IMPROVEMENT OF GENIPIN PRODUCTION PROCESS AND ITS APPLICATIONS TO DRUG ANALYSIS AND ENZYME IMMOBILIZATION

By

Mr. Weerapath Winotapun

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree

Doctor of Philosophy Program in Pharmaceutical Chemistry and Natural Products

Graduate School, Silpakorn University

Academic Year 2016

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การปรับปรุงกระบวนการผลิตเจนิพินและการประยุกต์ใช้สำหรับวิเคราะห์ยาและตรึงเอนไซม์

โดย

นาย วีรภัทร วิโนทพรรษ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรดุษฎีบัณฑิต

สาขาวิชาเภสัชเคมีและผลิตภัณฑ์ธรรมชาติ

บัณฑิตวิทยาลัย มหาวิทยาลัยศิลปากร

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The Graduate School, Silpakorn University has approved and accredited the thesis title of “Improvement of genipin production process and its applications to drug analysis and enzyme immobilization” submitted by Mr Weerapath Winotapun as a partial fulfillment of the requirements for the degree of Doctor of Philosophy in Pharmaceutical Chemistry and Natural Products.

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WEERAPATH WINOTAPUN : IMPROVEMENT OF GENIPIN PRODUCTION PROCESS AND ITS APPLICATIONS TO DRUG ANALYSIS AND ENZYME IMMOBILIZATION. THESIS ADVISORS : ASSOC. PROF. THEERASAK ROJANARATA, Ph.D. AND ASSOC. PROF. PRANEET OPANASOPIT, Ph.D. 145 pp.

Genipin is an iridoid aglycone found in fruit of *Gardenia jasminoides* Ellis. It has been used as food colorant, fabric dye and amine cross-linker and has been reported for several pharmacological activities such as anti-inflammation, anti-cancer, anti-fungal and neuroprotective. However, genipin is naturally present at a low concentration while its glycoside counterpart namely geniposide occurs more abundantly. Genipin is usually obtained by multi-step, time- and reagent-consuming preparation consisting of the initial extraction of plant materials, fractionation and purification of geniposide and final hydrolysis of geniposide to genipin. In this study, a direct route for the production of genipin was investigated by using cellulase as a single enzyme to disrupt plant cell wall for the efficient release of geniposide as well as to cleave off the sugar moiety from geniposide. Simultaneously, ethyl acetate was added as an extractant to extract genipin *in situ* into the organic phase, thus helping to drive the reaction equilibrium and obtain the product with the enhanced purity. Via this approach, the yield was achieved at 58.83 mg genipin starting from 1 g of the dry plant. In the aspect of pharmaceutical applications, genipin was used in this study as a colorimetric reagent for the analysis of drugs i.e. gabapentin, and as a cross-linker for enzyme immobilization. For the assay of gabapentin, the principle relied on the formation of blue product once genipin reacted with the primary amine group of gabapentin. The reaction was setup by mixing the solutions of gabapentin and 2 mM genipin solution at pH 7, followed by heating at 80 ºC for 1 h. Then, the absorbance values of the resultant solutions were measured spectrophotometrically at 590 nm. The proposed method showed an excellent linearity in the range of 0.15-0.50 mM gabapentin. It was accurate, precise and insensitive to the interferences from related impurities and common excipients. The method produced stable blue color and provided the assay results in agreement with the pharmacopeial HPLC method. Beyond the assay development, the treatment of waste from the laboratory was also designed based on the use of gypsum as an adsorbent. After the treatment, the blue product was dramatically removed from the waste solution and the treated waste was proven to be safe for tested aquatic organisms i.e. brine shrimps and guppy fishes. Since one molecule of genipin can react with two amine groups, it was used in this study to cross-link β-glucosidase with chitosan beads aimed for enzyme immobilization. Several methods with different mixing orders as well as parameters in the cross-link reaction were studied. It was found that the preparation of immobilized chitosan beads by mixing 0.5 mg/mL β-glucosidase with 0.1% genipin at 40 °C for 6 h showed the highest enzyme activity. By this approach, the immobilized enzyme showed greater pH and thermal stability than free enzyme and those prepared by using glutaraldehyde. The immobilized beads were tested for the reusability by using p-NPG as a substrate. The results showed that it still had the activity higher than 80% of its activity after 10 cycles used. Moreover, the immobilized beads were applied to hydrolyze a glycoside, namely genistin, and convert to genisetin which has the pharmacological activities. It showed a satisfactory reusability, retaining more than 70% of its activity after 5 cycles used. In addition, the residual activity of the cross-linked and immobilized chitosan beads remained more than 90% of its initial value after storage at 4 °C for 30 days.

Program of Pharmaceutical Chemistry and Natural Products Graduate School, Silpakorn University
Student's signature..... Academic Year 2016
Thesis Advisors' signature 1.....2.....

55356801 : สาขาวิชาเภสัชเคมีและผลิตภัณฑ์ธรรมชาติ
คำสำคัญ : เจนิพิน / กระบวนการผลิต / เซลลูเลส / การแยกผลิตภัณฑ์ / การวิเคราะห์ยา / กาบาเพนติน / การตรึงเอนไซม์ / ไคโตซาน / เบต้ากลูโคซิเดส / เจนิสตีน / เจนิสเตอิน

วีรภัทร วิโนทพรรษ์ : การปรับปรุงกระบวนการผลิตเจนิพินและการประยุกต์ใช้สำหรับวิเคราะห์ยาและตรึงเอนไซม์. อาจารย์ที่ปรึกษาวิทยานิพนธ์ : ภก.รศ.ดร.ธีรศักดิ์ โรจนราธา และ ภญ.รศ.ดร.ปราณีต โอปณะโสภิต. 145 หน้า.

เจนิพินเป็นสารในกลุ่มอิริดอยด์อะไกลโคนที่พบได้ในผลของพืชตระกูลพุด และถูกนำมาใช้เป็นสีผสมอาหาร สีย้อมผ้า และสารเชื่อมขวางสำหรับหมู่เอมีน รวมทั้งมีรายงานว่ามีฤทธิ์ทางเภสัชวิทยาที่หลากหลาย ได้แก่ ต้านการอักเสบ ต้านมะเร็ง ต้านเชื้อรา และปกป้องเซลล์สมอง อย่างไรก็ตาม ในธรรมชาติพบเจนิพินในปริมาณต่ำ แต่พบเจนิโพไซด์ซึ่งเป็นสารในรูปไกลโคไซด์ในปริมาณที่มากกว่า ดังนั้นการสกัดเจนิพินต้องใช้หลายขั้นตอน ซึ่งใช้เวลานานและสิ้นเปลืองสารเคมี ประกอบด้วยการเริ่มต้นสกัดจากพืช การแยกเจนิโพไซด์และการทำให้บริสุทธิ์ และขั้นตอนสุดท้ายโดยการไฮโดรไลซิสเจนิโพไซด์เพื่อเปลี่ยนเป็นเจนิพิน ในการศึกษานี้ศึกษาการผลิตเจนิพินโดยตรงจากผลพุดโดยนำเอนไซม์เซลลูเลส มาทำให้ผนังเซลล์พืชแตกออก ซึ่งสามารถเพิ่มการปลดปล่อยสารเจนิโพไซด์ออกจากผลพุดได้อย่างมีประสิทธิภาพ และเพื่อกำจัดหมู่น้ำตาลออกจากโครงสร้างของเจนิโพไซด์ พร้อมกับการใช้เอทิลอะซีเตทเป็นตัวทำละลายในการสกัดแยกเจนิพินมาสู่วัฏภาคอินทรีย์ ซึ่งช่วยผลักดันปฏิกิริยาให้เข้าสู่สมดุล และได้ผลิตภัณฑ์ที่มีความบริสุทธิ์เพิ่มขึ้น โดยวิธีดังกล่าว สามารถเตรียมเจนิพินได้ 58.83 มิลลิกรัม จากผลพุดแห้ง 1 กรัม ในด้านการนำมาประยุกต์ใช้ทางด้านเภสัชกรรม เจนิพินถูกนำมาใช้เป็นสารก่อสีสำหรับวิเคราะห์หาปริมาณยา ได้แก่ กาบาเพนติน และเป็นสารเชื่อมขวางสำหรับการตรึงเอนไซม์ สำหรับการวิเคราะห์หาปริมาณกาบาเพนติน อาศัยการทำปฏิกิริยาระหว่างเจนิพินกับหมู่เอมีนปฐมภูมิของตัวยา ทำให้เกิดผลิตภัณฑ์ที่มีสีน้ำเงิน ปฏิกิริยาเกิดขึ้นโดยผสมสารละลายกาบาเพนตินกับเจนิพินที่มีความเข้มข้น 2 มิลลิโมลาร์ ที่ค่าพีเอช 7 จากนั้นนำไปให้ความร้อนที่ 80 องศาเซลเซียส เป็นเวลา 1 ชั่วโมง แล้วนำสารละลายที่ได้ไปวัดค่าการดูดกลืนแสงที่ความยาวคลื่น 590 นาโนเมตร พบว่าวิธีนี้มีความสัมพันธ์เป็นเส้นตรงที่ดีมาก เมื่อใช้ในการวิเคราะห์หาปริมาณกาบาเพนตินในช่วงความเข้มข้น 0.15-0.50 มิลลิโมลาร์ มีความถูกต้อง แม่นยำ และมีความจำเพาะ โดยไม่ถูกรบกวนจากสารเจือปนที่มีโครงสร้างใกล้เคียงกับตัวยาและสารปรุงแต่งที่อยู่ในตำรับ วิธีนี้ทำให้เกิดสีที่มีความคงตัวดี และสามารถวิเคราะห์หาปริมาณตัวยาได้ไม่แตกต่างจากวิธีโครมาโทกราฟีชนิดของเหลวสมรรถนะสูงซึ่งเป็นวิธีที่เภสัชตำรับกำหนด นอกจากนี้ในงานวิจัยยังได้ออกแบบกรรมวิธีบำบัดของเสียที่เกิดขึ้น โดยใช้แร่ซิปโซ่มมาดูดซับสีและสารต่างๆ ในสารละลายของเสีย ซึ่งพบว่าของเสียที่ผ่านการบำบัดแล้วนั้นมีความปลอดภัยต่อสิ่งมีชีวิตที่นำมาใช้ทดสอบ ได้แก่ ไรกุ้งและปลาหางนกยูง จากการที่เจนิพิน 1 โมเลกุลสามารถทำปฏิกิริยากับหมู่เอมีนได้ 2 หมู่ ในงานวิจัยจึงนำเจนิพินมาใช้เป็นสารเชื่อมขวางเพื่อตรึงเอนไซม์เบต้ากลูโคซิเดสบนเม็ดไคโตซาน โดยศึกษาลำดับการผสมที่แตกต่างกันรวมทั้งปัจจัยที่ส่งผลต่อปฏิกิริยาการเชื่อมขวาง พบว่าการตรึงเอนไซม์เบต้ากลูโคซิเดสบนเม็ดไคโตซาน โดยผสมเอนไซม์เบต้ากลูโคซิเดสที่มีความเข้มข้น 0.5 มิลลิกรัมต่อมิลลิลิตร กับเจนิพินที่มีความเข้มข้นร้อยละ 0.1 เข้ากับเม็ดไคโตซานที่อุณหภูมิ 40 องศาเซลเซียส เป็นเวลา 6 ชั่วโมง ให้เอนไซม์ในรูปตรึงที่มีการทำงานของเอนไซม์ที่ดีที่สุด และมีความคงตัวต่ออุณหภูมิและพีเอชที่ดีกว่าเอนไซม์ในรูปอิสระและเอนไซม์ที่ถูกตรึงอยู่บนเม็ดไคโตซานที่ถูกเชื่อมขวางด้วยกลูตารัลดีไฮด์ เมื่อทดสอบประสิทธิภาพในการใช้ซ้ำโดยใช้ p-NPG เป็นสารตั้งต้น เอนไซม์ที่ถูกตรึงบนเม็ดไคโตซานมีความสามารถในการใช้ซ้ำที่ดี โดยมีค่าการทำงานของเอนไซม์ร้อยละ 80 หลังจากผ่านการใช้งานไป 10 ครั้ง นอกจากนี้ยังสามารถนำไปประยุกต์ใช้ในการไฮโดรไลซ์หมู่น้ำตาลที่อยู่บนโครงสร้างไกลโคไซด์ที่มีชื่อว่าเจนิสติน และเปลี่ยนเป็นเจนิสเตอิน ซึ่งเป็นสารที่มีฤทธิ์ทางเภสัชวิทยา โดยเอนไซม์ในรูปตรึงมีสมบัติในการใช้ซ้ำ โดยยังมีการทำงานของเอนไซม์ร้อยละ 70 หลังจากผ่านการใช้งานไป 5 ครั้ง เมื่อเทียบกับการใช้งานในครั้งแรก นอกจากนี้เอนไซม์ในรูปตรึงนี้ยังคงความสามารถในการทำงานของเอนไซม์มากกว่าร้อยละ 90 หลังจากเก็บไว้ที่อุณหภูมิ 4 องศาเซลเซียสเป็นเวลา 30 วัน

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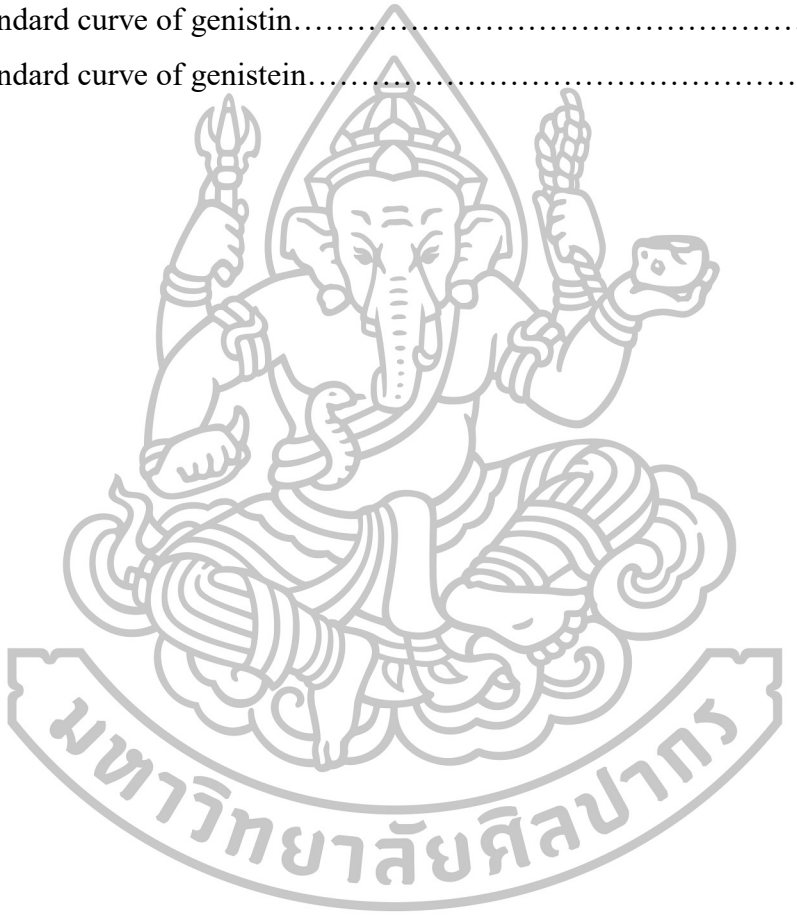

LIST OF ABBREVIATIONS

®	Registered trademark
% w/v	Percent weight by volume
% v/v	Percent volume by volume
% RSD	Percent relative standard deviation
^{o}C	Degree Celsius
µg	Microgram
µL	Microliter
µm	Micrometer
α	Alpha
β	Beta
γ	Gamme
Ɛ	Epsilon
A°	Angstrom
ANOVA	Analysis of variance
B	Boron
BSA	Bovine serum albumin
$CaSO_4$	Calcium sulphate
cm	Centimeter
Co.	Company
Da	Dalton
e.g.	exempli gratia (Latin); for example
et al.	And others
Fe(II)	Ferrous
Fe(III)	Ferric
g	Gram
GABA	Gamma amino butaric acid
h	Hour
H_2O	Water
HCl	Hydrochloride

