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

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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 optimize the extraction of stevioside from *Stevia rebaudiana* leaves by using the cellulase. A Central Composite Design (CCD) has been chosen to study the effect of enzyme concentration, incubation time and temperature on extraction of stevioside. 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. The *p*-value for cellulase concentration and incubation time was less than 0.05 indicate that these factors have significant effects on the extraction yield of stevioside. The extraction of stevioside is represented by a quadratic model with *p*-value <0.0001. From the experimental work, an enzymatic extraction method gives the highest concentration of stevioside (840.839 µg/ml) at 45°C as maximum temperature, concentration of cellulase at 1.75% (w/v) and the incubation times of 105 minutes gives the maximum time required to complete the extraction process of stevioside. The extraction stevioside yield in this experiment gets more than predicted values (835.49µg/ml). However the experimental value is not much difference compare to the predicted value. The extraction of stevioside from *Stevia rebaudiana* leaves using cellulase is a new efficient way of obtaining high concentration of stevioside and can minimize the use of solvent and energy consumption in degrading the cell wall.

ABSTRAK

Stevioside adalah salah satu komponen yang dikenali sebagai glycoside diterpene sedia ada dalam daun *Stevia rebaudiana* yang mempunyai 250 hingga 300 lebih manis daripada sukrosa pada kepekatan 0.4% (w / v). Ia digunakan sebagai ejen-ejen pemanis dan rasa pengubahsuai kebanyakannya dalam industri makanan. Selain itu, stevioside tidak mempunyai nilai kalori dan sesuai digunakan dalam terapi terutama dalam merawat orang kencing manis. Objektif kajian ini adalah untuk mengoptimumkan pengeluaran stevioside dari daun *Stevia rebaudiana* dengan menggunakan cellulase. Satu reka bentuk pusat komposit (CCD) telah dipilih untuk mengkaji kesan kepekatan enzim, masa inkubasi dan suhu ke atas pengeluaran stevioside. Medium untuk enzim yang digunakan adalah buffer asetat manakala etanol digunakan sebagai pelarut dalam pengeluaran enzim stevioside dengan melakukan pelbagai parameter seperti kepekatan enzim, masa pengeraman dan suhu. Nilai *p* untuk kepekatan sellulase dan masa pengeraman adalah kurang daripada 0.05 menunjukkan bahawa faktor ini mempunyai kesan signifikan pada hasil pengeluaran stevioside. Pengeluaran stevioside ditunjukkan oleh model kuadratik dengan nilai $p<0.0001$. Daripada kerja eksperimen, pengekstrakan enzim memberikan kepekatan tertinggi stevioside (840.839 µg/ml) pada 45 ° C dengan suhu maksimum, kepekatan selulase pada kadar 1.75% (w / v) dan masa inkubasi 105 minit memberikan masa maksimum yang diperlukan untuk melengkapkan proses pengekstrakan daripada stevioside. Pengekstrakan hasil stevioside di dalam eksperimen lebih tinggi daripada nilai ramalan. Walaubagaimanapun, nilai eksperimen tidak banyak perbezaan berbanding dengan nilai yang diramalkan. Pengekstrakan stevioside dari *Stevia rebaudiana* daun menggunakan selulase adalah cara berkesan yang baru mendapatkan kepekatan yang tinggi stevioside dan boleh mengurangkan penggunaan pelarut dan tenaga dalam menjatuhkan dinding sel.

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LIST OF ABBREVIATIONS

ºC	Degree Celcius
%	Percentage
w/v	Weight per volume
α	Alpha
β	Beta
mL	Millilitre
M	Molarity
µm	Micrometer
mg	Milligram
g	Gram
µg	Microgram
p	Significance level
R^2	Coefficient of determination
Xi	real value of an independent variable
Xo	real value of an independent variable at the center point
Y	predicted response (Stevioside yield)
β_0	coefficient of linear effect
βi	coefficient of linear effect
βii	coefficient of squared effect
βij	coefficient of interaction effect

1 INTRODUCTION

1.1 Background of research

The increasing consumption of sugar has resulted in several nutritional and medical problems such as obesity, cardiovascular diseases and diabetics (Puri et al., 2011) with more report on dental caries problem (Kim & Kinghorn, 2002). Increases in the rates of these diseases are linked to high consumption of sugar and high-calorie sweeteners. Figure 1-1 shows the increasing of the obesity statistics in four countries.

