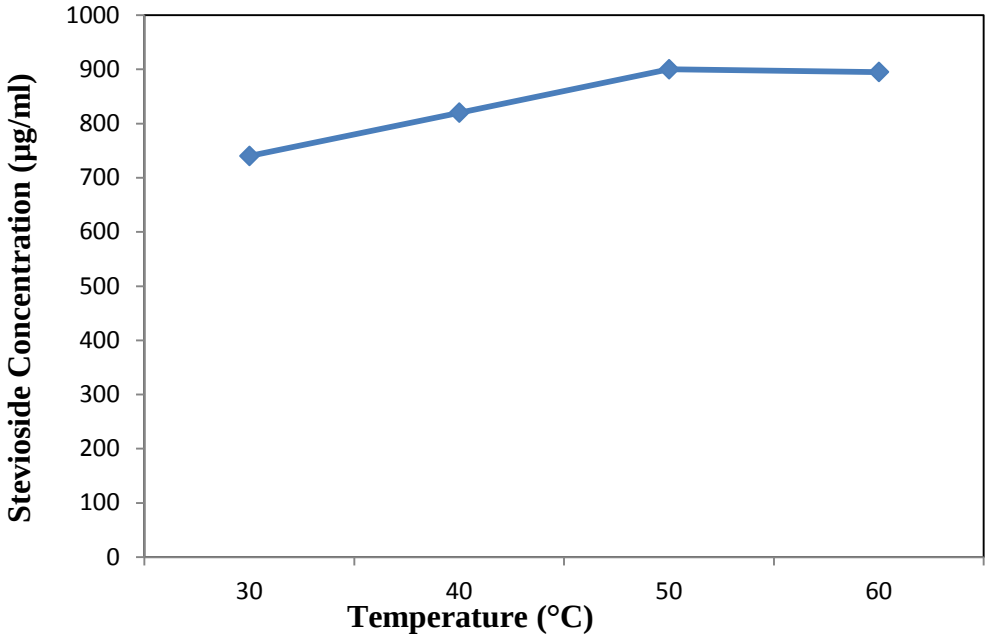

Figure 4.3 Correlation between stevioside concentration (µg/ml) and temperature (27-60°C) on Enzymatic Extraction

The figure 4.3 has shown the effect of using different temperatures (27, 40, 50, and 60°C) in enzymatic extraction at fixed concentration of enzyme (2% w/v) and incubation time for 60 minutes. The results illustrated that the maximum temperature required for the yield production of stevioside was said to be at highest peak which is at 50°C.

Based on the graph from figure 4.3, the stevioside yield was increased to maximum yield which is at 900µg/ml at temperature 50°C and slightly decreased by 26% when reached 60°C and the stevioside yield is also decreased from 900µg/ml to 895µg/ml since temperature is one of the factors for cellulase activity.

The enzyme activities at maximum temperature for cellulase activity is appeared to be 50°C since the yield of stevioside increased from 740µg/ml at room temperature. The enzyme activities at maximum temperature have made the degradation of the cell wall of polysaccharides and indirectly maximize the stevioside yield. Li et al., (2012) reported that the reaction of enzyme solution accelerated with increase of temperature to the highest temperature for enzyme activity and declined after because of denaturation of the enzyme at beyond the temperature range given. Hence, the stevioside yield became decreased at higher temperature than maximum temperature for the cellulase activity due to denaturation of cellulase enzyme. The activity of enzyme will be deactivated when high temperature is applied thus warm temperature between 50 to 55°C is the most suitable temperature for the cellulase enzyme activity.

The conventional method for *S.rebaudiana* extraction usually use high temperature in terms of thermal degradation, pressurize hot water extraction, supercritical fluid extraction and they also use solvent as chemical to extract the leaves in order to obtain the stevioside. This method may require high temperature and high energy when the extract is subjected to thermal degradation through overheating. Therefore, enzymatic extraction is the best solution for extracting the Stevia leaves because this method can reduce the energy consumption by lowering the temperature used as compared to conventional method.