Hg(II)	Mercury
HPLC	High performance liquid chromatography
I.D.	Internal diameter
i.e.	id est (Latin); that is
k	Kilo
KS	Kansas State
L	Litre
LC	Letal concentratioin
LOD	Limit of detection
LOQ	Limit of quantitation
Ltd.	Limited
MD	Maryland State
mg	Milligram
min	Minute
mL	Milliliter
mM	Millimolar
mm	Millimeter
MO	Missouri State
Mo(IV)	Molybdenum
MW	Molecular weight
Na_2CO_3	Sodium carbonate
NH_2	Primary amine
nm	Nanometer
NaOH	Sodium hydroxide
NY	Ney York State
pH	Potentia hydrogenii (latin); power of hydrogen
p-NPG	p-4-nitrophenol β-D-glucopyranoside
ppt	Part per thousand
rpm	Round per minute
SD	Standard deviation
SEM	Scanning electron microscopy

spp.	Species
TPP	Tripolyphosphate
TUa	Acute toxic unit
U	Unit
UK	United Kingdom
USA	United State of America
USP	United State of Pharmacopoiea
UV	Ultraviolet
Zr	Zirconium

CHAPTER 1

INTRODUCTION

1.1 Statements and significance of the problems

The fruit of gardenia from *Gardenia* spp. such as *Gardenia jasminoides* Ellis (Rubiaceae) is a natural source of iridoid glycosides, namely geniposide. Upon enzymatic hydrolysis, geniposide is cleaved the sugar moiety from its molecule and converted to aglycone compound known as genipin. Due to the specific reaction with primary amine ($-NH_2$) to form the blue colored products, genipin has been used as an alternative source for production the safe and edible pigments which are widely used in East Asia as food coloring agents and natural fabric dyes (Park et al., 2002). Moreover, it has been reported that genipin possesses many pharmacological activities such as hepatoprotective (Peng, Qian and Liu, 2003), anti-inflammatory (Wang et al., 2009), anticancer (Cao et al., 2010), antithrombotic (Suzuki et al., 2001), antifungal (Lelono, Tachibana and Itoh, 2009), anti-diabetic (Zhang et al., 2006) and neuroprotective (Yamazaki, Chiba and Yoshikawa, 2009). Apart from that, genipin has been used as a natural cross-linking agent with low cytotoxicity for primary amine containing polymers i.e. gelatin and chitosan in tissue engineering and drug delivery applications (Bigi et al., 2002; Muzzarelli, 2009; Harris, Lecumberri and Heras, 2010) and for the colorimetric assays of amino acids (Lee et al., 2003), free amino groups of water soluble chitin derivatives (Ramos-Ponce et al., 2010). Because of a variety of applications, genipin has been increasingly on demand and thus the more efficient, feasible and environmentally friendly production approaches are still needed to be developed since very low level of genipin (about 0.005-0.01%) is naturally found in gardenia fruit while geniposide is present more abundantly at about 3-10% (Cao et al., 2001; Pan et al., 2002; Yang, Liu and Gao, 2009; Zhou et al., 2012), depending on the cultivation location and climate as well as the harvesting condition and time.

The first report of genipin preparation relied on several steps starting from the initial extraction of geniposide from dry gardenia fruits with organic solvent. After that, the resultant purified geniposide was subjected to the hydrolysis process by β-glucosidase and then converted to genipin (Endo and Taguchi, 1973). Later, some method modifications have been made by other researchers including the improvement of the isolation step of geniposide from plant biomass (Yang, Liu and Gao, 2009; Zhou et al., 2012), reflux extraction, continuous circumfluence extraction and ultrasonic extraction (Yang and Lv, 2004). In addition, different enzyme platforms i.e. immobilized enzymes (Yang et al., 2011; Fugikawa et al., 1987) and whole cell biocatalysis using β-glucosidase-producing strains (Xu et al., 2008) have been studied and used to increase the yields of genipin. To the best of our knowledge, however, all the previous methods were based on the discrete process where the extraction and purification of geniposide step separated apart from the hydrolysis of the glycoside to aglycone. There has been no directly production method of genipin starting from dry fruits of gardenia and ending up with the desired product in one pot. In the field of natural product isolation and bioprocessing, "enzyme-assisted extraction" and "in situ product separation" are becoming increasingly attractive and challenging. The first approach involves the use of plant cell-degrading enzymes such as cellulase, hemicellulase and pectinase either alone or in combination to disrupt plant cell structures and to enhance the releasing of intracellular contents from plant sources (Puri, Sharma and Barrow, 2012). This environmentally friendly route has been shown to increase the extraction yields of several plant constituents such as oils (Qian et al., 2010), flavorants (Li, Smith and Hossain, 2006), colorants (Sowbhagya and Chitra, 2010) and bioactive compounds (Zu et al., 2009). For the latter strategy, it presents a form of process integration where the product is selectively removed from the vicinity of the biocatalyst by effective means such as in situ extraction or in situ adsorption. The technique helps alleviate product inhibition or toxicity, promote unwanted reaction equilibrium, minimize product degradation and reduce costs in downstream processing (Freeman, Woodley and Lilly, 1993). At present, in situ product separation has been implemented for the production of valuable chemicals in whole-cell and isolated-enzyme mediated biocatalysis with improved yields and/or

productivity (Jianlonga, Xianghua and Ding, 2000). However, few examples have been found so far for the convergence of both techniques.

In this study, enzyme-assisted extraction and in situ product separation were combined to develop a direct route for the production of genipin from dry gardenia fruit. Cellulase is less expensive than β-glucosidase (Zu et al., 2009) and it efficiently catalyzes the sugar moiety from geniposide with satisfactory extent of above 90%. Also, it could break down the plant cell wall. Thus, single cellulase was feasible for usable and adequate for dual roles in one-pot reactor. Additionally, the fact that geniposide was likely to be released into the water phase due to the solubility and underwent hydrolytic reaction which efficiently took place in aqueous medium whereas genipin formed favorably distributed in the organic phase had offered the possibility for the in situ recovery of product by using two-phase extraction. For this purpose, various parameters such as enzyme concentration, pH of reaction and time of incubation were optimized to achieve the highest yield of genipin in parallel with the satisfactory performance in terms of economic aspect and eco-friendliness.

In accordance with the principles of green analytical chemistry, the elimination or substitution of toxic reagents in the analysis with less harmful alternatives is available means to develop safe, green and sustainable assays (Rojanarata, 2012). Nowadays, a number of compounds originating from natural sources such as plants have been proven to be attractive options that can be used as the colorimetric agent with no or little purification or modification (Grudpan et al., 2010). For example, the extracted from *Curcuma longa* L. known as curcuminin was used as a colorimetric agent for the analysis of boron in sea water samples (Liu and Lee, 2009). Chlorophyll from pea plant was used in the fluorescence assay of mercury(II) (Gao et al., 2006).

Next, this study also investigated the novel green analytical procedure for primary amine-containing compound, using gabapentin as a model drug. Gabapentin (1-(aminomethyl) cyclohexaneacetic acid) is a neurotransmitter γ-aminobutyric acid (GABA) analogue which is used for the treatment of partial seizures and neuropathic pain (Petroff et al., 2000; Rowbotham et al., 1998). At present, the several methods have been developed for the quality control of this drug in bulk and pharmaceutical

formulations such as high performance liquid chromatography (HPLC) (Souri, Jalalizadeh and Shafiee, 2007; Ciavarella et al., 2007), capillary electrophoresis (Lin et al., 2004; Sekar and Azhaguvel, 2004), chemiluminometry (Manera et al., 2009), potentiometry (Jalalia, Arkana and Bahrami, 2007), voltammetry (Hegdea et al., 2009), spectrofluorimetry (Belal et al., 2002; Hassan et al., 2001) and spectrophotometry (Abdellatef and Khalil, 2003; Al-Zehouri, Al-Madi and Belal, 2001; Salem, 2008; Galande, Baheti and Dehghan, 2010). Among them, colorimetric assays are usually considered as simple, effective and rapid methods and require minimal and common, inexpensive instrumentation. From literatures, gabapentin which has very low UV absorption could be assayed on the visible spectrophotometry after it was derivatized via different reactions or reagents such as vanillin and ninhydrin. Recently, the formation of ion-pair complexes as a result of a proton transfer from picric acid or 2,4-dinitrophenol to the primary amino group of gabapentin was used as a basis for the colorimetric assay of this medicine (Abdulrahmanand and Basavaiah, 2011). Although these methods have shown several advantages, some chemical reagents and/or organic solvents with known or suspicious toxicity were employed in the assays. For instance, besides the organic solvents which potentially cause harmful effects on health and environment, a common derivatizing reagent, ninhydrin, is known to have the toxicity including somnolence, regional or general arteolar or venous dilation, dyspnea, skin irritation (Searle, 1984; Piirila et al., 1997) and potentially promotes tumor development on mouse skin (Shukla et al., 1994). By this reason, the use of genipin not only facile and efficient but also safe colorimetric assays for this drug is still on the demand.

This work had developed based on the colorimetric reaction with genipin. Unlike most other previous works which ended up once the satisfactorily accurate and reproducible methods were obtained. In this study, a simple and facile treatment of waste generated from the assay was further proposed and the ecological effects of the treated waste were evaluated to confirm its safety on two aquatic organisms i.e. brine shrimp and guppy fish. Therefore, this was the first report that not only presented the application of naturally derived genipin to drug analysis, but also fully illustrated the benign-by-design development of the analytical methodologies starting from the safe

source of reagents toward the safe sink when the wastes from the assay were released into the environment.

Enzymes are remarkable discovery in the field of bioprocess technology. It has been widely utilized in several fields owing to their ease of production, substrate specificity and green chemistry. Immobilized enzymes with functional efficiency and enhanced reproducibility are used as alternatives in spite of their expensiveness. Immobilized biocatalysts can either be enzymes or whole cells (Liang et al., 2004). Inert polymers and inorganic materials are usually used as carrier matrices such as chitosan which is one of the biodegradable and biocompatible natural polymers. Several methods are used for enzyme immobilization and various factors influence the performance of immobilized enzymes (Zhang, 2013). Being a non-toxic natural compound, genipin has valuable properties to be used as a cross-linking agent in the various applications i.e. tissue engineering (Zhang et al., 2010; Yan et al., 2010), medical and pharmaceutical aids (Muzzarelli, 2009). In general, the support activation with glutaraldehyde is a simple and efficient method that improves the stability of polymer and enzyme due to multipoint linkage or among the subunits of the enzyme (Betancor et al., 2006). There are many researchers that used glutaraldehyde in the cross-linking process.

In this study, genipin was used as an alternative cross-linking agent between primary amine-containing polymers and enzyme, compared with conventional cross-linking agent i.e. glutaraldehyde. β-glucosidase is enzymes that has the property for hydrolysis of glycosidic bonds to release the non-reducing terminal glucosyl residues from glycoside compounds and oligossaccharides. The objective of this study was to investigate the efficiency of genipin to act as a cross-linking agent to simultaneous increase the stability of supporter and to immobilize enzyme on chitosan beads. The β-glucosidase activity on chitosan beads was tested by using p-4-nitrophenol β-D-glucopyranoside (p-NPG) as a substrate. After that, the immobilized beads was applied to hydrolyze the sugar part from genistin to genistein. In addition, the various factors such as type of cross-linking agents, concentration of cross-linking agents, time of immobilization, optimal pH and temperature, pH and thermal stability and reusability were optimized.

1.2 Objectives of research

1.2.1 To improve the production process of genipin from dried gardenia fruits by using simultaneous enzyme-assisted extraction and cleavage of glucose from glycoside combined with in situ product separation.

1.2.2 To develop a green assay based on the use of genipin for amine-containing compound using gabapentin as a model.

1.2.3 To study the use of genipin as a cross-linking agent for enzyme immobilization.

1.3 Hypothesis to be tested

1.3.1 Enzyme-assisted extraction and cleavage of glucose from glycoside combined with in situ product separation is an effective way for the production and purification of genipin from dried gardenia fruits.

1.3.3 Some amine-containing compounds such as gabapentin can be assayed by using naturally derived genipin as colorimetric agent.

1.3.3 Genipin has a potential to be used as a cross-linking agent for the immobilization of enzyme on aminated matrix e.g. chitosan beads.

CHAPTER 2

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2.1 Green chemistry

The most important goals of sustainable development are reducing the adverse consequences of the substances that we use and generate. Worldwide demand for environmentally friendly chemical processes and products requires the development of novel and cost-effective approaches to pollution prevention. One of the most attractive concepts in chemistry for sustainability is “Green Chemistry”, which is the utilization of a set of principles according to Table 2.1 (Anastas and Warner, 1998). Although some of the principles seem to be common sense, their combined use as a designer framework frequently requires the redesign of chemical products or processes. It should be noted that the rapid development of Green Chemistry is due to the recognition that environmentally friendly products and processes will be economical on a long term.

It can be concluded that “Green Chemistry” is based on a set of principles that used in the design, development and implementation of chemical products and processes. This approach uses renewable, biodegradable materials which do not persist in the environment. Morover, it not only used the catalysis and biocatalysis to improve efficiency and conduct reactions at low or ambient temperatures but also reduced the use and generation of hazardous substances and offered a strategic path way to build a sustainable future.

Table 2.1 The principles of "Green Chemistry".

No.	Topic
1	Prevent waste
2	Atom economy
3	Less hazardous synthesis
4	Design benign chemicals
5	Benign solvents and auxiliaries
6	Design for energy efficiency
7	Use of renewable feedstocks
8	Reduce derivatives
9	Catalysis
10	Design for degradation
11	Real-time analysis for pollution prevention
12	Inherently benign chemical for accident prevention

Source: Available from: URL: https://www.acs.org/content/dam/acsorg/greenchemistry/resources/the-12-principles-of-green-chemistry-pocket-guide.pdf (accessed on 28.03,2017).

2.2 Genipin

2.2.1 Introduction

Genipin is a hydrolytic product of geniposide (Figure 2.1), which is found in the fruit of *Gardenia jasminoides* Ellis (Park et al., 2002). The structure of genipin was discovered in the 1960 using NMR spectroscopic data and chemical degradation experiments (Djerassi et al., 1960; Djerassi et al., 1961). It possesses the molecular formula $C_{11}H_{14}O_5$ and contains a dihydropyran ring (Djerassi et al., 1961) in its structure. From the literature reviews, genipin is a safe agent that can be used as a blue colorant by food industries in East Asia (Park et al., 2002) and traditional Chinese medicine (Tsai et al., 2002). Sung and co-workers (Sung et al., 1999) have undertaken the investigation on the cytotoxicity, feasibility, and biocompatibility of genipin for tissue fixation, and found that genipin was 10,000 times less cytotoxic than commonly used glutaraldehyde. Therefore, it was used as a cross-linking agent to increase the biomechanical strength which is safe to cornea and lens (Avila and Narvaez, 2012). In addition, it did not affected the proliferation of osteoblasts and

chondrocytes when it was used in the tissue engineering applications (Wang et al., 2011).

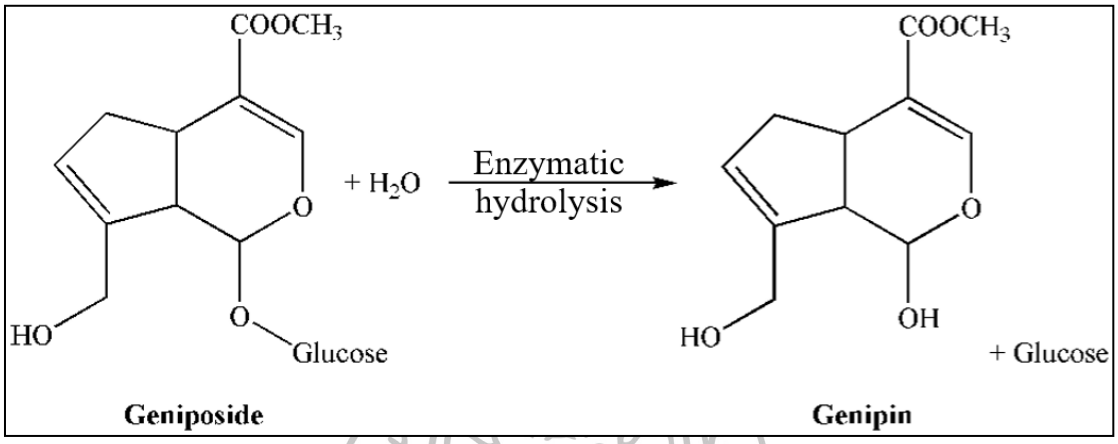

Figure 2.1 The conversion of geniposide into genipin by the hrdrolysis reaction.