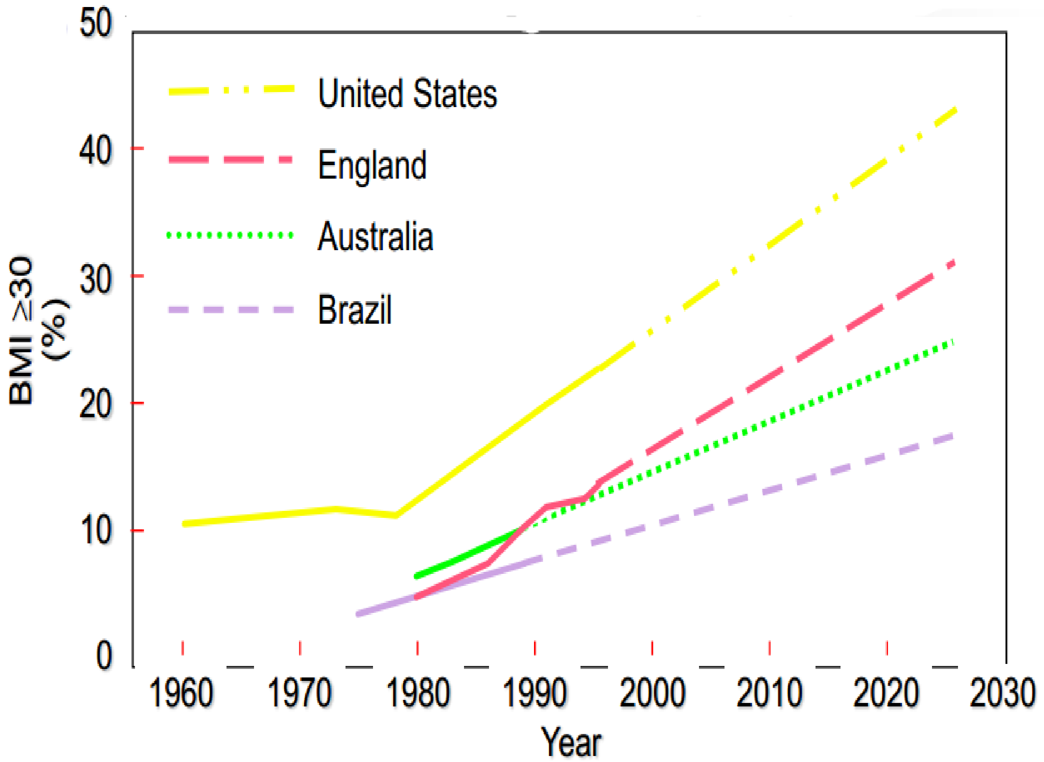

Figure 1-1 : Obesity statistics in four countries (Stevia First Corporation, 2013).

World Health Organization (WHO) reported that 347 million people worldwide have diabetes in 2008 ("350 Million Adults have Diabetes: Study Reveals the Scale of Global Epidemic", 2011). The estimated number of diabetics was considerably higher than a previous study in 2009 which put the number worldwide at 285 million. Every year, the number of diabetic patients increases rapidly and studies on 2011 state that more than 371 million people worldwide have diabetes. There are about 4.8 million people died due to diabetes stated in 2011 (Castillo, 2012).

Ministry of Health revealed that in 2012, 2.6 million Malaysians have diabetes. This data is not included undiagnosed people. One out of five people above the age of 30 suffer from diabetes. In 2020, it is predicted that there will be 4.5 million diabetics in Malaysia above the age of 18 ("We've highest diabetes rate in the region,"2012). Besides diabetes diseases, obesity or overweight also is one of the problems facing by Malaysian. This is due to excessive sugar intake in daily life.

Stevia is a perennial shrub member of the aster family (Asteraceae), better known as the sunflower family, and it is related to other herbs and flowers including sunflowers, chrysanthemums, daisies, lettuce, endive, tarragon, and chamomile. There are 154 various species of stevia including *Stevia eupatoria*, *Stevia ovata*, *Stevia plummerae*, *Stevia rebaudiana*, *Stevia salicifolia* and *Stevia serrata* have been identified (Chattopadhya, 2007). *Stevia rebaudiana bertoni* and in some species had been found to produce sweet steviol glycosides (Brandle et al., 1998). The leaves contain a complex mixture of natural sweet diterpene glycosides. There are stevioside (4-13% dry weight), steviolbioside (trace), the rebaudiosides A (24%), B (trace), C (1-2%), D (trace), E (trace) and dulcoside A (0.4-0.7%).

