EXTRACTION OF STEVIOSIDE FROM *STEVIA REBAUDIANA* LEAVES USING CELLULASE

NOR HAYATI BT SHAHIDAN

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UNIVERSITY MALAYSIA PAHANG

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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.

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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).

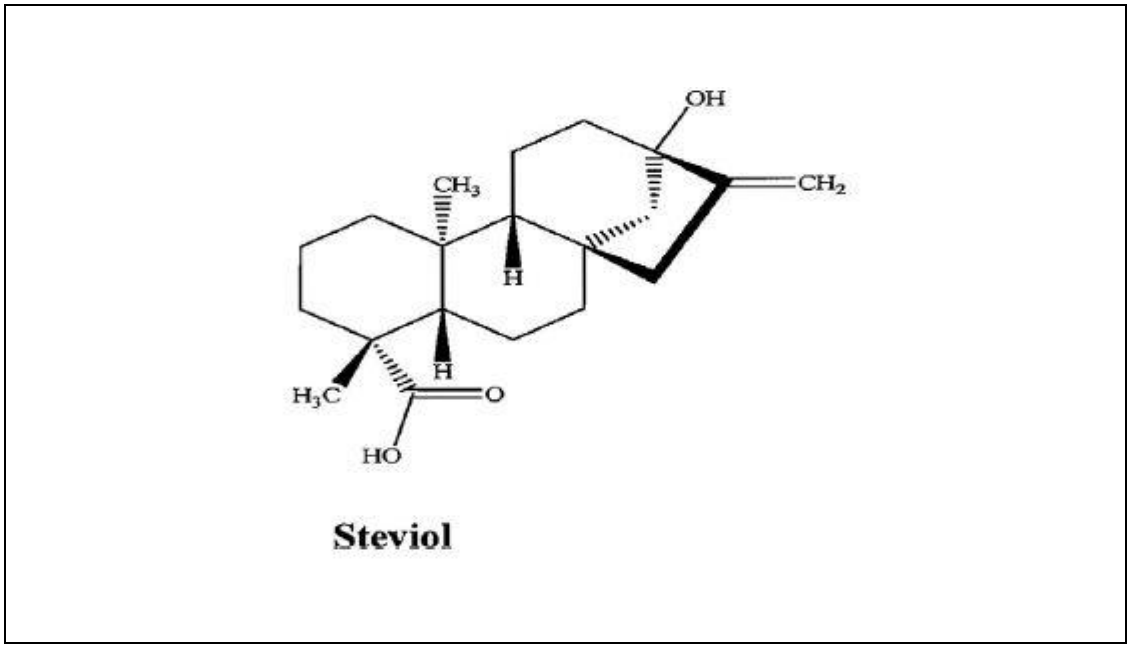

Figure 2.1: Structure of Steviol Glycoside

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).

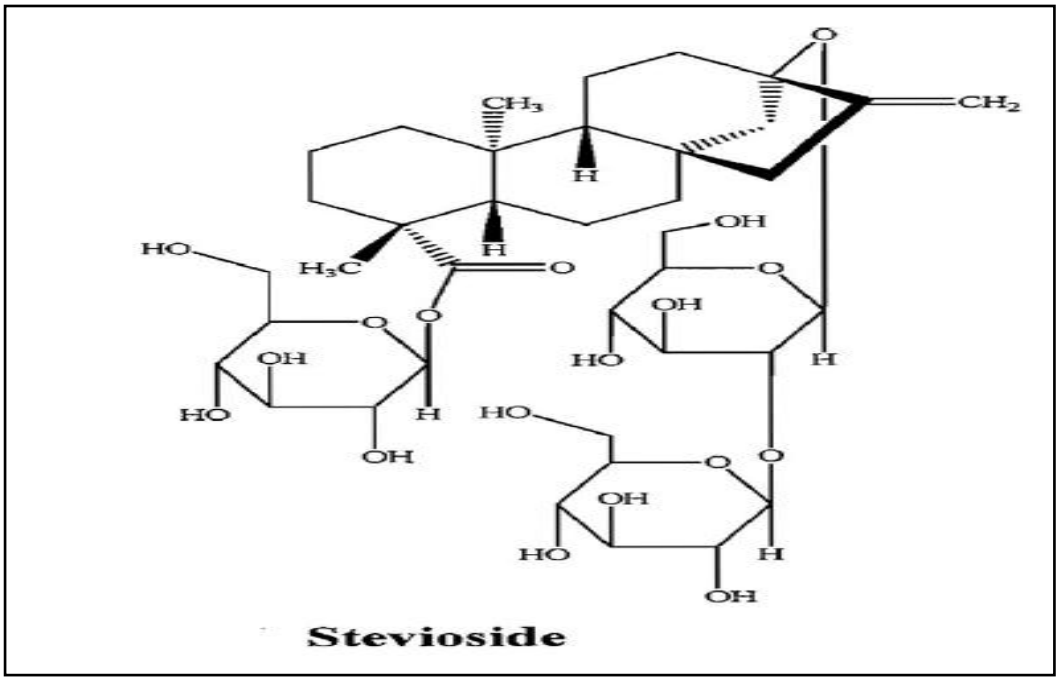

Figure 2.2: Structure of stevioside molecular bonding

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).