2.2.2 Botanical data of *Gadenia jasminoides* Ellis

The dry fruit of *Gardenia jasminoides* Ellis (Figure 2.2) is the natural plant source of geniposide. It is from the plant family Rubiaceace and is native to China. It is an evergreen shrub that grows four to eight feet tall and wide. The fruit are oval in shape about one-half inch to one inch long and orange in color. The major component of the fruit of *Gardenia jasminoides* Ellis is the iridoid glycoside geniposide (see Figure 2.1).

Figure 2.2 The dry fruits of gardenia from *gardenia jasminoides* Ellis.

Source: Available from: URL: http://www.pipitech.com/product/gardeniayellow /gardeniayellowRD.htm2011 (accessed on 20.03,2017).

2.2.3 Genipin production

From the literature reviews, genipin can be prepared by several methods. The first report about the preparatioin of genipin is relyied on several steps starting from the initial extraction of geniposide from dry gardenia fruits with organic solvent. After that, the resultant purified geniposide was subjected to the hydrolysis process by β-glucosidase and then convert to genipin (Endo and Taguchi, 1973). Later, some method modifications have been made by other researchers including the improvement of the isolation step of geniposide from plant biomass which using the response surface methodology to predict the extraction process of bioactive compounds from gardenia fruits (Yang, Liu and Gao, 2009)

In addition, other extraction methods have been applied for the genipin extraction to increase the extraction yield or to pretreat the sample prior extraction process such as reflux extraction, continuous circumfluence extraction and ultrasonic extraction (Yang and Lv, 2004). In addition, different enzyme platforms i.e. immobilized enzymes (Yang et al., 2011; Fugikawa et al., 1987) and whole cell biocatalysis using β-glucosidase-producing strains e.g. *Penicillium nigricans* (Xu et al., 2008) have been studied and used to increase the yields of genipin.

2.2.4 Applications of genipin

Because it is a naturally occurring, biodegradable molecule with low cytotoxicity, genipin has a lot of pharmacological activities and can be used as a safe pigment in food industry and natural fabric dye. Morover, it has recently been investigated to be used in a variety of applications such as the use of gienipin as a cross-linking agent in many biological applications (Bigi et al., 2002; Muzzarelli, 2009; Harris, Lecumberri and Heras, 2010) and drug delivery (Manickham, Sreedharan and Elumalai, 2014; Khan, Shukla and Bajpai, 2016; Zhang et al., 2017).

2.2.4.1 Pharmacological activities

Genipin has long been used in oriental traditional medicines for the treatment of various disorders such as inflammation, hepatic disease and abdominal pain due to dysentery (Tang and Eisenbrand, 1992). In addition, it has been currently reported that genipin possesses many curative and pharmacological actions such as hepatoprotective, choleretic (Peng et al., 2003), anti-inflammatory (Wang, Wu and Rao, 2009), anticancer (Cao et al., 2010), antithrombotic (Suzuki et al., 2001), antibacterial (Lelono, Tachibana and Itoh, 2009), gastritis curative (Lee, Lee and Jeong, 2009), anti-diabetic (Zhang et al., 2006), neuroprotective (Yamazaki, Chiba and Yoshikawa, 2009) and antidepressant-like effects (Tian et al., 2010).

For instance, genipin showed concentration-dependent inhibition on lipid peroxidation induced by Fe^{2+}/ascorbate in rat brain homogenate. It exhibited significant topical anti-inflammatory effect shown as an inhibition of croton oil-induced ear edema in mice (Koo et al., 2004). In addition, the study of hepatoprotective of genipin was carried out by Kim, J. (2013). The results showed that geniposide and genipin offered significant hepatoprotection against I/R injury by reducing oxidative stress and apoptotic cell death. Since geniposide goes through intestinal metabolism before being converting into genipin, the protective effect revealed in this study is likely to be drawn from genipin itself. However considering the superior stability and solubility of geniposide, it should be given priority as a candidate for potential therapeutic agent on I/R injury (Kim, Kim and Lee, 2013)

2.2.4.2 Bioadhesives and wound-dressing materials

Recent explorations into the use of genipin cross-linked gelatin for the use as a bioadhesive, wound dressing, and as bone substitutes, have shown it to have potential as a new and safe cross-linking agent (Chang et al., 2003). The most common cross-linking agent has been glutaraldehyde, but because of concerns about its toxicity new methods are being tested like genipin.

Bioadhesives are used in surgery for tissue adhesion and also for stopping the flow of blood. They are important for sealing air leaks and body fluid

links in organs and tissues that are resistant to suture or stapling techniques. The current bioadhesive being used is gelatin cross-linked with glutaraldehyde (Sung et al., 1999). However, as stated earlier the toxicity of glutaraldehyde has led to the investigation of the use of other cross-linking agents. Sung and his co-workers conducted an experiment to evaluate gelatin cross-linked with genipin glue to create a bioadhesive (Sung et al., 1999). They used the gelatin cross-linked with glutaraldehyde glue has a control for comparison. They compared the cytotoxicity, the gelation time, bonding strength, and flexibility. They found gelatin cross-linked with genipin glue to be much less cytotoxic about 10,000 times less then gelatin cross-linked with glutaraldehyde glue. The gelation time for the gelatin cross-linked with genipin glue was longer than that of the gelatin cross-linked with glutaraldehyde glue. The bonding strength of the gelatin cross-linked with glutaraldehyde glue was greater than the gelatin cross-linked with genipin glue but still comparable. The gelatin cross-linked with genipin glue was found to be more flexible than the gelatin cross-linked with glutaraldehyde glue.

Sung and his co-workers also conducted an in vivo experiment to test the ability of gelatin cross-linked with genipin glue as a bioadhesive for closing skin wounds (Sung et al., 1999). The healing of skin wounds is currently done by the use of sutures but sutures increase the risk of infection and require the use of local or general anesthesia. The in vivo test compared the use of sutures, gelatin cross-linked with glutaraldehyde glue, and gelatin cross-linked with genipin glue. They found that the tensile strength of the skin across the wounds increased with time for both the glues and the suture. All three techniques did not interfere with the healing of the wound and no calcification was seen. It took six days for the suture to be reabsorbed while it took 9 days for the gelatin cross-linked with genipin glue to be reabsorbed. The gelatin cross-linked with glutaraldehyde glue took 14 days to be completely reabsorbed. The healing process of the suture was more rapid then the glues. The wounds treated with gelatin cross-linked with genipin glue healed faster and less inflammation was seen then in the wound treated with gelatin cross-linked with glutaraldehyde glue. Both of these experiments, indicate that further investigation into the use of gelatin cross-linked with genipin glue as a bioadhesive is warranted.

The results of the previous two studies encouraged Chang and his colleagues to develop a genipin cross-linked gelatin membrane to use as a wound dressing material (Chang et al., 2003). They used a glutaraldehyde cross-linked gelatin membrane as the control. Their in vitro study indicated that the degree of cross-linking for both was equivalent. The tensile strength of the genipin cross-linked gelatin membrane was much greater than the glutaraldehyde cross-linked gelatin membrane; however, the strain at fracture was much less in the genipin cross-linked gelatin membrane than the glutaraldehyde cross-linked membrane. The swelling ratio of the genipin cross-linked membrane was smaller than the swelling ratio for the glutaraldehyde cross-linked gelatin membrane. The genipin cross-linked membrane degraded at a slower rate than the glutaraldehyde cross-linked gelatin membrane when exposed to collagenase solution. This can be attributed to the higher stereohindrance of the cyclic genipin cross-linked gelatin membrane. In the in vivo study, they found that the inflammation in the genipin cross-linked gelatin membrane was less severe throughout the course of the experiment which was 21 days. This indicates the genipin cross-linked gelatin membrane has a better biocompatibility then the genipin cross-linked gelatin membrane. The healing rate of the wound using the genipin cross-linked gelatin membrane was also much faster than the healing time of the wound for the genipin cross-linked gelatin membrane.

2.2.4.3 Fingerprint reagent and quantitation of amino acid

In the area of forensic science, genipin is being examined as a new way of developing latent fingerprints on paper products (Almog et al., 2004; Levinton-Shamuilov et al., 2005). Genipin is also being investigated in the field of forensic science as a fingerprint reagent to develop latent fingerprints on paper products. In an article by Lee and his collogues, they investigated the use of genipin as a way to development amino acid stains (Lee et al., 2003). They compared genipin ability to develop amino acid stains with ninhydrin ability. The product of the reaction of genipin with amino acids was very stable and lasted 7 months, but the ninhydrin product disappeared much sooner than the genipin product. The sensitivity was also greater with genipin then ninhydrin as seen through the molar absorptivities. Because of the properties genipin showed in this study, Almog and collogues investigated

genipin as a fingerprint reagent (Almog et al., 2004). Ninhydrin is the current reagent used to develop latent fingerprints on paper products. They define an ideal reagent for developing latent fingerprints as a reagent that produces color and also fluorescent. It must also be a simple reaction that can be done easily at a crime scene. Lastly, it must be highly sensitive. They found that the fingerprints developed with genipin were blue in color and showed clear ridge detail. They were also able to visualize weak fingerprints using fluorescence and genipin shifted the excitation-emission domain toward the longer wavelength which produces less background. They concluded that genipin meets the requirements for a fingerprint reagent and should be considered as an alternate to ninhydrin. In a second study, Almog and co-workers showed the advantages of genipin over ninhydrin (Levinton-Shamuilov et al., 2005). Genipin has the advantage of the combination of both color and fluorescence in a single reaction and as stated early it has a low background when fluoresced because it is viewed at longer wavelengths. Genipin is also safe and environment friendly because it is a natural plant product. The safety of genipin is very important because ninhydrin has recently been reported to potentially cause health hazards. Besides the cost of genipin they concluded that it has great potential to be used as a fingerprint reagent.

Based on the reaction of genipin with amino acid, it was applied for the determination of amino acid on the thin layer chromatography plates. Thin layer chromatographic analysis showed that the genipin reaction produces clear and stable colored spots. The blue ninhydrin reaction spots were usually bleached in 24 h at room temperature, while the genipin reaction spots remained unchanged for several months (Lee et al., 2003). Apart from that, it can be reacted with the free amino groups in sample of water soluble chitin derivatives. The blue colored adduct formed during genipin reaction with free amino groups was measured at about 589 nm and Beer-Lambert's law obeyed over the concentration range of 50 to 300 mg/L. The colorimetric method with genipin was proved to be a rapid and efficient technique to determine the free amino groups in water soluble chitin derivatives. This method can also be applied for the detection of the enzymatic activity of chitindeacetylase (Ramos-Ponce et al., 2010).

2.2.4.4 Cross-linking for pharmaceutical formulations

One of the popular approaches in controlling drug delivery from the polymeric carriers is suitably achieved by the inclusion of cross-linking agents into the formulations at different concentrations. Nevertheless, addition of the chemical cross-linkers such as glutaraldehyde, formaldehyde etc, used in the drug delivery systems causes very serious cytotoxic reactions. These chemical cross-linking agents did not offer any significant advantageous effects when compared to the natural cross-linking agents for instance genipin, which is quite less toxic, biocompatible and offers very stable cross-linked products. So, genipin was utilized as a cross-linking agent in controlling the drug delivery from the various formulations (Manickham, Sreedharan and Elumalai, 2014).

Recent advances in hydrogel technology have focused on finding more biocompatible, non-toxic materials intended for pharmaceutical and biomedical applications. The using of naturally occurring genipin for cross-linking of casein protein in aqueous system was reported for the formation of novel hydrogel materials by Song et al., 2009. The results showed that the casein hydrogel cross-linked with genipin have various swelling and drug release characteristics in simulated gastrointestinal tract conditions. They suggest that the genipin-cross-linked casein hydrogel may be used as a suitable carrier for protein drug delivery (Song et al., 2009). Moreover, genipin can be used as a cross-linking agent for buccal drug delivery (Jinke et al., 2015) and intestine drug delivery (Chen et al., 2004).

2.3 Enzyme-assisted extraction of bioactive compounds from plants

Bioactives are metabolites synthesized by plants for self defence and other purposes. It have the potential to be used by humans for a variety of applications including to promote optimal health and to reduce the risk of chronic diseases such as cancer, coronary heart disease, stroke and Alzheimer's disease (Biesalski et al., 2009). Bioactives are obtained selectively from plants as specialty chemicals and can be used as nutraceuticals, processed foods to complement a balanced diet or as drug leads. Essential and non-essential bioactives are presented in a vast range of foods (such as

fruits, vegetables and grains) and consumed as part of the human diet. Bioactive compounds in plants are typically present at low concentrations (Stafford, 2002). Unfortunately, solvent-based extraction of bioactives often suffers from low extraction yields, requires long extraction times and the final product often contains residual organic solvents, which decrease the product quality (Yang et al., 2011). Thus, the development of an effective and selective method for bioactive compounds extraction is important. Methods such as cold pressing, super-critical fluid and solvent extraction are used to extract bioactives from plants (Table 2.2) However, the use of organic solvents for the recovery of natural products has several drawbacks, including safety hazards, high energy input, low product quality, environment risk and toxicological effects (Teo et al., 2010). There is a need to develop optimized and comprehensive protocols for enhancement the yield of bioactive compounds, particularly from plants where the cell wall can inhibit extraction efficiency.

Table 2.2 Solvents used for the extraction of bioactive compounds from plants.

Polarity of solvents	**Solvent used**	**Product**
Apolar	Cyclohexane, hexane, toluene, benzene, ether, chloroform, ethyl acetate	Alkaloids, terpenoids, coumarins, fatty acids, flavanoids
Polar	Acetone, acetonitrile, butanol, propanol, ethanol, methane	Flavanols, lectins, alkaloids, quassinoids, flavones, polyphenols, tannins, saponins

Source: Puri, M., D. Sharma and C. J. Barrow. "Enzyme-assisted extraction of bioactives from plants." **Trends in Biotechnology** 30, 1: 37-44.

Over the last decade, a novel alternative method for extraction of bioactive compounds from plants by using enzyme-assisted extraction has been used increasingly. This method expected to break down the compositions of plant cell wall such as cellulose, hemicelluloses and pectin into small pieces and ready to facilitate the flow of active constituent yield prior the extraction by conventional extraction method. The use of enzymes in extraction process appears potentially attractive compared to the conventional method since it offers many advantages. For instance, it

decreases the solvent consumption with reportedly may also lower the investment costs and energy requirements (Rosenthal et al., 1996).

2.3.1 Composition of plant cell wall

The cell wall of most of the vegetable matrixes is composed of three different layers i.e. the primary cell wall, the middle lamella and the secondary cell wall (Figure 2.3).