4.2.3 Effect on Incubation Time

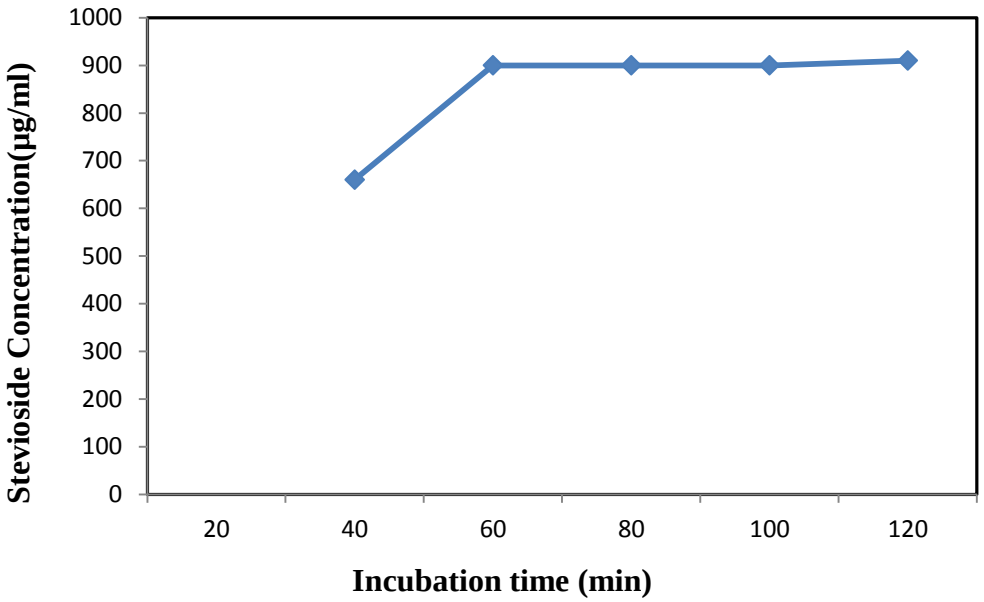

Figure 4.4 Correlation between stevioside concentration (µg/ml) and Incubation Time (30-60 minutes)

The figure 4.4 has shown the effect of incubation time for 30, 60 and 120 minutes on fixed temperature at 50°C in unchanging concentration of enzyme (2% w/v). According to the graph above, an incubation period of 60 minutes was found to be optimum for cellulase in enzymatic extraction for stevioside. Cellulase attack on cellulose which exists in the primary wall beneath the first layer of middle lamella of plant cell wall, and this catalyse breakdown into glucose.

There was a large increase in yield of stevioside starting at 30 minutes (660µgml) to 60 minutes which was 900µg/ml. Increasing extraction times to 120 minutes did not further increase in extraction yield. The yield of stevioside remain constant until reached 120 minutes. This is because the cellulase has been fully utilized in degrading the cell wall during the extraction process. The yield will only be increased when the right duration of incubation time and maximum temperature for cellulase activity in degrading the cell wall is performed.

However, the yield of stevioside will not increase when the extraction process does not applied in the right temperature even if in fixed duration of incubation time. In addition, the yield will only decrease when extending the duration time of incubation with the maximum temperature of cellulase used because the activity of cellulase reduced due to deactivation at high temperature. Hence, the reaction rate will also be decreased.

Shorter incubation time will only release partially yield of stevioside based on the graph in Figure 4.4 whereby the yield obtained is 660µg/ml when 30 minutes of incubation time was performed. Thus, enzymatic extraction is more efficient compared to conventional method due to required less time consuming which only provide 60 minutes rather than 12 hours for extraction of stevioside (Puri *et al*., 2012).

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The extraction of stevioside from *Stevia rebaudiana* leaves using cellulase was completed successfully. The parameter that affects the cellulase in releasing the stevioside such as temperature, concentration of cellulase used and also incubation time were determined. The maximum concentration of cellulase that suitable for the extraction of stevia in achieving the highest yield of stevioside was 2% w/v since only small extraction scale was used in this present study. Meanwhile, the maximum temperature for cellulase activity was found to be at 50℃. Then, the incubation time taken for the extraction to complete was investigated which is at 60 minutes. The highest yield produced from the extraction was determined at 900µg/ml under optimum conditions for cellulase activity.

Therefore, 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 and also can be highly efficient where the solvent can be minimized in degrading the cell wall. Thus, enzymatic extraction can be used an alternative method in replacing conventional method that utilizes high energy and high volume of solvent for the extraction.

5.2 Recommendation

The research project can be improved by following several recommendations for this study. Firstly, the cellulase enzyme can be replaced by using immobilized cellulase enzyme. This will be very economical since enzyme nowadays is quite expensive. Furthermore, immobilized enzyme can be easily separated from the product. Secondly, it is highly recommended to measure the approximate amount of stevioside according to percentage by using brix meter or refractometer.

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APPENDIX A

APPENDIX A.1

Figure A.1 illustrated the standard calibration curve of absorbance that was analysed using UV-Vis spectrophotometer (Hitachi) at wavelength of 628 nm.