Stevia has been used to sweeten foods and beverages for hundreds of years in many parts of the world because of its natural plant extract, calorie-free sweetening ingredients, commercially available. Figure 1-2 shows that the percentages from different country that used stevia in food and beverages. Table 1-1 shows the maximum stevioside levels permitted in various foods.

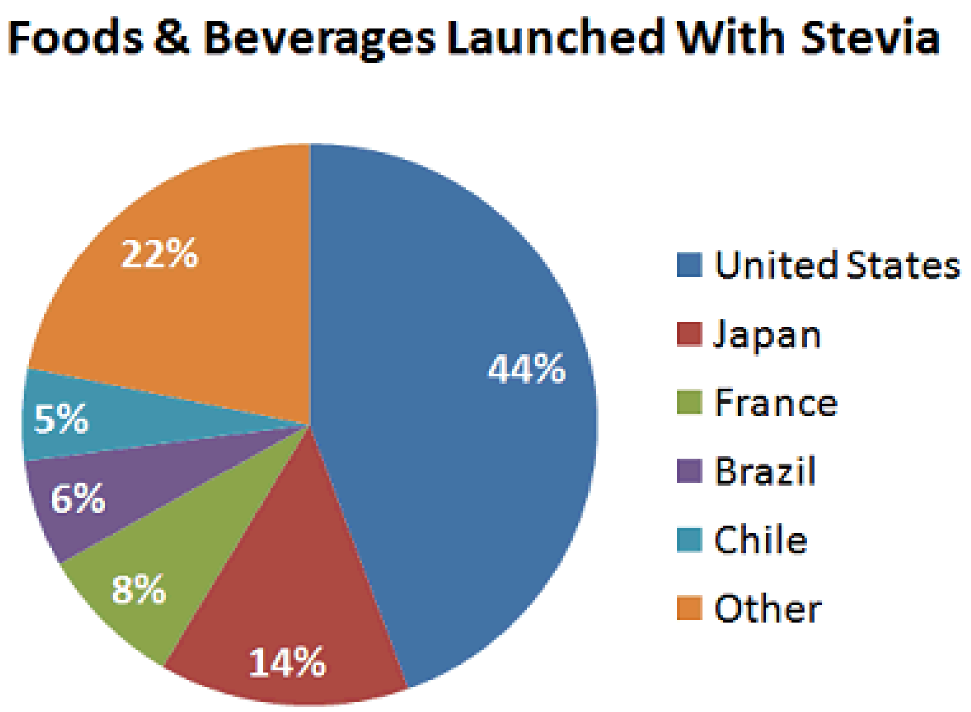

Figure 1-2 : Percentages from different country used stevia in food and beverages (Sabato, 2012).

Table 1-1: Maximum stevioside levels permitted in various foods (Puri et al., 2012).

Food type	Stevioside level (mg/kg)
Beverages	500
Dessert	500
Yogurts	500
Cold confectionary	500
Sauces	1000
Pickles	1000
Delicacies	1000
Sweet corn	200
Bread	160
Biscuits	300

The stevia plant extract is mainly used as an artificial sweetener. Stevia is a new alternative to other artificial sweeteners such as saccharin and aspartame which known as carcinogens. In the worldwide artificial sweetener market, the most commonly used is high intensity sweeteners artificial sugar. The worldwide demands for it good benefit has encouraged growing interest in stevia. Figure 1-1 shows the forecast over the next 30 years on the obesity rates. These forecast including four country only which is United States (US), England, Australia and Brazil. From the forecast, it can be conclude that the obesity among people in the world will increase rapidly. The worldwide demand on the stevia is expected to increase due to its benefits. Stevia naturally reduce sugars and calories can be as part of a healthful diet (Cohen, 2013). Consumers love stevia because it is natural, healthy, delicious and sweet, and calorie free. Stevia is also safe for diabetics, does not cause tooth decay, and halal (Pol et al., 2007).

Stevia become as the popular demand for sweetener when steviol glycosides, the sweetening components of the leaves, were declared to be safe and Rebaudioside A is one particular steviol glycosides was granted Generally Recognized as Safe (GRAS) status by US Food and Drug Administration (FDA). Figure 1-3 show products with stevia launched per year. The approval from US Food and Drug Administration (FDA) have contributed to an increased growth of stevia as sweetener foods and beverages.