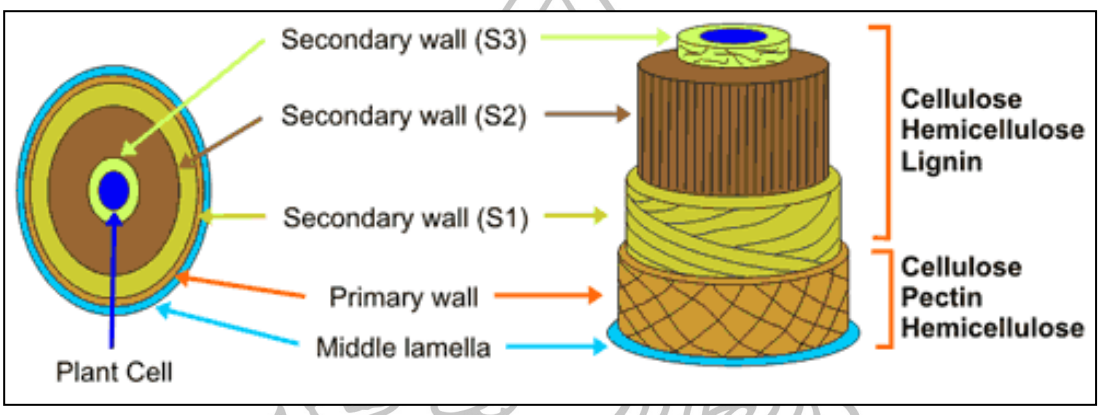

Figure 2.3 Plant cell wall structure.

Source: Available from: URL: http://bioenergy.ccrc.uga.edu/Background/background.htm (accessed on 20.03,2017).

The primary cell wall, generally a thin, flexible and extensible layer formed while the cell is growing. The main chemical components of the primary plant cell wall compose of cellulose (a polysaccharide consisting of a linear chain of several hundred to many thousands of β(1→4) linked D-glucose units, pectin (a family of complex polysaccharides that all contain 1,4-linked α-D-galacturonic acid) and hemicelluloses, branched polysaccharides that are structurally homologous to cellulose because they have a backbone composed of 1,4-linked β-D-hexosyl residues. The predominant hemicellulose in many primary walls is xyloglucan. Organized into a network with the cellulose microfibrils, the cross-linking glycans increase the tensile strength of the cellulose, whereas the coextensive network of pectins provides the cell wall with the ability to resist compression. In addition to these networks, a small amount of protein can be found in all plant primary cell walls.

Some of this protein is thought to increase mechanical strength and part of it consists of enzymes, which initiate reactions that form, remodel, or breakdown the structural networks of the wall. The middle lamella is a layer rich in pectins. This outermost layer forms the interface between adjacent plant cells and glues them together (see Figure 2.3). The middle lamella is shared by neighboring cells and cements them firmly together. Positioned in such a manner, cells are able to communicate with one another and share their contents through special conduits.

The secondary cell wall, (see Figure 2.3) a thick layer formed inside the primary cell wall after the cell is fully grown. It is not found in all cell types. Some cells, such as the conducting cells in xylem, possess a secondary wall containing lignin, which strengthens and waterproofs the wall. The secondary plant cell wall, which is often deposited inside the primary cell wall as a cell matures, sometimes has a composition nearly identical to that of the earlier-developed wall. More commonly, however, additional substances, especially lignin, are found in the secondary wall. Lignin is the general name for a group of polymers of aromatic alcohols that are hard and impart considerable strength to the structure of the secondary wall. Lignin is what provides the favorable characteristics of wood to the fiber cells of woody tissues and is also common in the secondary walls of xylem vessels, which are central in providing structural support to plants. Lignin also makes plant cell walls less vulnerable to attack by fungi or bacteria, as do cutin, suberin, and other waxy materials that are sometimes found in plant cell walls.

2.3.2 Cell wall degrading enzymes

The application of enzymes for extraction of bioactive compounds from plants is an attractive proposition since the compositions of plant cell wall were studied. Cellulolytic, pectinolytic or other enzymes able to catalyst the bonds in plant cell resulting in the decomposition of the structure. Many researchers reported the significant differences in the amount of total bioactive compound when cell wall degrading enzymes were used in pretreatment step prior the conventional extraction. Table 2.3 shows the list of bioactive compounds of industrial importance obtained by enzyme-assisted extraction from plants (Puri, Sharma and Barrow, 2012).

To date, cellulase, hemicellulase and pectinase have a wide range of potential applications in enzyme-assisted extraction of bioactive compounds from plants. These enzymes account for approximately 20% of the world enzyme market (Bhat, M.K., 2000).

2.3.2.1 Cellulase

Cellulase enzymes are produced primarily by fungi, bacteria, and protozoans. Cellulases are a mixture of slightly different enzymes that include endo-1,4-β-glucanase enzymes, which attack internal bonds, exo-1,4-β-glucanase enzymes, which cleave two to four units from the ends of cellulose strands and cellobiase, which cleaves the disaccharide cellobiose into two glucose moieties. Cellulase enzyme catalyzes the hydrolysis of cellulose into glucose (Figure 2.4 and 2.5).

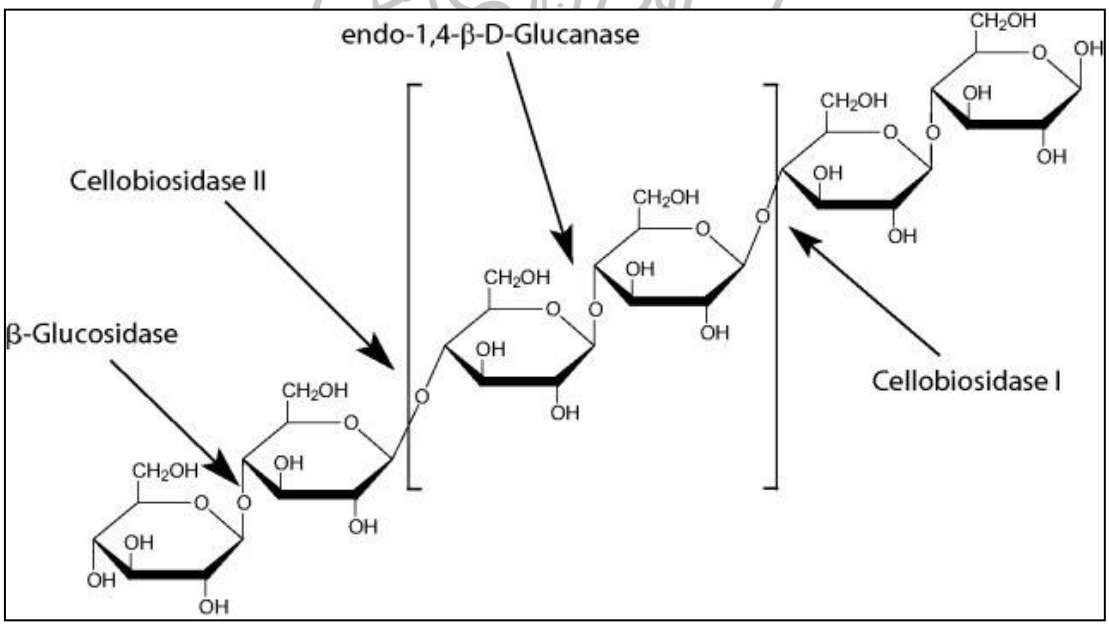

Figure 2.4 Structure of cellulose and its digestion to glucose.

Source: Available from: URL: http://www.sigmaaldrich.com/life-science/metabolomics/enzyme-explorer/analytical-enzymes/enzymes-for-aer.html (accessed on 20.03,2017).

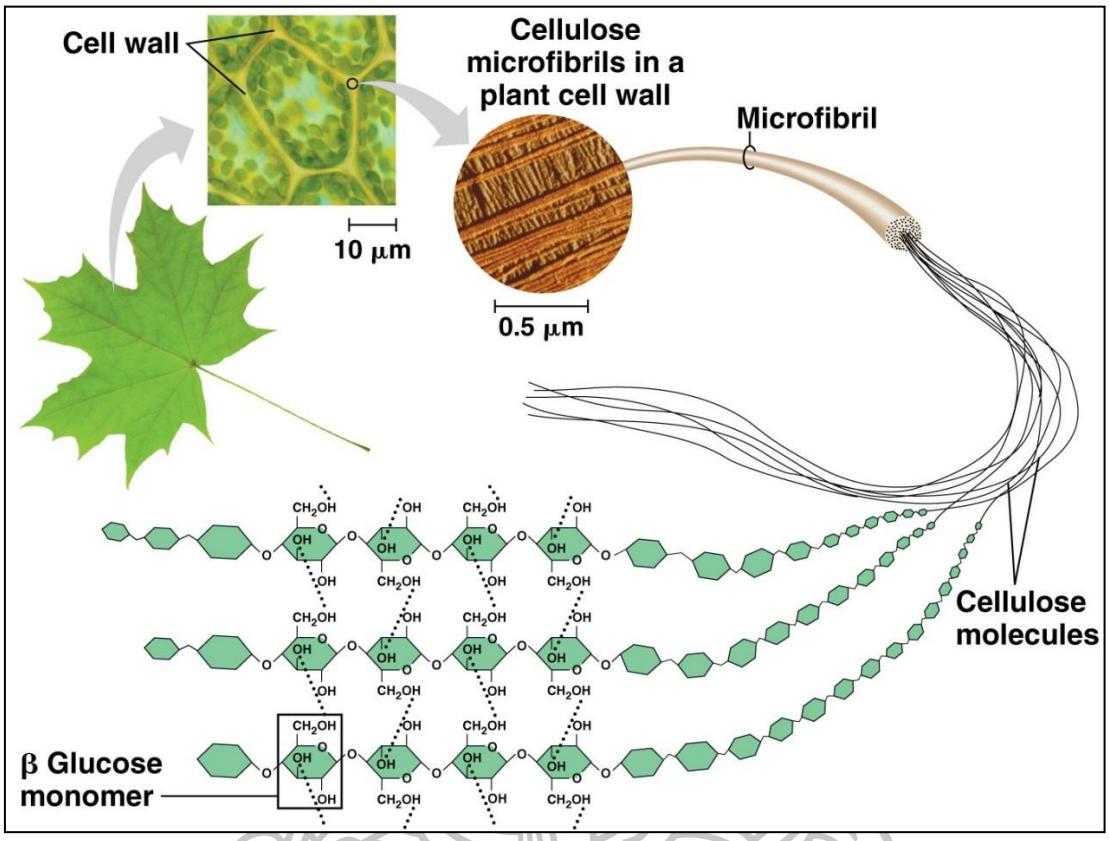

Figure 2.5 Hydrolysis of cellulose microfibrils of cell wall.

Source: Available from: URL: https://www.slideshare.net/kindarspirit/05-the-structure-and-function-of-large-biological-molecules (accessed on 20.03,2017)

2.3.2.2 Hemicellulase

Hemicellulase is a group of enzymes as a mixture of glycolytic enzymes usually containing xylanase, mananase and other activities that degrade the plant cell wall hemicellulose. Hemicellulase enzymes are produced primarily by fungi.

2.3.2.3 Pectinase

Pectinase enzymes are produced primarily by fungi and bacteria. Pectinase catalyzes the random hydrolysis of 1,4-α-D-galactosiduronic linkages in pectin and other galacturonans.

Table 2.3 List of bioactive compounds obtained by enzyme-assisted extraction from plants.

Product type	**Product**	**Source**	**Enzyme used**	**Maximum yield (%)**
Oil and carotenoids	Oil	Grape seed	Cellulase, protease and xylase	17.5
	Volatile oil	Mandarin peel	Xylan-degrading enzymes	15.0
	Colorant	Pitaya	Pectinolytic, hemicellulytic and cellulytic enzymes	83.5
	Lycopene	Tomato	Cellulase and pectinase	206.0
Glycosides	Oligosaccharide	Rice bran	Cellulase	39.0
	Starch	Cassava	Pectinase	45.6
Others	Polyphenols	Grape pomace	Pectinolytic	98.1
	Catechins	Tea beverage	Pepsin	80.0
	Soluble fibre	Carrot pomace	Cellulase-rice crude preparation	77.3

Source: Puri, M., D. Sharma and C. J. Barrow. "Enzyme-assisted extraction of bioactives from plants." **Trends in Biotechnology** 30, 1: 37-44.

2.3.3 Factors effecting the efficiency of the enzyme-assisted extraction of bioactive compounds from plants

There are several factors effect to the activity of enzymes on the degradation of the cell walls, and release of the bioactive compounds. The time-temperature regime of the enzymatic treatment is possibly one of the most important; to perform the enzymatic treatment at optimum conditions of temperature and also pH will result in the maximal activity of enzyme. Different enzyme treatments with mixed enzyme preparations have also been show to affect differently the efficiency of the extraction bioactive from plants. The type of extraction solvent employed as well as the enzyme/plant ratio is one of the most studied variables (Pinelo, Sineiro and Nunez, 2006).

From the literatures, the pH value can affect enzyme activity because different enzymes have their own optimal pH, which might be because a change in pH affects the spatial structure of enzymes. Thus, the enzyme conformation and enzymatic activity can be altered by changes in pH (Yin, You and Jiang, 2011). Yin and co-workers reported that the extraction yield of polysaccharides by using enzyme form *Tricholoma matsutake* assisted-extraction continued to increase with the increase of pH value (2.5-4.0) and reached the peak value (6.73%) at pH value 4. However, the extraction yield of polysaccharides no longer increased when the pH value exceeded 4. The possible reason for this phenomenon may be that the appropriate pH of the complex enzyme was in the range of 2.5-4.0, and when the pH value exceeded 4 the activity of the complex enzyme was decreased. They also investigated the effect of temperature on the yield of extraction. The possible reason for this result may be the complex effect of the following two aspect: firstly, higher temperature can enhance the mass transfer and accelerate the extraction speed; secondly, the complex enzymes have a suitable effect temperatures, when the temperature was under the suitable effect temperatures, the enzyme activity increased with the increase of temperature, when the temperature was higher than the suitable effect temperatures, the enzyme activity decreased with the increase of temperature. This tendency is in agreement with reports of other authors' in extracting polysaccharides (Zou and Guo, 2010)

Passos and co-workers measured the effect of reaction time, temperature, pH of reaction and enzyme concentration on the enzyme activities. The following set of parameters was optimized to enhanced the yield of grape seed oil as; time of reaction was 24 h, pH of reaction was 4, temperature was 30-40 °C, and cocktail solution combination of cellulase, protease, xylanase and pectinase at the ration of 29:1191:21:569 unit/g, respectively. The extraction yield was 13.7% which represents an increment 106% over non-treated sample. For 120 h the yield achieved was 17.5%, and increment reach 163% indicated that a prolonged enzymatic treatment may certainly be used to enhance oil extraction (Passos et al., 2009).

2.3.4 Benefits and limitations of enzyme-assisted extraction

The application of enzymes for complete extraction of bioactives without the use of solvents is an attractive proposition. Enzyme pretreatment of raw material normally results in a reduction in extraction time, minimizes usage of solvents and provides increased yield and quality of product (Meyer, 2010; Sowbhagya and Chitra, 2010). Prior knowledge of the cell wall composition of the raw materials helps in the selection of an enzyme or enzymes useful for pretreatment. Decreased solvent use during extraction is particularly important for both regulatory and environmental reasons, providing a 'greener' option than traditional non-enzymatic extraction. Enzyme-assisted extraction of bioactive compounds from plants has potential commercial and technical limitations: (i) the cost of enzymes is relatively expensive for processing large volumes of raw material; (ii) currently available enzyme preparations cannot completely hydrolyze plant cell walls, limiting extraction yields of compounds, including the extraction of stevioside; (iii) enzyme-assisted extraction can be difficult to scale up to industrial scale because enzymes behave differently as environmental conditions such as the percentage of dissolved oxygen, temperature and nutrient availability vary. However, if the above limitations can be overcome, then enzyme-based extraction could provide an opportunity to not only increase extraction yields, but also to enhance product quality by enabling the use of milder processing conditions such as lower extraction temperatures.

2.4 In situ product separation

2.4.1 The separation techniques

Several separation steps are necessary in a biotransformation process to recover and purify the desired product. The ease or otherwise of the separation will depend upon the type of reaction being performed, the biocatalyst form (whole cell, immobilized enzyme or free enzyme) and the design and operation of the bioreactor. These factors will determine whether or not there are undesirable regio- or stereoisomers of the product to be removed, whether or not there are compounds resulting from the decomposition or further reaction of the product that need to be

separated and, ultimately, the relative concentrations of substrate and product molecules in the reactor at the end of the reaction. The last point is particularly important, because the substrate and product molecules of a biocatalytic process will generally be structurally and chemically very similar to each other and hence extremely difficult to separate.