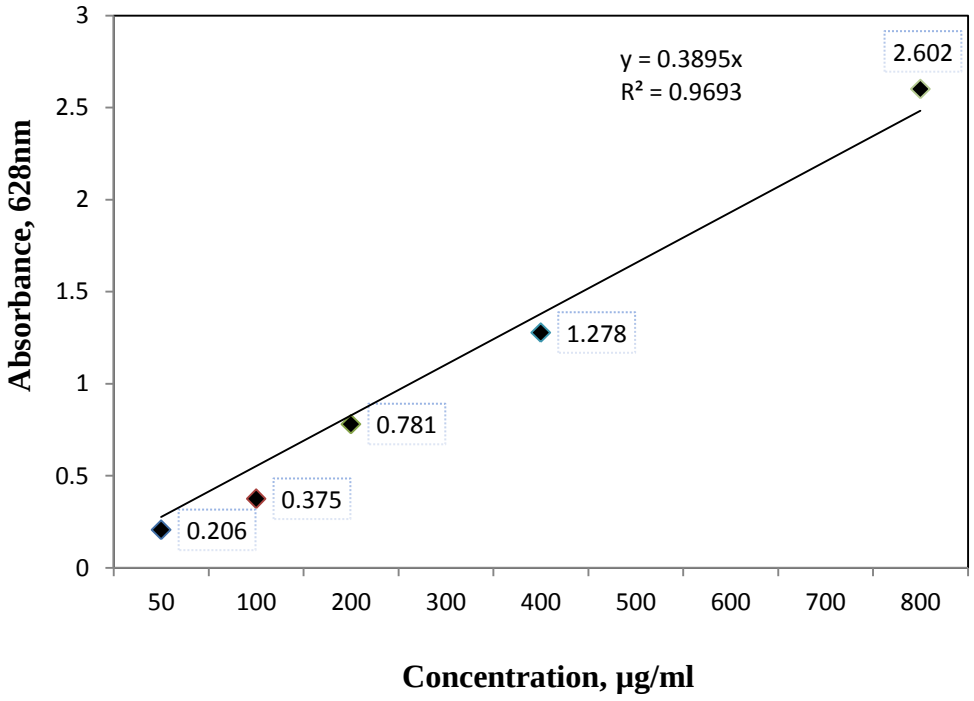

Figure A.1 Correlation between absorbance (628 nm) and concentration of stevioside Hydrate (50-800 µg/ml)

APPENDIX A.2

Table A.2.1 demonstrated the results of absorbance values with different concentration of enzymes by which the temperature at 50°C the incubation of time for 60 minutes and the temperature and the stevioside yield obtained by referring to figure A.1.

Table A2.1 Results of absorbance value and stevioside yield at different concentration of enzyme for 60 minutes

Stevioside yield ,µg/ml	Enzyme Concentration, (%, w/v)	Absorbance value, (nm)
460	0.5	1.523
660	1	1.979
700	2	2.210
590	4	1.857

Table A.2.2 demonstrated the results of absorbance values with different temperature by which the incubation of time for 60 minutes and the concentration of enzyme was at 2% (w/v) while the stevioside yield obtained by referring to figure A.1.

Table A2.2 Results of absorbance value and stevioside yield at different temperatures for 60 minutes and concentration of enzyme at 2% (w/v)

Stevioside yield ,µg/ml	Temperature, (°C)	Absorbance value, (nm)
740	27	2.301
820	40	2.570
900	50	2.699
895	60	2.693

Table A.2.3 demonstrated the results of absorbance values with different incubation of time meanwhile the temperature at 50°C and the enzyme concentration at 2% (w/v) and the stevioside yield was obtained by referring to figure A.1.

Table A.2.3 Results of absorbance value and stevioside yield at different incubation of time for 50°C and concentration of enzyme at 2% (w/v)

Stevioside yield ,μg/ml	Incubation time (mins)	Absorbance value, (nm)
660	30	1.979
900	60	2.699
900	50	2.593

APPENDIX B

GANTT CHART

Project Title (PSM): Enzymatic Extraction of Stevioside from *Stevia rebaudiana* leaves by using Cellulase										
Year	PSM I: 2012					PSM II: 2012-2013				
Month	Feb	Mac	Apr	May	Jun	Sep	Oct	Nov	Dec	Jan
Project Tasks / Weeks	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4
Identify project (problem to investigate) and scope of research	x									
Plan work schedule		x								
Review related literature		x x x x								
Determine methodology			x x							
Write proposal and abstract (summary of proposal)			x x							
Present and defend proposal in oral presentation				x						
Submit written research proposal and abstract					x					
Collect and analyse data						x x x x	x			
Interpret results							x x	x		
Evaluate results**:**								x x	x	
Achieve research objectives/ milestones								x	x	
Draw conclusions and **suggest** recommendations									x x	x
Revise and edit first draft of Introduction, Literature Review and Methodology (from proposal)										
Write first draft of Results & Discussions, Conclusions & Recommendations									x x	x
Revise and edit abstract (from proposal)										x
Compile entire final report and revised abstract										x
Present and defend final report in oral presentation										x
Submit written final report and abstract										x