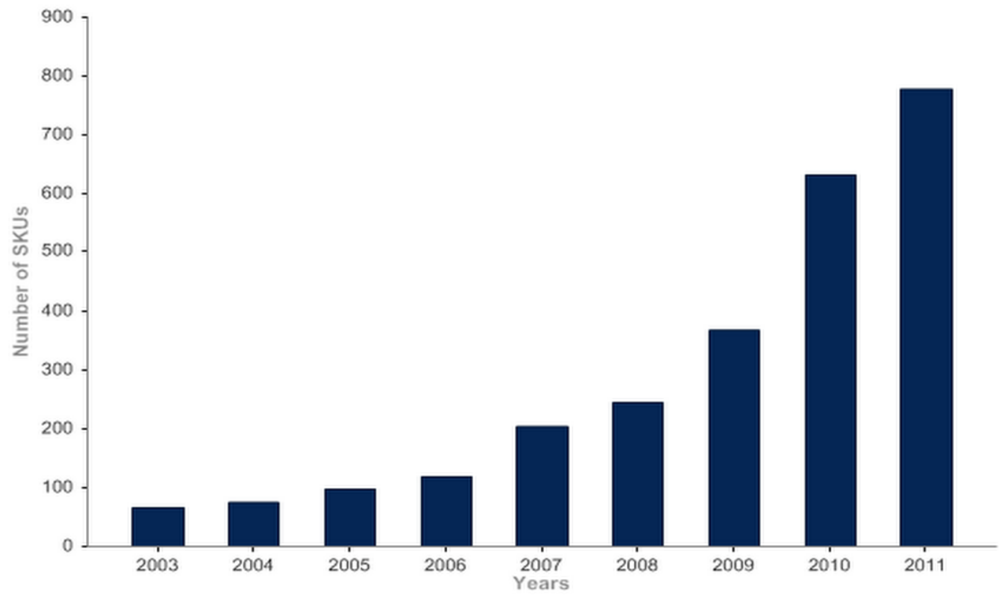

Figure 1-3: Products with stevia launched per year (Global Stevia Institute, 2011).

1.2 Problem statement

The method such as cold pressing, super-critical fluid and solvent extraction are used to extract bioactive from plant stevia (Puri et al., 2012). However, the use of organic solvents for the recovery of natural products has several weakness including safety hazards, high energy input, low product quality, environment risk and toxicological effects. Solvent extraction method always suffer low extraction yields, requires long extraction times and these will affect the final product. The final product contains traces of organic solvents, which decrease the product quality (Puri et al., 2012).

The conventional method is hot-water treatment but this method takes a very long time and high temperature in order to possess a quality and yield of target product. Therefore enzymatic extraction method is a potential alternative to other conventional method. Enzymatic extraction method offer green option in order to minimize the usage of organic solvents.

The conventional method of the optimization has been carried out by studying one variable at a time on an experimental response while only one parameter is changed, other are kept at a constant level (Puri et al., 2011). This technique called one-variable-at-a-time. This technique fails to interact with several variables. Other disadvantage of one-variable-at-a-time technique is duration of time and expenses will increase due to increase in the number of experiments necessary to conduct the research (Khuri & Mukhopadhyay, 2010).

The performance of a system, process or product can be improved by optimization in order to obtain maximum benefit from it. In extraction processes, there are multiple independent variables affecting the responding factors, it is likely that the operational variables interact and influence each other's effects on the response. Therefore to overcome this problem, the optimization of analytical procedures is necessary to determine many factors and possible interactions between these independent variables by using multivariate statistic techniques, so that a set of optimal experimental conditions can be determined (Bezerra et al., 2008).

Among the most relevant multivariate techniques used in analytical optimization is response surface methodology (RSM). Response surface methodology (RSM) is a collection of mathematical and statistical techniques for empirical model building

(Khuri & Mukhopadhyay, 2010). It is widely used food industry to determine the effects of several variables and to optimize conditions (Puri et al., 2011). The main advantage of Response Surface Methodology (RSM) is to reduce the number of experimental trials needed to evaluate multiple variables & their interactions, and provide the information necessary for design and process optimization.

1.3 Objective

The objective of this research is to optimize the extraction of stevioside from *Stevia rebaudiana* leaves by using the cellulase. Central composite designs (CCD) were used for experimental design and analysis of the results to obtain optimal extraction conditions.