In terms of the sequence in which the various separation steps are carried out, the ‘rule of thumb’ is generally to perform first those operations for which the separation driving force is largest; that is, where there is the greatest difference in physicochemical properties between the species to be separated. Given the large size difference between the biocatalyst and substrate and product molecules, especially when using immobilized- enzyme or whole-cell biocatalysts, the first separation step will logically be some form of solid-liquid separation such as filtration or centrifugation.

There are various physical and chemical methods for the subsequent separation of substrate and product molecules, and these can be classified according to the basis on which the separation is achieved (Table 2.4). The most appropriate techniques are those that involve partitioning of the product into a second liquid phase or the adsorption onto or generation of a solid phase. Although examples of product recovery using distillation do exist, even with in situ applications (Paiva and Malcata, 1994; Paiva and Malcata, 1997), such techniques are often not sufficiently selective and are only applicable to limited classes of biocatalytic process, such as those with small and highly volatile molecules. The selectivity and capacity requirements of the various separation techniques will be discussed later.

Table 2.4 Classification of key substrate and product separation techniques according to the basis of separation.

Separation basis (driving force)	**Comments**	**Example techniques**
Physical properties		
Volatility	Few examples with these properties.	Distillation
		Gas stripping
Molecular weight or size	The difference between substrate and product is frequently too small.	Centrifugation
		Size exclusion
Solubility	High capacity but generally selectivity.	Extraction
		Precipitation
		Crystallization
Chemical properties		
Charge	High selectivity but generally low capacity.	Ion-exchange, electrodialysis
Hydrophobictity		Adsorption
Specific elements		Reversible complexation
		Affinity methods

Source: Gary, J. L. and J. M. Woodley. "Application of in situ product-removal techniques to biocatalytic processes." **Trends in Biotechnology** 17, 10: 395-402.

The mode of operation of in situ product separation techniques (i.e. whether they are batch, fed-batch or continuous and whether the separation step occurs within the bioreactor or outside it) Much of the work reported was, however, concerned with the recovery of ethanol or organic acids from fermentation processes. Other reviews on process integration have been confined to a particular class of biocatalytic reaction, such as those involving lipases (Paiva and Malcata, 1997). The following examples show how some of the recovery and separation techniques listed in Table 2.4 have been applied to a range of biocatalytic processes, with varying degrees of success and using different in situ product separation configurations.

2.4.2 Limitations of biocatalytic processes and the role of in situ product separation

The productivity of biocatalytic processes is frequently limited by the need to operate the reaction under conditions unsuited to the biocatalyst. Indeed, this differentiates biological from chemical catalysis, in that the optimal environment for the biological catalyst has been carefully evolved for operation under natural physiological conditions, whereas chemical catalysts are designed for a specific conversion at user-defined conditions. Inevitably, the need for high process productivities outside the environment for which the biocatalyst was evolved leads to compromises over bioreactor design and operation. In several cases, however, compromises are not sufficient and it is necessary to maintain one environment around the biocatalyst while the bulk of the reactor operates under different conditions.

This philosophy of compartmentalization is, in principle, an effective strategy to overcome the problem of low productivities. It has already been successfully applied to cases where the pH (Byers et al., 1993) or substrate concentration (van den Tweel et al., 1988) need to be different for the reactor and the biocatalyst. A further need is for compartmentalization of the product owing to inhibitory (reversible loss of catalytic activity) or toxic (irreversible loss of catalytic activity) effects on the biocatalyst, to product degradation and/or to unfavourable reaction equilibria. These limitations to productivity in the presence of the product have been well documented in the past (Mattiasson and Holst, 1991; Roffler, Blanch and Wilke, 1984).

In situ product separation methods address these limitations by selectively removing the product from the vicinity of the biocatalyst as soon as it is formed and can also provide further benefits for the subsequent downstream processing (Freeman, Woodley and Lily, 1993; Szathmary and Grandics, 1990; Daugulis, 1988). In situ product separation methods can increase the productivity or yield of a given biocatalytic reaction by any of the following means (Chanhan and Woodley, 1997): (i) overcoming inhibitory or toxic effects; (ii) shifting unfavourable

reaction equilibria; (iii) minimizing product losses owing to degradation or uncontrolled release; and (iv) reducing the total number of downstream-processing steps. Despite these advantages, however, the application of in situ product separation to the production of small molecules has been slow except in a limited number of well-known cases. The primary examples are of low-value, high-volume products, such as the removal of organic acids and solvents from fermentation processes (Freeman, Woodley and Lilly, 1993).

2.5 Colorimetric agent derived from natural compounds

2.5.1 Green analytical chemistry

The late 1900's and early 2000's have seen the growth of interest in so-called Green Chemistry, (Anastas and Warner, 1998) which has been driven by concerns regarding the environmental impact and sustainability of chemistry, and to some extent by the poor public perceptions of the discipline of chemistry and the chemical industry in general. Implicit in the Green Chemistry paradigm is the need for the development and adoption of green analytical techniques and procedures (Anastas, 1999; Armenta, Garrigues and Guardia, 2008). These include the reduction of reagent and solvent usage, the minimization of solid, liquid and gaseous materials produced by analytical processes, the replacement of reagents and solvents of high occupational or environmental toxicity with much more innocuous materials, and the reduction of energy use in analytical processes (Namiesnik, 2001; Keith Gron and Young, 2007). As Armenta et al. have pointed out in their review on Green Analytical Chemistry, (Garrigues and Guardia, 2008) flow analytical techniques are inherently green, because they involve much smaller volumes of reagents and sample, produce much less waste, and offer the possibility of both efficient on-line sample pretreatment and even analytical waste treatment. However, a principle of Green Analytical Chemistry that apparently has not been widely annunciated is the potential and desirable use of natural or unrefined reagents as a replacement for highly refined or purified reagents, in analytical processes (Garrigues and Guardia, 2008). This suggestion may at first seem to be counter to the best-held principles of analytical chemistry, in which reagents of the highest available purity are used in order to

minimize reagent blanks and potential interferences. However, when a reagent is only required in excess, or a crude extract contains the necessary active reagent, then in some cases these may be substituted for more purified reagents without compromising the success of the determination. Clearly the use of such natural reagents is highly desirable because of the simplicity of their preparation and the inherent energy and chemical waste savings.

2.5.2 The use of natural compounds as detection reagents i.e. indicators, chromo-, fluoro, and lumino-gens

There is a wealth of high quality chemical information on potential natural analytical reagents in the compendia of natural product and food chemistry. For example the indicator and fluorescent properties of anthocyanins in crude extracts from plants such as red cabbage (*Brassica oleracea*) (Sapers, Taffer and Ross, 1981) red grapes (Heredia et al., 1998) and Morning Glory flowers (Asen, Stewart and Norris, 1977) are well documented, but they are not widely used in analysis despite ready availablity and low cost. It has been demonstrated that crude extracts of the flowers of azalea (*Rhododendron simsii*) and quaresmeira (*Tibouchina granulosa*) used as pH indicators gave endpoints within 2% of those obtained by potentiometry (Cortes, Ramos and Cavalheiro, 2007). There are also reports of the use of extracts of Chinese hibiscus (*Hibiscusrosa sinensis* L.) and Indian shot (*Canna indica* L.) being used as simple visual γ-ray dosimetry indicators (Saisomboon and Siri-Upathum, 1987).

Other natural reagents were historically used either for spot tests, e.g. cinchonine from Calisaya (*Cinchona calisaya*, Wedd) was used for the detection of bismuth, while salicylate, derived from willow bark (*Salix* spp.), was known as an effective reagent for iron (Stephen, 1977)

Table 2.5 lists some natural indicators, as well as examples of some plant-based materials that have been used for spectrophotometric and fluorimetric determinations. For example, curcumin, which is obtained by extraction from the rhizomes of turmeric (*Curcuma longa* L.) has long been known as a suitable reagent

for the determination of boron (Liu and Lee, 2009). Similarly, aqueous extracts of guava leaves have been shown to be useful in the flow injection analysis spectrophotometric determination of Fe (Settheeworrarit et al., 2005). Three flow injection configurations were tested, a normal flow injection analysis mode in which sample was injected into a flowing stream of guava extract, a reactor containing guava leaves for on-line leaching of the extract, and a reverse flow injection analysis configuration in which extract was injected into a stream of sample. The latter mode was preferred because it minimized reagent use and gave greater sensitivity. However, all modes required the incorporation of a washing step to remove an unidentified precipitate that formed. If performed in batch mode, precipitate formation would be problematic, and may mean that the analytical procedure is unviable, whereas when performed in flow analysis mode, washing to remove precipitate can be performed in a controlled and highly reproducible manner.

Some plants contain fluorescent constituents, e.g. quinine is naturally derived from *Cinchona* bark, and ripe banana skins are known to fluoresce under UV light, due to the break down products of chlorophyll (Moser et al., 2008). The determination of mercury by fluorescence quenching of chlorophyll (Gao et al., 2006) is a particularly good example of a natural reagent-based determination, in that it is simple, selective and very sensitive, with a limit of detection of *ca*. 1 µg L^{-1}. In a method for the speciation of Fe(II)/Fe(III) that has very good green chemistry credentials, Pulido-Tofino et al. described a flow injection method based on a fluorescent siderophore, pyoverdin, that is biosynthesised by *Pseudomonas fluorescens*. Pyoverdin was immobilized on controlled pore glass and used repeatedly, while peroxydisulfate for Fe(II) oxidation was immobilized on Dowex resin and used with a switching valve to enable determination of either Fe(III) or total Fe (Pulido-Tofino, Barrero-Moreno and Perez-Conde, (2000). Methods based on fluorescence measurement such as these certainly warrant further investigation and validation.

Table 2.5 Natural pH indicators, chromogenic and fluorogenic substances.

Natural pH indicators			
Analyte reagent	**Source**	**Sample type**	**Method**
pH	Roselle (*Hibiscus subdariffa*)	-	Acid-base titration
pH	Crude extracted of flowers from Azalea (*Rhododendron simsii*)	-	Acid-base titration
Natural chromogenic and fluorogenic reagents			
B	Curcumin from turmeric (Curcuma Longa L.)	Sea water	Spectrophotometry
Fe	Guava leaf extracted (*Psidium guajava* L.)	Extracted	Flow injection analysis-spectrophotometry
Mo(VI)	Slippery elm leaf extracted (Ulmus rubra)	Waters sample	Spectrophotometry
Hg(II)	Chlorophyll from pea plant	Extracted	Fluorescence inhibition
Zr	Crude plant extracted from oriental plane tree (*Platanus orientalis*)	Tap, wasterwater, well water samples	Spectrophotometry

Source: Modified from Grudpan, K. et al. "The case for the use of unrefined natural reagents in analytical chemistry-A green chemical perspective." **Analytical Method** 11, 2: 1651-1661.

2.6 Enzyme immobilization

2.6.1 Introduction

An enzyme derived from an organism or cell culture that catalyses metabolic reaction in living organisms and/or substrate conversions in various chemical reactions. The enzymes are the potential catalyst works in mild temperature, pressure, pH, substrate specificity under suitable reaction conditions and for the production of desired products without any intermediate products as contaminations for these advantages enzyme are used in variety of applications. The biotechnological method of producing enzyme is expensive; hence new methods have been

implemented to reduce the cost. The enzymes have various other limitations such as low stability, highly sensitive to the process conditions and these problems can be overcome by the immobilization techniques (Cao, 2005; Hernandez and Fernandez-Lafuente, 2011; Krajewska, 2004). Immobilized enzymes are being used since 1916, when Nelson and Griffin discovered that invertase when absorbed to charcoal has the ability to hydrolyse the sucrose (Nelson and Griffin, 1916). In earlier days the immobilization of enzymes are carried out using biotechnological method, but now due to rapid growth of nanotechnology, the synergistic interaction of two technologies, the development of immobilized enzymes on various nanomaterials using conventional methods such as covalent and adsorption attachment (Kim, Grate and Wang, 2008). Brena and Batista have classified enzyme immobilization as irreversible enzyme immobilization and reversible enzyme immobilization methods. Irreversible enzyme immobilization includes covalent binding and Entrapment. Reversible enzyme immobilization includes adsorption, ionic binding, affinity binding and metal binding. Immobilization of enzyme by enzymatic process is recently identified by researchers in order to avoid harsh immobilization.

2.6.2 Enzyme immobilization methods

Several methods are used for immobilization and various factors influence the performance of immobilized enzymes according to Table 2.6

2.6.2.1 Covalent binding

Covalent binding is a conventional method for immobilization; it can be achieved by direct attachment with the enzyme and the material through the covalent linkage (Wong, Thirlway and Micklefield, 2008). The covalent linkage is strong and stable and the support material of enzymes includes polyacrylamide, porous glass, agarose and porous silica (Ghous, 2001). Covalent method of immobilization is mainly used when a reaction process does not require enzyme in the product, this is the criteria to choose covalent immobilization method. This covalent binding of the enzyme with the support material involves two main steps such as, the activation of

the support material by the addition of the reactive compound and the second one is the modification of the polymer backbone to activate the matrix (Figure 2.6).

Table 2.6 Factors influencing performance of immobilized enzymes

Factors	**Implications of immobilziation**
Hydrophobic partition	Enhancement of reaction rate of hydrophobic substrate
Microenvironment of carrier	Hydrophobic nature stabilizes enzyme
Multipoint attachment of carrier	Enhancement of enzyme thermal stability
Spacer or arm of various types of immobilized enzymes	Prevents enzyme deactivation
Diffusion constraints	Enzyme activity decreases and stability increases
Presence of substrates or inhibitors	Higher activity retention
Physical post-treatments	Improvement of enzyme performance
Difference binding mode	Activity and stability can be affected
Physical structure of the carrier such as pore size	Activity retention was often pore-size dependent
Physical nature of the carrier	Carriers with large pore size mitigate diffusion limitation, leading to higher activity retention

Source: Cao, L. (2006). **Carrier-bound immobilized enzymes: principles, application and design.** Weinheim: Wiley-VCH Verlag GmBH & Co. KGaA.

2.6.2.2 Entrapment

Enzymes are occluded in the synthetic or natural polymeric networks, it is a permeable membrane which allows the substrates and the products to pass, but it retains the enzyme inside the network, the entrapment can be achieved by the gel, fibre entrapping and microencapsulation (Bernfeld and Wan, 1963). The advantage of entrapment of enzyme immobilization is fast, cheap and mild conditions required for

reaction process. The disadvantage is that limitation in mass transfer. The support matrix protects the enzymes from microbial contamination, proteins and enzymes in the microenvironment. Microencapsulation method is that the enzyme molecules are capsulated within spherical semipermeable membranes with a selective controlled permeability. This method provides the large surface area between polymeric material and the enzyme. The drawback of this method is inactivation of enzyme during encapsulation (Bernfeld and Wan, 1963).

2.6.2.3 Adsorption

This is a simple method of preparing an immobilized enzymes and the materials used for adsorption are activated charcoal, alumina, ion exchange resins, this method is cheap and easy for use and the disadvantage is a weak binding force between the carrier and the enzyme (Brady and Jordan, 2009). This method comes under carrier bound immobilization and the process of immobilization is reversible. Adsorption is the easiest and oldest immobilization techniques (Tanyolac, Yuruksoy and Ozdural, 1998). The interaction between the enzyme and the surface of the matrix through weak forces by salt linkage, hydrogen bonds, hydrophobic bonds, ionic bonds and van der waals forces. Based on the charges of the matrix and the protein arrangements the strongly bound, but not distorted enzyme will be formed. The advantage of enzyme adsorption is minimum activation step and as a result of minimum activation, no reagents required. It is cheap and easy way of immobilization.

2.6.2.4 Ionic binding

The bonding involved between the enzyme and the support material is salt linkages. The nature of this non-covalent immobilization the process will be reversed by changing the temperature polarity and ionic strength conditions. This principle is similar to protein-ligand interactions principles used in chromatography (Guisan et al., 1997).