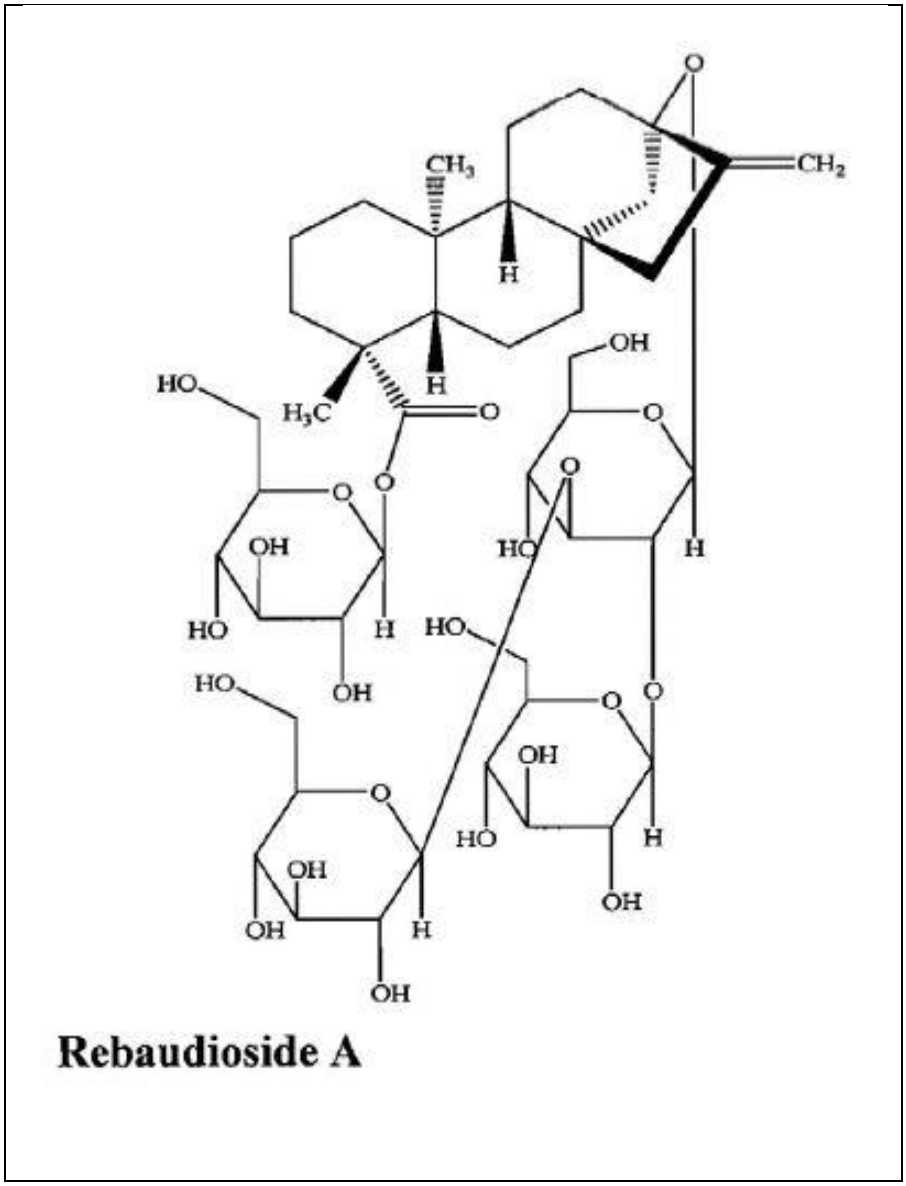

Figure 2.3: Structure of Rebaudioside A

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.

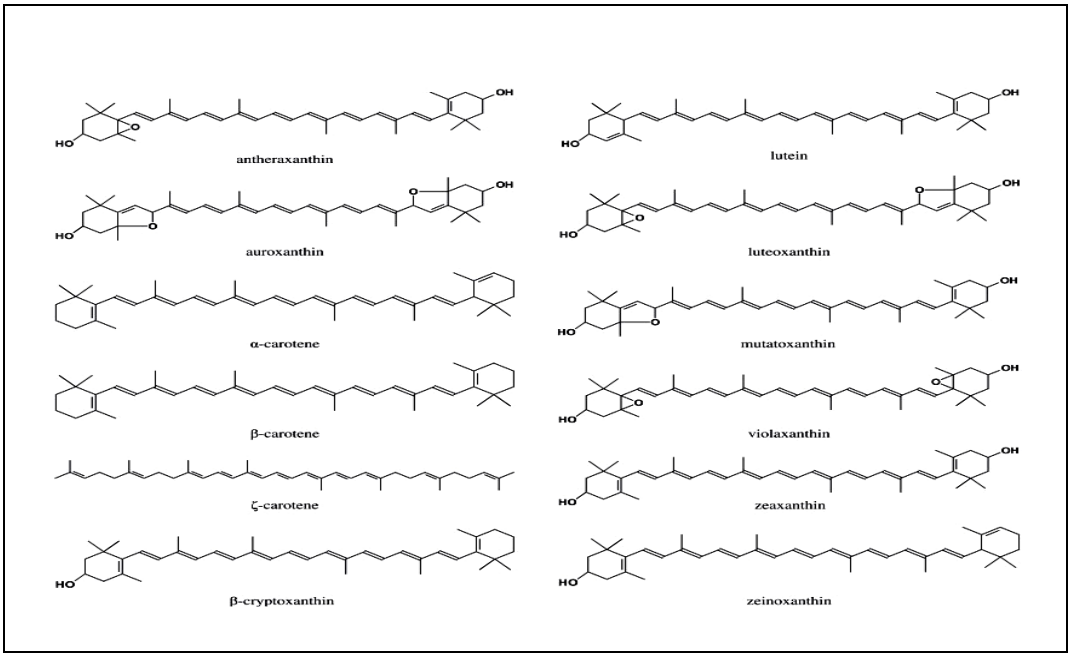

Figure 2.4: Chemical structure of orange juices carotenoids.

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.