1.4 Scope of study

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

i. To carry out optimization using RSM on the incubation time, temperature and concentration of enzyme (cellulose).

i. To evaluate the extraction time on the stevioside yield (30 min, 60 min, 120 min and 180 min)

ii. To identify the extraction temperature on the stevioside (30 ^{o}C, 40 ^{o}C, 50^{o}C, 60^{o}C)

iii. To evaluate the effect of enzyme concentration on the stevioside yield (0.5%, 1%, 2% and 3%).

1.5 Rationale and significance

This study is very important for people having medical problems such as obesity, cardiovascular diseases and diabetics. The increasing number of diabetic and obesity people in the worldwide and also in Malaysia need to be concern. Stevia is expected to play a major role in the battle against obesity related problems like heart diseases and diabetes. Stevia has taken a role as sweetener to replace chemical sweeteners like Aspartame, Saccharine which can harm human health. Research has shown that a whole leaf concentrate has a regulating effect on the pancreas and helps stabilize blood sugar levels. Stevia as sweeteners have been the only sugar alternative for those with diabetes.

A few researches on enzymatic extraction of stevioside have been done. Puri et al (2011) investigate the extraction stevioside yield based on incubation time, temperature and concentration of enzyme (cellulase). The highest yield of stevioside was achieved in one hour (359 µg). The extraction yield was highest at 2% cellulase concentration, with stevioside yield of 359.6 µg at 50^{o}C. The maximum stevioside yield (230 µg) achieved at 50^{O}C (Puri et al, 2011).

Another research done by Nor (2013), shows that the highest concentration of stevioside (900 µg/ml) at 50^{o}C as a maximum temperature. The concentrations of cellulase at 2% (w/v) and at 60 minutes of incubation time contribute to the highest yield of stevioside (830µg/ml).

This study is to optimize the extraction of stevioside from *Stevia rebaudiana* leaves by using the cellulase. Therefore these studies can improve the stevioside yield by using previous study on the extraction condition. The extraction condition consists of three parameter which is the incubation time (30 min, 60 min, 120 min and 180 min), temperature (30 ^{o}C, 40 ^{o}C, 50 ^{o}C, 60^{o}C) and concentration of enzyme (0.5%, 1%, 2% and 3%). The extraction conditions were optimized using response surface methodology (RSM). Central composite designs (CCD) were used for experimental design and analysis of the results to obtain optimal extraction conditions.

2 LITERATURE REVIEW

2.1 Overview

This thesis presents an optimization of enzymatic extraction of stevia from *Stevia rebaudiana* by using cellulase. This study was carried out in order to find the optimum parameter that influences the yield of stevioside extract. The effects of variety of parameters on the extraction yield of stevioside from *Stevia rebaudiana* leaves were studied. This chapter covered on sweeteners, *Stevia rebaudiana bertoni*, stevioside, process recovery and cellulase enzyme.

2.2 Introduction

Stevia is a plant origin from Brazil and Paraguay (Chattopadhya, 2007). Stevia leaves had been approve as non-caloric sweetener which contribute good benefit to human health (Gardana et al., 2010). It is approximately 200–300 times the relative sweetness intensity of 0.4% w/v, a sweetening power similar to that of aspartame (Erkucuk et al., 2009).

2.3 Sweeteners

A sugar is a carbohydrate that is soluble in water. Sugars are crystalline and have a sweet taste. By a photosynthesis, the process by which plants convert the sunlight into their food and energy supply producing sugar. Once photosynthesis creates sugar, plants have the unique ability to change sugar to starch and starch to various sugars for storage. This diversity provides us with a wide variety of tasty fruits and vegetables, from the starchy potato to the sweet carrot. Sugar cane and sugar beet used as commercial sources of sugar because they contain sucrose in large quantities. A stalk of the cane plant contains about 14% sugar. Sugar beets contain about 16% sugar (The Sugar Association, 2005). Sugar it is always refers to sucrose which is derived from sugar cane or sugar beet. During processing, preparation or before consumption, syrups were added to foods (Fitch & Keim, 2012).

The two main categories of sugars are monosaccharide and disaccharides. Monosaccharide also called as simple sugars. Monosaccharide is common base unit of all carbohydrate molecules. The characteristics of monosaccharide are soluble in waters and sweetness variable (with individual monosaccharide). Two monosaccharides joined together called as disaccharides. The characteristics of disaccharides are soluble in water and must be broken into monosaccharides before they can be absorbed into the body (IvyRose, 2013).