2.6.2.5 Affinity binding

The immobilization of enzyme linked to the matrix through the specific interactions. The Two methods are being followed in affinity immobilization. The first method is the activation of the support material which contains the coupled affinity ligand, so that the enzyme will be added. The advantage of this method is the enzyme is not exposed to any harsh chemicals conditions. The second method, the enzyme modified to another molecule which has the ability to bind towards a matrix (Porath, 1992).

2.6.2.6 Metal linked immobilization

In metal linked enzyme immobilization, the metal salts are precipitated over the surface of the carriers and it has the potential to bind with the nucleophilic groups on the matrix. The precipitation of the meatl ion on the carrier can be achieved by heating. This method is simple and the activity of the immobilized enzymes is relatively high (30-80%). The carrier and the enzyme can be separated by decreasing the pH, hence it is a reversible process (Yucel, 2011).The matrix and the enzyme can be regenerated, by the process.

2.6.3 Matrices and cross-linking agents for enzyme immobilization

2.6.3.1 Natural polymers

2.6.3.1.1 Alginate

Alginate derived from cell walls of brown algae are calcium, magnesium and sodium salts of alginic acid and have been extensively used for immobilization as xanthan-alginate beads, alginate-polyacrylamide gels and calcium alginate beads with enhanced enzyme activity and reusability. Cross-linking of alginate with divalent ions (like Calcium) and glutaraldehyde improves the stability of enzymes (Elcin, 1995; Flores-Maltos et al., 2011).

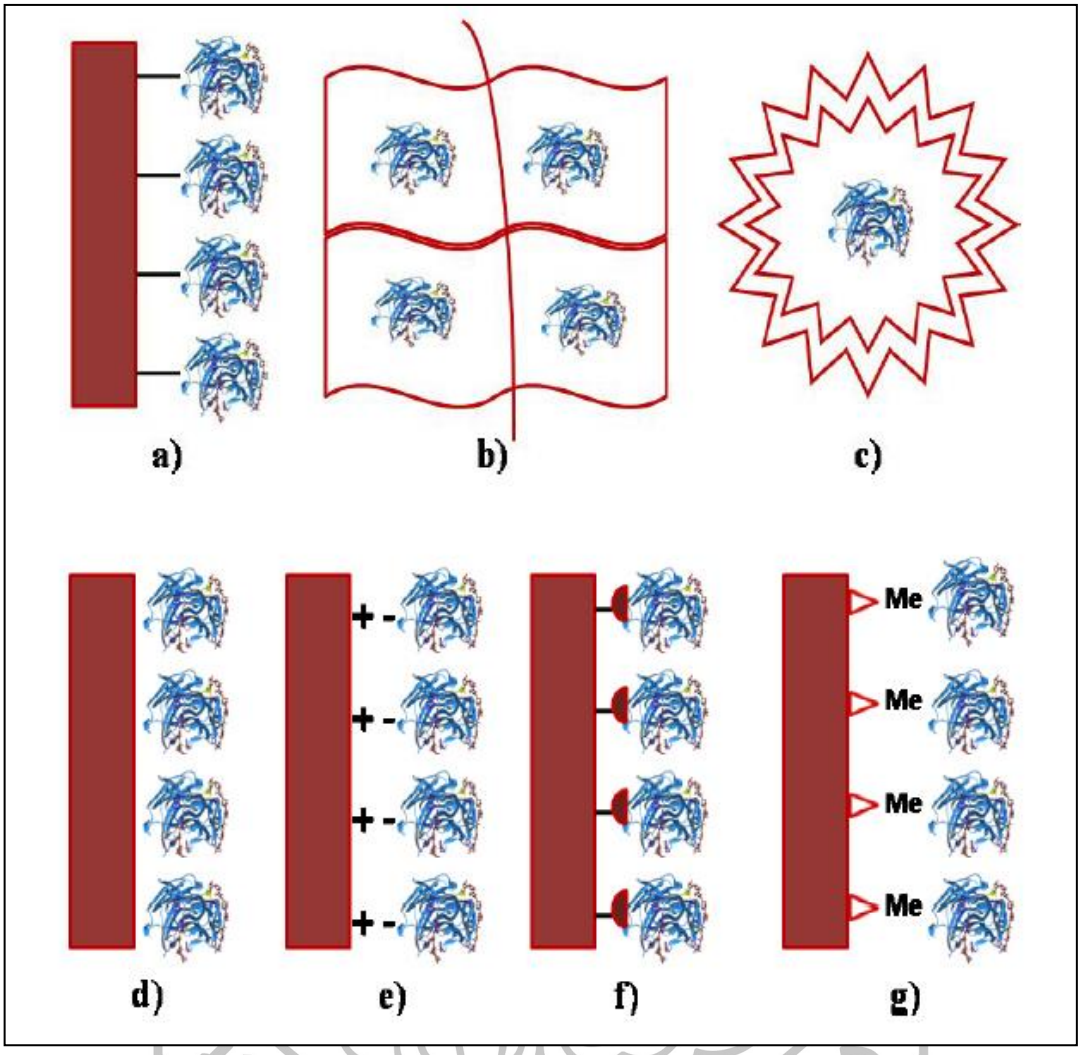

Figure 2.6 Shows pictorial representation of different enzyme immobilization methods a) covalent Binding b) entrapment c) encapsulation d) adsorption e) ionic binding f) affinity Binding and g) metal ion immobilization

Source: Nisha, S., S. Arun Kathick and N. Gobi. "A review on methods, application and properties of immobilized enzyme." **Chemical Science Review and Letters 1**, 3: 148-155.

2.6.3.1.2 Chitosan and chitin

Natural polymers like chitin and chitosan have been used as supports for immobilization (Vaillant et al., 2000; Kapoor and Kuhad, 2007). The protein or carbohydrate moieties of enzymes are used for binding them to chitosan (Hsieh, Liu and Liao, 2000). Chitosan has been used in combination with alginate where chitosan-coated enzymes had less leaching effect compared to alginate owing to the physical and ionic interactions between the enzyme and support (Betigeri and Neau, 2002). Similarly, a wet composite of chitosan and clay proved to be more reliable for enzyme trapping, because it has hydroxyl and amino groups, which easily link with enzymes, together with good hydrophilicity and high porosity. Chitosan in the form of beads can entrap twice as much of the enzymes (Chang and Juang, 2007). According to Chern and Chao (2005), the chitin-binding domain of chitinase A1 from Bacillus circulans has a high affinity to chitin; so, this property has been exploited to retain D-hydantoinase.

2.6.3.1.3 Gelatin

Gelatin is a hydrocolloid material, high in amino acids, and can adsorb up to ten times its weight in water. Its indefinite shelf life has attracted attention for enzyme immobilization. Gelatin has been utilized in mixed carrier system with polyacrylamide where cross-linking with chromium (III) acetate proved better than chromium (III) sulfate and potassium chromium (III) sulfate (Emregul, Sungur and Akbulut, 2006). Calcium alginate with gelatin forms a good template for calcium phosphate deposition for enzyme immobilization, and gelatin in combination with polyester films promoted 75% loading efficiency, compared to previous studies which had 50% loading efficiency (Shen et al., 2011; Ates and Dogan, 2010).

2.6.3.1.4 Pectin

This structural heteropolysaccharide along with 0.2–0.7% glycerol acts as plasticizer to reduce brittleness of support and has been used to immobilize papain and for development of new materials for skin injury treatment (Ceniceros et al., 2003). Pectin-chitin and pectin-calcium alginate support have

enhanced thermal and denaturant resistance and catalytic properties of entrapped enzymes due to the formation of high stable polyelectrolyte complexes between the enzyme and the pectin-coated support (Gomez et al., 2006; Satar, Matto and Husain, 2008).

2.6.3.2 Synthetic compounds

2.6.3.2.1 Silica derivatives

Enzymes like lignin peroxidase and horseradish peroxidase immobilized on activated silica have been effectively used for the removal of chlorolignins from eucalyptus kraft effluent (Dezott, Innocentini-Mei and Duran, 1995). α-Amylase immobilized on silica nanoparticles improves cleaning performance of detergents. They have been used because of their nano-sized structures with high surface area, ordered arrangement and high stability to chemical and mechanical forces (Soleimani, Khani and Najafzadeh, 2011). Surface modifications of silica by amination of hydroxyl and reactive siloxane groups and addition of methyl or polyvinyl alcohol groups strengthen enzyme and support bonds (Rao, Kembhavi and Pant, 2000; Shioji et al., 2003; Pogorilyi et al., 2007).

2.6.3.2.2 Activated carbon derivatives

Both natural and hydrochloric acid-modified activated carbon has provided valuable support for enzyme adsorption (Alkan et al., 2009). Lately, mesoporous-activated carbon particles containing large contact sites for enzyme immobilization have been used for immobilizing acid protease and acidic lipases where catalytic efficiency has been significantly maintained after 21 cycles of reuse (Kumar et al., 2010; Ramani et al., 2011). It was also found that activated carbon with a high surface area (600-1,000 m^2g^{-1}) and a significant fraction of its pore volume in the 300-1,000 A° range was suitable for enzyme immobilization (Daoud, Kaddour and Sadoun, 2010).

2.6.3.2.3 Charcoal derivatives

Chemical modification of charcoal by adsorbing papain with sulfhydryl groups increased the number of active sites and has been utilized for recovery of mercury from aqueous solution and efficiently employed for industrial wastewater treatment (Dutta et al., 2009). Charcoal supports have been also used in food industries for immobilizing amyloglucosidase for starch hydrolysis without any cross-linking agent and has 90% catalytic activity (Rani, Das and Satyanarayana, 2000). As reported earlier by Kibarer and Akovali (1996), charcoal is an excellent adsorbent with high adsorptive capacity and minimum fine particulate matter release.

2.6.4 Application of the Immobilized Enzymes

2.6.4.1 Pharmaceutical applications

Chemical immobilization of proteins and enzymes was first attempted in 1960s, and it is an emerging approach to new drug therapies. Immobilization means the enzymes with restricted mobility or rendered less motile by chemical or physical treatment. It was first prepared by loading to polymeric matrices or binding onto carrier materials. Industrial use of enzymes is greatly limited because they are relatively unstable, have a very high cost of purification, and have cumbersome process of recovery of active enzyme from reaction mixture after the completion of catalytic process. Immobilized enzymes are more stable to pH and temperature stress and less susceptible to the denaturing agents. In addition, an immobilized enzyme should have long-term stability and unaltered sensitivity and biological activity after attachment to the matrix than free enzyme when used as therapeutic purpose (Klein and Langer, 1986). Immobilization has been successfully utilized for studies with such enzymes, as cytochrome P-450, UDP-glucuronosyltransferases, glutathione S-transferases, S-methyltransferases, and N-acetyltransferases (Dulik and Fenselau, 1998).

One of the major applications of immobilized enzymes in pharmaceutical industry is the production of 6-aminopenicillanic acid (6-APA) by deacylation of the side chain in either penicillin G or V, using penicillin acylase

(penicillin amidase). Today more than 50 % of 6-APA is enzymatically produced using the immobilized route which is core of penicillin antibiotic. Penicillin amidase from *Escherichia coli* is immobilized on cellulose triacetate fibers for producing 6-APA from penicillin G (Alvaro et al., 1990). Similarly for producing 6-APA from penicillin V, penicillin amidase is immobilized by covalent binding to Amberlite XAD-7 with glutaraldehyde through physical adsorption to bentonite or by ionic binding to DEAE-Sephadex and also by covalent binding to a copolymer of acrylamide and maleic anhydride (Arshad, Farooq and Ali, 2007). The major reasons for its success is in obtaining a pure product, thereby minimizing the purification costs (Giordano et al., 2006). This process of the immobilized enzyme technology was also approved in India. The first industrial process for the production of 6-APA was started in 1970s by Astra, Sweden, and Riga Biochemical Plant (former USSR).

Immobilization has also been used for the production of 7-aminodeacetoxycephalosporanic acid, an intermediate in the production of semisynthetic cephalosporins. Conversion of 7-amino-3-deacetoxy-cephalosporanic acid (7-ADCA) to cephalexin by immobilized penicillin G acylase (IMPGA) has been investigated. It was observed that under optimized conditions, IMPGA can attain 85% conversion of 7-ADCA to cephalexin. Furthermore, IMPGA can be reused for about ten cycles (Maladkar, 1994). Production of cefazolin by immobilized cefazolin synthetase from E. coli as a biocatalyst has been possible. The complex of the physicochemical studies makes it possible to design a highly efficient technological process for production of cefazolin (Kurochkina and Nys, 1999). Macrolide antibiotics tylosin and nikkomycin also can be produced by *Streptomycin* spp., immobilized with calcium alginate.

2.6.4.2 Biomedical application

Immobilized enzymes are used in medicine from 1990 (Tischer and Wedekind, 1993), immobilized enzymes are used for diagnosis and treatment of diseases in the medical field. The inborn metabolic deficiency can be overcome by replacing the encapsulated enzymes (i.e., enzymes encapsulated by erythrocytes) instead of waste metabolites, the RBC acts as a carrier for the exogenous enzyme

drugs and the enzymes are biocompatable in nature, hence there is no immune response (Johnson et al., 1998). The enzyme encapsulation through the electroporation is an easiest way of immobilization in the biomedical field and it is a reversible process for which enzyme can be regenerated. The enzymes when combined with the biomaterials it provides biological and functional systems. The biomaterials are used in tissue engineering application for repair of the defect. The advantage of the enzyme immobilization in biomedical is that the free enzymes are consumed by the cells and not active for prolonged use, hence the immobilized enzymes remains stable, to stimulate the growth and to repair the defect. The cancer therapy is delivery of enzymes to the oncogenic sites have been improved with new methods. The nanoparticles and nanospheres are often used as enzyme carriers for the delivery of therapeutic agents.

2.6.4.3 Food industry application

In food industry, the purified enzymes are used but during the purification the enzymes will denature. Hence the immobilization technique makes the enzymes stable. The immobilized enzymes are used for the production of syrups. Immobilized beta-galactosidase used for lactose hydrolysis in whey for the production of bakers yeast. The enzyme is linked to porous silica matrix through covalent linkage. This method is not preferably used due to its cost and the other technique developed by Valio in 1980, the enzyme galactosidase was linked to resin (food grade) through cross linking. This method was used for the various purposes such as confectionaries and icecreams (Bozoglu, Swaisgood and Adams, 1984).

2.6.4.4 Others

2.6.4.4.1 Biodiesel production

Biodiesel is monoalkyl esters of long chain fatty acids. Biodiesel is produced through triglycerides (vegetable oil, animal fat) with esterification of alcohol (methanol, ethanol) in the presence of the catalyst. The production of catalyst is a drawback of high energy requirements, recovery of glycerol and side reaction which may affect the pollution. Hence the biological

production of liquid fuel with lipases nowadays has a great consideration with a rapid improvement (Fjerbaek, Christensen and Norddahl, 2009). Lipase catalyses the reaction with less energy requirements and mild conditions required. But the production of lipase is of high cost, hence the immobilization of lipase which results in repeated use and stability (Cao, 2005; Salis et al., 2008). The immobilization of lipase includes several methods entrapment, encapsulation, cross-linking, adsorption and covalent bonding. Adsortion method of immobilization is widely used in recent years when compared to covalent bond, entrapment and cross-linking (Jegannathan et al., 2008). In the biological production of biodiesel the methanol inactivates the the lipase, hence the immobilization method is an advantage for the biodiesel production (Shimada et al., 2002). The low cost of lipase, candida spp. as origin is of more industrial use (Tan et al., 2010). The nanostructured carriers are with high porosity (Sakai et al., 2010), natural material activated carbon (Ji et al., 2010), celite, zeolite (Yagiz, Kazan and Nilgun Akin, 2007). The carriers for lipase immobilization by covalent attachment of olive pomace (Yucel, 2011), resins (Mendes et al., 2011), Polyurethane foam (Dizge, Keskinler and Tanriseven, 2009), chitosan (Shao et al., 2008), silica (Lee et al., 2008) and magnetic nanostructures (Dussan et al., 2010), When compared to the natural support material chitosan is used for enzyme binding, the immobilized lipase retains its stability for 10 cycles of pomace oil, esterification, while maintaining 80% residual activity.

2.6.4.4.2 Wastewater treatment

The increasing consumption of fresh water and water bodies are mixed up with polluted industrial wastewater and the wastewater treatments are necessary at present. The sources of dye effluents are textile industry, paper industry, leather industry and the effluents are rich in dye colorants. These effluents are threat to the environment and even in low concentration it is carcinogenic. Nowadays enzymes are used to degrade the dye stuffs. The enzymes used in the wastewater treatments are preoxidases, laccase, azo reductases. These enzymes due to harsh conditions like extreme temperature, low or high pH and high ionic strength may lose its activity; to overcome this problem immobilized enzymes are used. The horseradish peroxidases are entrapped in calcium alginate beads, this method is still in lab scale

research (Omar, 2008). The immobilized laccase enzyme has the ability to degrade dyes anthracinoid dye, Lancet blue and Ponceau red (Wu et al., 1998). Adsorption method is widely used because of its easy regeneration. During the covalent method of immobilization the conformational change in the enzyme occurs which will affect the activity of the enzyme (Watlington, 2005). In Single Enzyme Nanoparticle, the enzyme is protected by a nanometer thick substance as it provides the large surface area. SEN has the ability to retain its activity during the extreme conditions. SEN is also used in the removal of heavy metals from the waste water (Rinzema et al., 1994).

Lipase has the ability to hydrolyse oil and fats to long chain fatty acid and glycerol. The immobilized lipase is of high interest for the hydrolysis of oils and fats for treating the wastewater from the food industry. The drawback of the conventional treatment methods is slow biodegradability, oil and fats are absorbed on the surface of sludge. Researchers are now focusing on the treatment with immobilized lipase. Lipase immobilized on the sol gel / calcium alginate with the size of 82 μm, immobilized lipase. Immobilized lipase operated for 100 days in continuous sludge without any problem, does not produced foam in the reactor (Jeganathan and Nakhla, 2007).

2.6.4.4.3 Textile industry

The enzymes derived from microbial origin are of great interest in textile industry. The enzymes such as cellulase, amylase, liccase, pectinase, cutinase etc and these are used for various textile applications such as scouring bio-polishing, desizing, denim finishing, treating wools etc. Among these enzymes cellulase has been widely used from the older period to till now. The textile industries now turned to enzyme process instead of using harsh chemical which affects the pollution and cause damage to the fabrics (Araujo, Casal and Cavaco-Paulo, 2008). The processing of fabrics with enzymes requires high temperatures and increased pH, the free enzymes does not able to withstand the extreme conditions. Hence, enzyme immobilization for this process able to withstand at extreme and able to maintains its activity for more than 5-6 cycles. Poly Methyl Methacrylateis linked with cellulase covalently. In this method the nanoparticle is synthesized with cellulase as core

particle (Olsen and Falholt, 1998; Heikinheimo et al., 2000). Endoglucanase is a component of cellulase enzyme, endoglucanase is microencapsulated with Arabic Gum is a natural polymer with the biodegradable property is used as a matrix for encapsulation of endoglucanase. Encapsulation of endoglucanase prevented it to retain its activity in the presence of detergents (Ramakrishnan et al., 2007).

2.6.4.4.4 Detergent industry

The detergent industry also employs enzymes for removal of stains. The enzymes used in detergent industry are protease which is used to remove the stains of blood, egg, grass and human sweat. Amylase used to remove the starch based stains like potatoes, gravies, chocolate. Lipase used to remove the stains of oil and fats and also used to remove the stains in cuffs and collars. Cellulase is used for cotton based fabrics in order to improve softening, colour brightening and to remove soil stains (Koch-Schmidt and Mosbach, 1997). Nowadays Biotech cleaning agents are widely used in the detergent industries. When compared to synthetic detergents the biobased detergents have good cleaning property (Hasan et al., 2010).

The enzymes based detergents can be used in low quantity when compared to the synthetic detergent, it has increased biodegradability, does not affect the environment works well in low temperature, and these are the advantages of enzymes in detergent industry. The immobilized enzymes are also used in immobilized enzymes. Proteases hydrolyse the proteins, and protease cannot be used for keratin based fabric wool and silk which cause adverse damage to the garment. So protease directly cannot be used for wool and silk garments, protease loses its stability in the presence of surfactants and oxidizing agents, hence protease is immobilized by covalently linking with Eudragit S-100 using carbodiimide coupling. The immobilized protease treated with wool for 72 h with 100U at 40 °C the free enzyme was degraded the wool but the immobilized enzyme retained 76% of the tensile strength of wool (Silva, Gubitz and Cavaco-Paulo, 2006). Protease also immobilized by entrapment method with polyacryamide gel, the enzyme retained its activity for about 6 cycles with incubation time of 20 min at 55 °C for each cycles, by this immobilization method, protease activity is retained for about 83%of the initial

activity after 6 cycles (Vasconcelos et al., 2006). The lipase immobilization is carried in order to prevent lipase from protease action and surfactant inhibition lipase is immobilized on acrylamine glass beads coated with zirconia with the size of 55nm. The maximum activity was found for immobilized lipase at the pH of 6.5 and for free enzyme it is 7.5 (Najafi, Deobagkar and Deobagkar, 2005).

CHAPTER 3

MATERIALS AND METHODS

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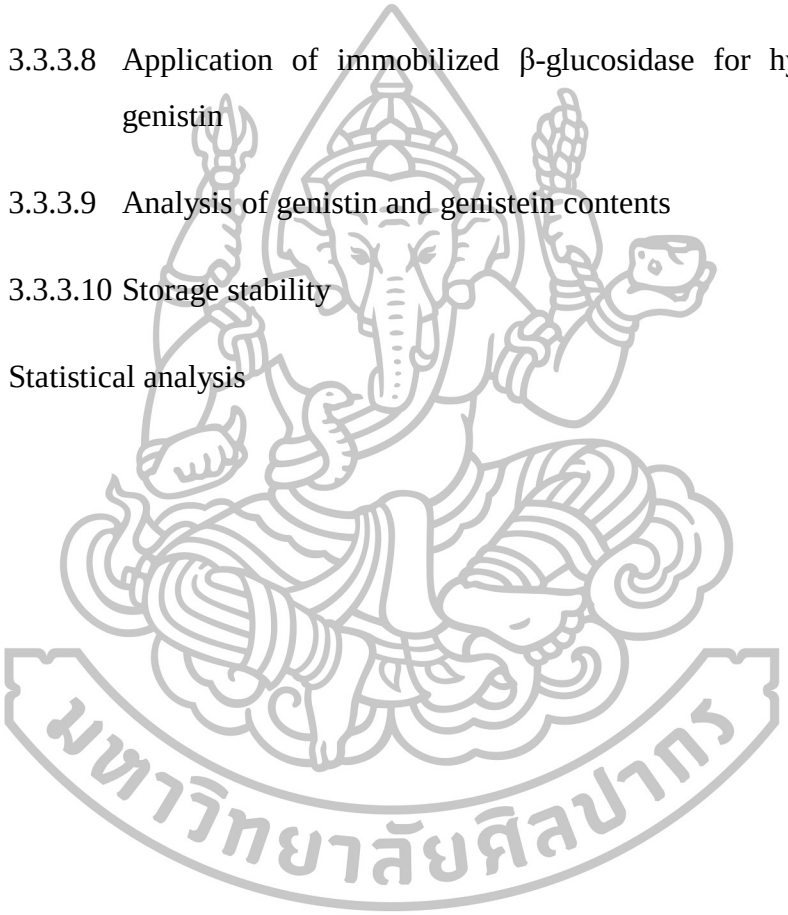

3.1 Materials

Bovine serum albumin (Sigma-Aldrich ®, St. Louis, MO, USA)

Chitosan (375 kDa, >75% degree of deacetylation (Sigma-Aldrich ®, St. Louis, MO, USA)

Commercial β-glucosidase from almonds (*Prunus dulcis*) (Sigma-Aldrich ®, St. Louis, MO, USA)

Commercial cellulase from *Aspergillus niger* (Sigma-Aldrich ®, St. Louis, MO, USA)

Dry fruit of *Gardenia jasminoides* Ellis (Local market, Nakhon Pathom, Thailand)

Gabapentin capsules (Local drug store, Thailand)

Gabapentin related compound A, B D and E (USP, Rock-ville, MD)

Genipin (Challenge Bioproducts Co., Ltd., Taichung, Taiwan)

Geniposide (Shaanxi Taiji Huqing Technology Co., Ltd., Shaanxi, China)

Genistein (Shaanxi Taiji Huqing Technology Co., Ltd., Shaanxi, China)

Genistin (Shaanxi Taiji Huqing Technology Co., Ltd., Shaanxi, China)

Glutaraldehyde solution (50%, Sigma-Aldrich ®, St. Louis, MO, USA)

Nitrophenol (Sigma-Aldrich ®, St. Louis, MO, USA)

Nitrophenyl β-D-glucopyranoside (Sigma-Aldrich ®, St. Louis, MO, USA)

Reference standard gabapentin (USP, Rock-ville, MD)

Sodium tripolyphosphate (Sigma-Aldrich ®, St. Louis, MO, USA)

All other chemicals

Acetic acid (RCI Labscan Limited, Bangkok, Thailand)

Acetone (RCI Labscan Limited, Bangkok, Thailand)

Acetonitrile HPLC grade (RCI Labscan Limited, Bangkok, Thailand)

Calcium sulfate (Fischer Scientific, UK Limited, UK)

Citric acid (Sigma-Aldrich ®, St. Louis, MO, USA)

Ethanol (RCI Labscan Limited, Bangkok, Thailand)

Ethyl acetate (RCI Labscan Limited, Bangkok, Thailand)

Hydrochloric acid (RCI Labscan Limited, Bangkok, Thailand)

Methanol (RCI Labscan Limited, Bangkok, Thailand)

Phosphoric acid (Fischer Scientific, UK Limited, UK)

Potassium hydrogen phosphate (Fischer Scientific, UK Limited, UK)

Potassium hydrogen phthalate (Fischer Scientific, UK Limited, UK)

Sodium acetate (Fischer Scientific, UK Limited, UK)

Sodium carbonate (Fischer Scientific, UK Limited, UK)

Sodium citrate (Sigma-Aldrich ®, St. Louis, MO, USA)

Sodium dihydrogen phosphate (Ajax Finechem Australia, New Zealand)

Sodium hydroxide pellet (Ajax Finechem Australia, New Zealand)

Triethanolamine (Ajax Finechem Australia, New Zealand)

Tris(hydroxymethyl)aminomethane (Fischer Scientific, UK Limited, UK)

Trichloroacetic acid (Sigma-Aldrich ®, St. Louis, MO, USA)

3.2 Equipment

1.5 mL microcentrifuge tube (Eppendorf®, Corning Incorporated, NY, USA)

2.0 mL microcentrifuge tube (Eppendorf®, Corning Incorporated, NY, USA)

15 mL centrifuge tube (Biologix Research Company, KS, USA)

50 mL centrifuge tube (Biologix Research Company, KS, USA)

Analytical Balance (Sartorious CP224S and CP3202S; Scientific Promotion Co., Ltd., Bangkok, Thailand)

Beaker 50, 100, 250, 500, 1000 and 5,000 mL (Pyrex®, USA)

C_{18} column (Luna 5u, Phenomenex®, CA, USA)

Centrifuge (Hermle Z300K: Labnet; Lab Focus Co., Ltd., Bangkok, Thailand)

Digital dry block heaters (Bang Trading, Bangkok, Thailand)

High performance liquid chromatography (HPLC) instrument (Agilent® 1100 series, Agilent Technologies, USA)

Image analysis software (JMicrovision V.1.2.7, Switzerland)

Magnetic bar

Magnetic stirrer (Framo, Germany)

Micropipette 2-20 µL (Finnpipette®, Thermo Fisher Scientific, Finland)

Micropipette 20-200 µL (Finnpipette®, Thermo Fisher Scientific, Finland)

Micropipette 100-1000 µL (Finnpipette®, Thermo Fisher Scientific, Finland)

Micropipette 0.5-5 mL (Finnpipette®, Thermo Fisher Scientific, Finland)

Micropipette tip (Bang Trading, Bangkok, Thailand)

pH meter (Horiba compact pH meter B-212, Japan)

Sartorious membrane filter 0.45 µm

Scanning electron microscope (SEM, Camscan MX-2000, UK)

Shaking incubator (Shel Lab, Model: SI4, Gibthai Co., Ltd., Bangkok, Thailand)

SPSS version 16.0 for Windows (SPSS Inc., Chicago, IL)

UV-visible spectrophotometer with diode-array detector (Agilent, USA)

Quartz cuvette (Bang Trading, Bangkok, Thailand)

Volumetric flask 50 and 100 mL (Pyrex®, USA)

Vortex mixer (Model: Labnet, USA)

Water bath (Hetofrig CB60; Heto High Technology of Scandinevia, Birkerod, Denmark)

Whatman filter paper No.1 (GE Healthcare UK Limited, UK)

Microscopy (DinoCapture, Naarden, Netherlands)

3.3 Method

3.3.1 Improvement of genipin production process by using simultaneous enzyme-assisted extraction and cleavage of glucose from glycoside combined with in situ product separation

3.3.1.1 Enzyme-assisted extraction and optimization

Dry gardenia fruit was finely ground and sieved through 150 mesh. The resulting powder was then dried at 50 °C for 12 h and kept in a desiccator until further used. To perform the enzyme-assisted extraction and to investigate the optimal conditions, cellulose was prepared as the solutions in 50 mM citric acid-sodium hydroxide buffer with different pH (3.0, 3.5, 4.0, 4.5 and 5.0) at various cellulase concentrations (2.5, 5, 10 and 50 mg/mL). Dry plant powder was weighed about 100 mg and placed in a 2-mL microcentrifuge tube. Then, 1.5 mL of cellulase solution was added by using micropipette (Finnpipette, Finland). The reaction was shaken on a shaking incubator (Shel Lab, Model: SI4, Bangkok, Thailand) at 300 rpm for a period of time (12, 18, 24, 30 and 36 h) at 50 °C. After the treatment, the suspension was centrifuged (Hermle Z300K, Bangkok, Thailand) at 14,500 × g for 20 min to sediment the plant debris. The liquid phase was transferred to a new microcentrifuge tube and 1/10 × volume of 50% trichloroacetic acid was added to stop the enzymatic reaction. The sample solution was centrifuged to precipitate the residual enzyme and then filtered through the membrane filter (0.45 μm pore size) before analyzed by high performance liquid chromatography (HPLC). All the experiments were performed in triplicate.

3.3.1.2 In situ product separation in two-phase system

After the optimal conditions for enzymatic reaction were established, in situ product separation by using two-phase system was incorporated into the production process. Via this strategy, the enzymatic treatment was done in the same way as described in Section 3.3.1.1 using the optimal conditions, except that the quantity of starting plant materials was increased by 10 folds. In addition, 15 mL ethyl acetate was added on top of the 15 mL plant-enzyme slurry (water phase). According

to the extraction of genipin product from the water phase, ethyl acetate (organic phase) was added along with the undergoing enzymatic extraction and biotransformation. For the comparison, the reactions were performed with three different methods including in situ product separation without cellulase treatment (T1), cellulase treatment without in situ product separation (T2) and in situ product separation combined with cellulase treatment (T3). Once completed, the reaction was centrifuged (Hermle Z300K, Bangkok, Thailand) at 14,500 × g for 20 min to sediment the plant particles and to separate the liquids into two phases. Then, all of upper organic phase was collected, evaporated, re-dissolved with 45% methanol and analyzed for the contents. For the water phase, the solution was transferred to a 1.5 mL microcentrifuge tube, and 1/10 × volume of 50% trichloroacetic acid was added to stop the reaction. The samples were centrifuged and filtered through 0.45 μm membrane filter prior to analysis for the contents by HPLC.

3.3.1.3 Analysis of geniposide and genipin content

The HPLC analysis for the content of geniposide and genipin was carried out by using the system consisting of an Agilent 1100 series equipped with a diode array detector (Agilent, USA) and Luna 5u C_{18} column (250 mm × 4.6 mm, 5 μm). The chromatographic conditions followed the method by Xu (2008) (Xu et al., 2008). The retention times of geniposide and genipin were 4.3 and 6.3 min, respectively.