Sweeteners can be classifying into two categories which is nutritive and non-nutritive sweeteners as shown in Figure 2-1. Nutritive sweeteners include sugar sweetener namely refined sugars, high fructose corn syrup, crystalline fructose, glucose, dextrose, corn sweeteners, honey, lactose, maltose, and reduced-energy polyols or sugar alcohol such as sorbitol, xylitol, mannitol, lactitol, maltitol and isomalt (Herman, 1914). Non-nutritive sweeteeners can be further subdivided into natural and artificial sweeteeners. From Figure 2-2, there are six non-nutritive sweeteners has been approved by The Food and Drug Administration (FDA) which is acesulfame-K, aspartame, neotame, saccharin, sucralose and stevia (Fitch & Keim, 2012).

Sweeteners acts as a preservative in jams and jellies by inhibit microbial growth, and a flavour enhancer in processed meats. Besides that, when sweeteners are added in a food it gives a sweet flavour. Other than that, it maintains freshness, natural colour, texture, shape of preserved fruit and product quality. Sweeteners also serve fermentation for breads and pickles, bulk to ice cream, and body to carbonated sodas. Sweeteners also contribute volume in ice-cream, baked goods and jams. Enhance the creamy consistency of frozen desserts and also the crystallization of confectionary products will be increase in addition of sugars. Sugars also balance the acidity in salad dressings, sauces, and condiments (Fitch & Keim, 2012).

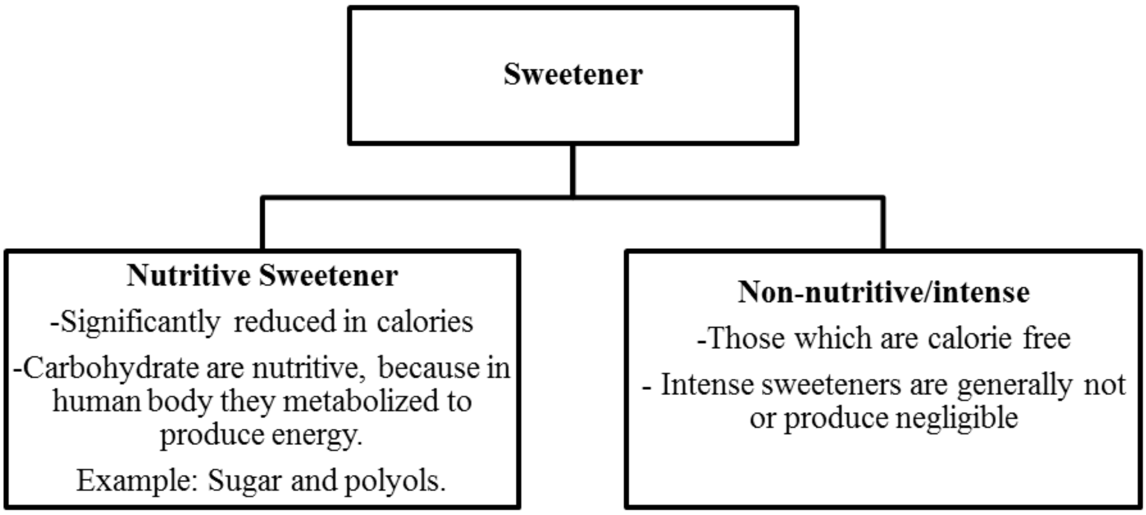

Figure 2-1: The type of sweeteners (Chattopadhya, 2007).

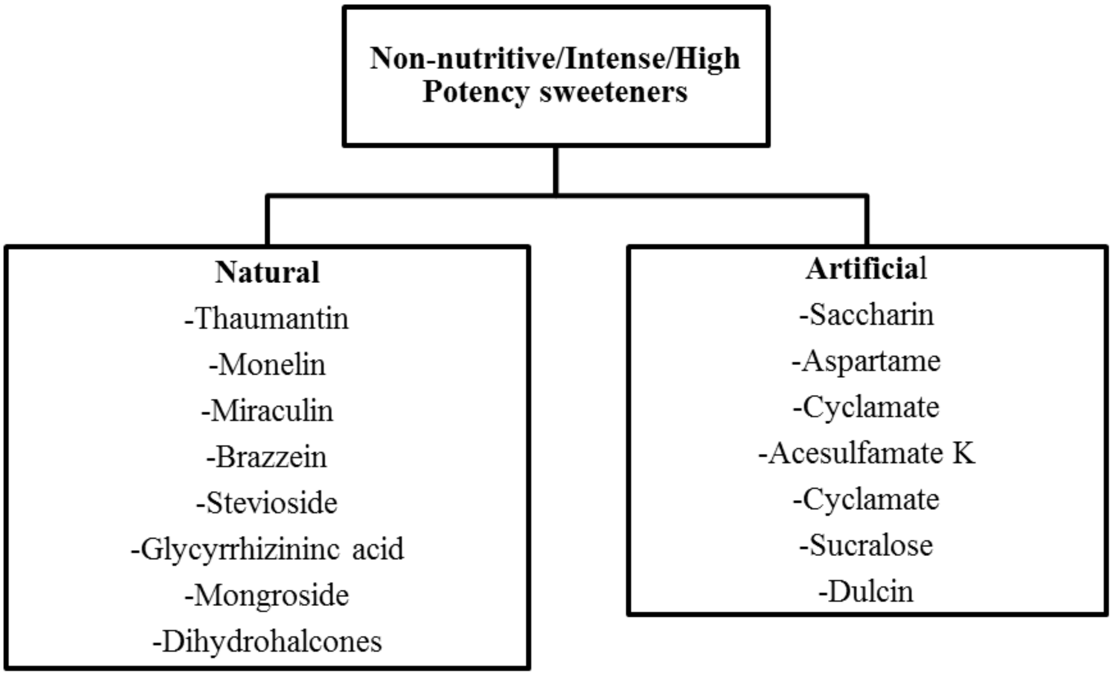

Figure 2-2: The non-nutritive sweeteners (Chattopadhya, 2007).

2.3.1 *Nutritive sweetener*

Nutritive sweetener is any sweetener that adds some energy value to food and it is known as natural sugar. Natural sugars, which are also called primary sweeteners are found naturally in food include glucose, fructose, galactose, sucrose, maltose, corn-based sweetener and agave nectar (Fitch & Keim, 2012).

Glucose is a monosaccharide and the primary source of energy for body cells. Fructose is a monosaccharide available in fruit, honey, and some vegetables. In nature, it is linked with glucose as the disaccharide sucrose. Fructose can be used as nutritive sweetener. Galactose is monosaccharide found in dairy products and some plants. Sucrose is a type of disaccharide, combination of glucose and fructose naturally presence in fruit and vegetables. It is used as nutritive sweetener and for its other functional properties. Sucrose is a disaccharide and carbohydrate, stored by plants as a reserve energy source to be used when needed (IvyRose, 2013). Humans cannot directly use sucrose, and it is metabolized in the body to glucose which needs the hormone insulin to help convert it into energy.

Maltose is found in molasses and is used for fermentation. Maltose is a disaccharide composed of two glucose units. Corn-based sweetener refers to many products made from corn. Corn-based sweetener may be composed primarily of glucose, fructose, or any combination of the two. High-fructose corn syrup (HFCS) is a mixture of glucose and fructose and is only available to food manufacturers. Agave nectar is a nutritive sweetener that contains fructans, oligosaccharides of fructose and glucose, and monosccharides of fructose and glucose (Fitch & Keim, 2012).

Nutritive sweeteners classify as foods called "generally recognized as safe" (GRAS) by the Food and Drug Administration (FDA). Nutritive sweeteners provide calories or energy. Although they may be safe, however excessive consumption nutritive sweetener or natural sugar can harm consumer health. The Institute of Medicine (IOM) suggests a maximum intake level of nutritive sweeteners of 25% of total energy intake because dietary quality, and ultimately your health, suffers with an intake above that level.

2.3.2 Non-nutritive sweetener

2.3.2.1 Artificial sweeteners

The American Heart Association labels low-calorie sweeteners, artificial sweeteners, and non-caloric sweeteners as non-nutritive sweeteners, since none nutritional benefits such as vitamins and minerals. Non-nutritive sweeteners, more commonly referred to as artificial sweeteners, have been used for decades to add a sweet flavour to foods without adding significant calories or promoting tooth decay. Artificial sweeteners are popular among diabetics and dieters alike, and new types of artificial sweeteners have been introduced to meet this growing demand. Currently there are five non-nutritive sweeteners that have been approved by the FDA which is acesulfame K, aspartame, neotame, saccharin, and sucralose (Jones, 2010).

2.4 *Stevia rebaudiana bertoni*

2.4.1 Introduction

Stevia rebaudiana bertoni leaves contain non-cariogenic and non-caloric sweeteners (steviol-glycosides) which contribute good benefit to human health (Gardana et al., 2010). It is approximately 200–300 times the relative sweetness intensity of 0.4% w/v, a sweetening power similar to that of aspartame (Erkucuk et al., 2009).

Stevia rebaudiana bertoni is tropical perennial and herbaceous shrub, which is belonging to the Aster (Asteraceae) or Chrysanthemum family of plants (Bawane et al., 2012). There are 154 various species of stevia including *Stevia eupatoria*, *Stevia ovata, Stevia plummerae, Stevia rebaudiana, Stevia salicifolia* and *Stevia serrata* have been identified (Chattopadhya, 2007). *Stevia rebaudiana Bertoni* and in some species had been found produce sweet steviol glycosides (Brandle et al., 1998). The leaves contain a complex mixture of natural sweet diterpene glycosides. These are stevioside (4-13% dry weight), steviolbioside (trace), the rebaudiosides A (24%), B (trace), C (1-2%), D (trace), E (trace) and dulcoside A (0.4-0.7%) (Opinion on *Stevia rebaudiana Bertoni* plants and leaves, 1999). Stevioside and rebaudioside A are the main sweetening compounds of interest.

Figure 2-3 : *Stevia rebaudiana bertoni* leaves (Madan et al., 2009).

2.4.2 The species of stevia

There are few species of Stevia which is:-

I. *Stevia ivifolia*

About 2 ft. height, erect, Stem shaggy-hairy, corymbosely branched at top, leaves rhomboid-lanceolate, deeply sharply toothed, and upper sessile. Flower heads white, in fastigiated corymbs; involucre & florets glandular & downy.

II. *Stevia ovata*

About 2 ft. height, Stem erect, Leaves ovate, toothed, wedge shaped at base; upper oblong, sub entire, flower-heads white, in rather compact fastigiated corymbs.

III. *Stevia purpurea*

About 18 inches height, erect stem velvery hairy much branched. Leaves lanceolate, alternate, lower obovate, channeled, narrowed to stalk, toothed at apex. Flowerheads purple in somewhat fastigiated corymbs, involucre pale greenish.

IV. *Stevia rebaudiana*

Annual herb, 1-1½ feet height stem puberulous leaves opposite, oblanceolate, crenulate flower heads very small, whitish in a corymb Leaves have a sugary flavour.

Synonym:-*Eupatorium rebaudianum*.

V. *Stevia salicifolia*

Glabrous shurb, 18 in height leaves opposite, narrow, lanceolate, nearly or quite entire, very shortly stalked, almost connate. Flower-heads white in spreading corymbs.

VI. *Stevia serrata*

About 18 inch height stem erect, branched, hairy leaves alternate, somewhat clustered, linear lanceolate, almost glabrous, toothed, entire at base, contracted to stalk. Flower heads white or pink, in fastigiated corymbs. The *Stevia rebaudiana* leaves measure from 2-3 inches long & up to 1 inch wide. When the plant reaches maturity, it is about 2-3 feet tall (Bawane et al., 2012).

2.4.3 The beginning spread of stevia

Stevia is widely used in many parts of the world. Stevia sweeteners are probably best known from their use in Japan. Stevia was first commercially adopted by Japan in the 1970s and the country continues to be the largest and most diverse user of stevia in the world.

In 1971, the first company in the world to commercialize the stevia sweetener was Morita Kagaku Kogyo Co. Ltd. of Japan. Morita Kagaku Kogyo Co. Ltd. starts to develop their own "Rebaudio" (rebaudioside) variety. They represented approximately 41% of the market share potently sweet substances consumed in Japan (Dounis, 2008). The whole process from cultivation of the stevia plants to extraction and refinement of the rebaudiside glycosides was fully control and operate by Morita (Dounis, 2008).

When the table-top sweetener like the packets of saccharin ("Sweet-n-Low") and aspartame ("Equal") widespread in the United States, stevia was also used by the Japanese to sweeten a variety of food products, including ice cream, bread, candies, pickles, seafood, vegetables, and soft drinks (Gates,2000). The Japanese have been using Stevia for over 30 years with no adverse effects. In Japan, stevia used to sweeten Coca-Cola, other soft drinks, teas and food. After that, Japan stand as the largest consumer of stevia in the world. In 1980, Brazil approved *Stevia rebaudiana* products. China began using stevia in 1984 and China is the world's largest exporter of stevioside. Since 1995, stevia has been used in powder and extract form in the United States (Dounis, 2008).