3.3.2 Application of genipin for colorimetric assay of gabapentin bulk drug and capsules

3.3.2.1 Preparation of gabapentin standard solution

The reference standard of gabapentin was accurately weighed about 0.085 g then transferred into a 20 mL volumetric flask and dissolved with distilled water to prepare 25 mM standard gabapentin solution. After that, 0.4 mL of the resultant solution was diluted with distilled water in a new 20 mL volumetric flask to obtain a final concentration of 0.5 mM which was used for the preparation of standard calibration curve.

3.3.2.2 Preparation of sample solutions from gabapentin bulk drug and capsules

To assay the raw material, bulk drug was prepared as a working solution containing 0.5 mM gabapentin by following the same procedure that was used to prepare the standard solution as described in section 3.3.2.1. For the capsules formulation, the contents of twenty capsules and powder equivalent to 0.085 g gabapentin were accurately weighed and transferred into a 20 mL volumetric flask. About 15 mL of water was added and the content was sonicated for 10 min to obtain the complete dissolution. The mixture was then added up to 20 mL with distilled water and filtered through 0.45 µm membrane filter, discarding the first portion of the filtrate. Further dilutions were made to give a nominal concentration of 0.5 mM gabapentin which was ready for the analysis.

3.3.2.3 Preparation of genipin cocktail solution

Genipin solution was prepared as a cocktail with buffer solution. This was done by adding 0.009 g of genipin and 10 mL of 100 mM potassium phosphate buffer, pH 7.0 into a 20 mL volumetric flask. Distilled water was used to dissolve the content and made up to the volume, resulting in the stock cocktail composed of 2 mM genipin in 50 mM potassium phosphate buffer, pH 7.0.

3.3.2.4 Assay procedure and optimization

Different amounts (180, 240, 360, 480 and 600 µL) of 0.5 mM standard gabapentin solution or 420 µl of sample solution were accurately transferred into a series of 1.5 mL microcentrifuge tubes. Distilled water in different volumes was precisely added into each to bring the total volume of 600 µL. This resulted in a set of standard gabapentin solution with the concentration of 0.15, 0.20, 0.30, 0.40, and 0.50 mM and the sample solution with the concentration of 0.35 mM. Subsequently, 600 µL of genipin cocktail was accurately pipetted into each tube. All tightly closed tubes were placed in a water bath and heated at 80 °C for 60 min. After the reaction was completed, the tubes were cooled by plugging in an ice bath for 5 min and then it was left at room temperature for 5 min. The absorbance of the solutions was read at 590

nm. The reagent blank composed of 600 µL of distilled water and 600 µl of genipin cocktail and run by the same assay procedure was also prepared and its absorbance was used to subtract the absorbance value obtained from the reactions which contained gabapentin. A standard curve was constructed by plotting the blank-corrected absorbance values versus gabapentin concentration. The concentration of the unknown sample was computed from the regression equation derived from the Beer's law data.

The experimental parameters that could effect to the formation of the blue products (genipin-gabapentin complex) including pH, genipin concentration, heating temperature and heating time were optimized independently. These studies aimed to obtain the most efficient, economical and time-saving assay.

3.3.2.4.1 Optimal pH

The pH influencing on the formation of the blue products was studied by setting up the reaction in various buffer solutions i.e. potassium hydrogen phthalate-HCl (pH 4.0), potassium hydrogen phthalate-NaOH (pH 5.0), potassium hydrogen phosphate-NaOH (pH 6.0 and 7.0), and Tris-HCl (pH 8.0 and 9.0). The protocol for determining the content of gabapentin was the same as mentioned above.

3.3.2.4.2 Optimal genipin concentration

The optimal concentration of genipin was examined by using the varied concentrations of genipin (1, 2, 4, 6 and 8 mM) for the construction of standard curves over the gabapentin concentration range of 0.2-0.5 mM. The protocol for determining the content of gabapentin was the same as mentioned above.

3.3.2.4.3 Optimal heating temperature

The different temperatures, 60, 70, 80 and 90 °C were varied while the heating time was fixed at 60 min to investigate the optimum heating temperature. The protocol for determining the content of gabapentin was the same as mentioned above.

3.3.2.4.4 Optimal heating time

The various heating times ranging from 30-90 min were investigated for the effect of heating time on the formation of genipin-gabapentin complex. The heating temperature was fixed at 80 °C. The protocol for determining the content of gabapentin was the same as mentioned above.

3.3.2.5 Method validation and comparison to the standard method

3.3.2.5.1 Specificity

To investigate the specificity of the proposed method, the interferences from the gabapentin related compounds specified in the USP including Compound A, B, D and E (Figure 3.1) as well as the excipients commonly used in capsule i.e. lactose, microcrystalline cellulose and colloidal silicon dioxide (Figure 3.2) were studied and the recovery percentage was determined**.**

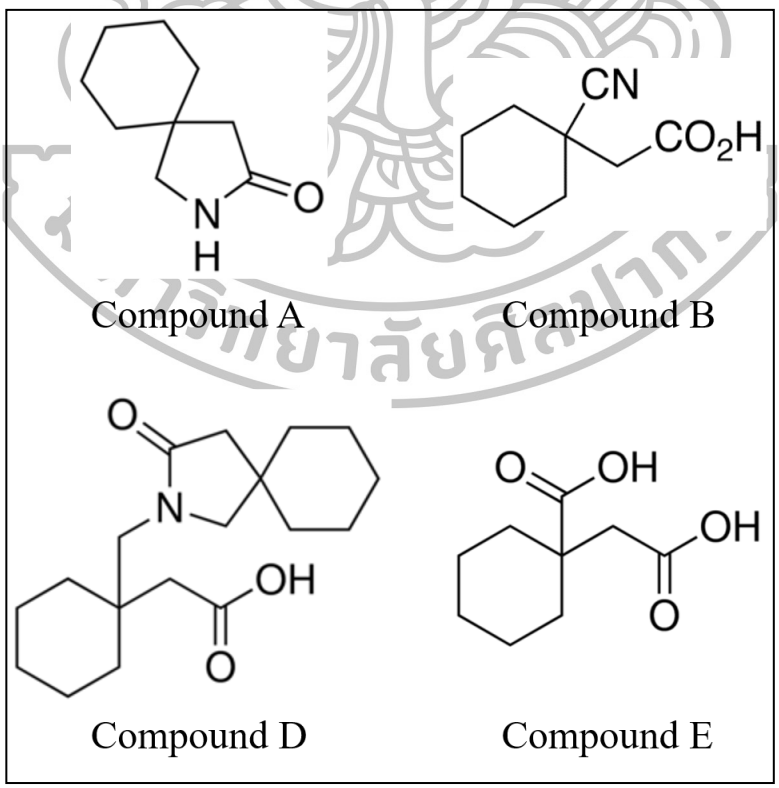

Figure 3.1 Gabapentin retated compounds.

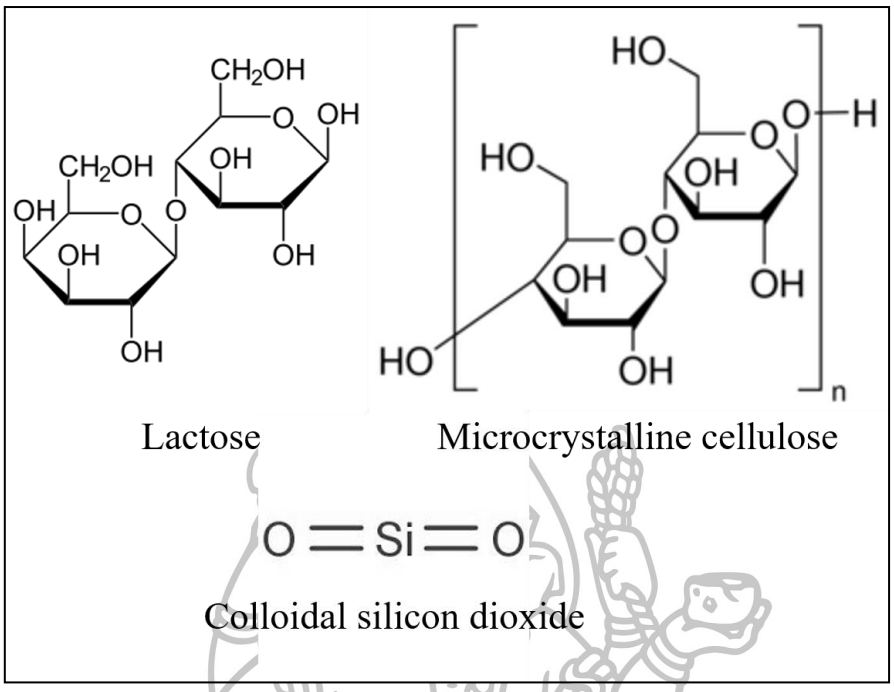

Figure 3.2 Excipients commonly used in capsules.

3.3.2.5.2 Accuracy

The accuracy of the proposed method was determined by spiking three different levels of standard gabapentin (0.15, 0.20, 0.25 mM) into the pre-analyzed drug solutions (0.15 mM) prepared from the capsules and determining the added concentration by the proposed method.

3.3.2.5.3 Precision

To determine the precision, repeatability (intra-days) and intermediate precision analysis (inter-days) were computed with % relative standard deviation (%RSD) for replicate samples (n = 3) using concentration of 0.35 mM. The repeatability and intermediate precision samples were calibrated with standard curve concurrently prepared in the same day of analysis.

3.3.2.5.4 Linearity and range

To establish the range of linearity, the standard gabapentin concentrations of 0.15-0.50 mM were used. Three replicates of sample were measured.

3.3.2.5.5 Limit of detection and quantitation

The limits of detection (LOD) and limit of quantitation (LOQ) were calculated from the standard calibration curve by using the following equations;

$$LOD = 3.3\ \sigma / S \quad \text{(Eq. 1)}$$

$$LOQ = 10\ \sigma / S \quad \text{(Eq. 2)}$$

Where; σ is the standard deviation of Y-intercept from linear regression.

S is the slope of the standard calibration plot.

3.3.2.6 Procedure for the treatment of laboratory waste

After the analytical process was completed, the waste was treated with five different absorbents available in laboratory including gypsum ($CaSO_4.H_2O$), charcoal, chitosan, palm fiber and $CaCO_3$ and then the efficiency of adsorbents was evaluated by measured the residual absorbance of wasted solution after pretreatment process.

The treatment of whole waste generated from the assay was carried out in a 50 mL conical tube by adding 0.5 g of adsorbent into 25 mL of waste solution (ratio of 1:50). The tube was vortexed for 2 min at the ambient temperature and the mixture was then filtered through a Whatman filter paper No.1. Since the pH of the solution decreased after the adsorption step, the filtrate obtained was neutralized with sodium hydroxide solution to pH 7.0 and the mixture was finally filtered to obtain a clear, colorless and neutral filtrate which was considered as the treated waste.

3.3.2.7 Acute toxicity assessment of treated laboratory waste

The waste after the treatment was evaluated for the acute toxicity to the two aquatic orgamisms namely brine shrimp (*Artemia salina*) and male, adult guppies (*Poecilia reticulate*). Prior to the test, brine shrimp cysts were hatched for 24-36 h in 35 ppt salinity artificial sea water which was well aerated at room temperature (25 ^oC). The treated waste was diluted with artificial sea water where the final salinity of

all test solutions was adjusted to 35 ppt. (for test on brine shrimp) or distilled water (for test on guppies) to different concentrations i.e. 0 (control), 6.25, 12.5, 25, 50 and 100% by volume of waste water. At test initiation, ten test organisms were randomly added to each replicate test chamber (n=2). The tests on brine shrimp were conducted under static conditions in 6 well polystyrene plates. In each well, 5 mL of waste solutions with different concentrations were added. The test on guppies was carried out in 1 L beakers containing 500 mL of waste solutions. The organisms were not fed during the test. After the exposure period of 24 h, the test organisms were observed and the number of live organisms per replicate was recorded. The test results were acceptable if control survival equaled to or exceeded 90%. The data analysis consisted of transforming the observed percentage mortalities with a probit transformation and transforming the waste water concentrations to log 10. The mean lethal concentration which caused 50% mortality of the test organisms (LC50) was determined at the probit value of 5 and the acute toxic unit (TUa) defined as the following equation;

$$TUa = 100 / LC50 \quad \text{(Eq. 3)}$$

Where; TUa is acute toxic unit.

The criterion of toxicity is based on the Aquatic Toxicity Testing Guideline for Waste Effluents: TUa value < 3 (non-toxic), 3-10 (slightly toxic), 10-50 (toxic), 50-100 (very toxic) and > 100 (extremely toxic). (Shannon Aquatic Toxicity Laboratory. Aquatic Toxicity Testing in Ireland (online). Available from: URL: /http://www.envirocentre.ie/includes/documents/Aquatic%20Toxicity%20Testing%20in%20Ireland%202011%20ver%205.pdfS, 2011 (accessed on 08.03,17).

3.3.3 Application of genipin as cross-linking agent for immobilization of β-glucosidase on chitosan beads

3.3.3.1 Preparation of chitosan beads

2% w/v chitosan solution was prepared by dissolving 0.2 g of chitosan powder in 10 ml of 1.5% v/v acetic acid solution and stirred overnight. The viscous solution was manually dropped through a syringe, at a constant rate, into 1% w/v

sodium tripolyphosphate (TPP) solution to solidified bead form. The prepared beads were washed with deionized water until the chitosan beads was neutral. The chitosan beads were kept in 2-8 °C until further used.

3.3.3.2 Preparation of cross-linked and immobilized chitosan beads

Enzyme immobilization on chitosan beads can be prepared by three different ways. The first method was based on the initial pre-activation of chitosan beads followed by the enzyme immobilization. In the second procedure, beads were firstly mixed with enzyme and then genipin was added. For the last mean, beads, enzyme and genipin were simultaneously mixed together. To prepare the pre-activation and enzyme immobilized beads, genipin at the various concentrations (0.1, 0.5 and 1.0%) was chosen as a cross-linking agent.

For the first type of beads, five chitosan beads were incubated with 1 mL of 50 mM phosphate buffer (pH 7.0) containing genipin and placed in a 2 mL microcentrifuge tube. The solution was shaken at 200 rpm and 40 °C for 3 h. After pre-activation process, the cross-linked chitosan beads were immobilized with 0.5 mg/mL β-glucosidase and shaken at 200 rpm and 40 °C at the different times (3, 6, 12, and 18 h) (Figure 3.3(a)).

For the second type of beads, five chitosan beads were firstly incubated with 0.5 mg/mL β-glucosidase at the different times (3, 6, 12, and 18 h). After that, five immobilized beads were cross-linked with genipin and shaken at 200 rpm and 40 °C for 3 h (Figure 3.3(b)).

For the third type of beads, immobilized chitosan beads were prepared by simultaneously incubating five chitosan beads with 0.5 mg/mL β-glucosidase and genipin (0.1, 0.5 and 1.0%), then it was shaken at 200 rpm and 40 °C for a period of time (3, 6, 12, and 18 h) (Figure 3.3(c)).

For the comparison, a common cross-linking agent i.e. glutaraldehyde was used in the process instead of genipin. Similarly, immobilized chitosan beads were prepared by incubated five chitosan beads with 1 ml of 50 mM phosphate buffer

(pH 7.0) containing glutaraldehyde at different concentrations (1, 2.5 and 5 %) were placed in a 2 mL microcentrifuge tube. Subsequently, it was shaken at 200 rpm and 25 °C for 3 h for activation the chitosan polymer before immobilized with β-glucosidase. After activation process, the activated chitosan beads were immobilized with 0.5 mg/mL β-glucosidase at the various times (3, 6, 12, and 18 h) (Figure 3.3(d)).

In addition, the control chitosan beads were prepared by incubating chitosan beads with 0.5 mg/mL β-glucosidase at the various times (3, 6, 12, and 18 h) without cross-linking agent, namely adsorption bead.

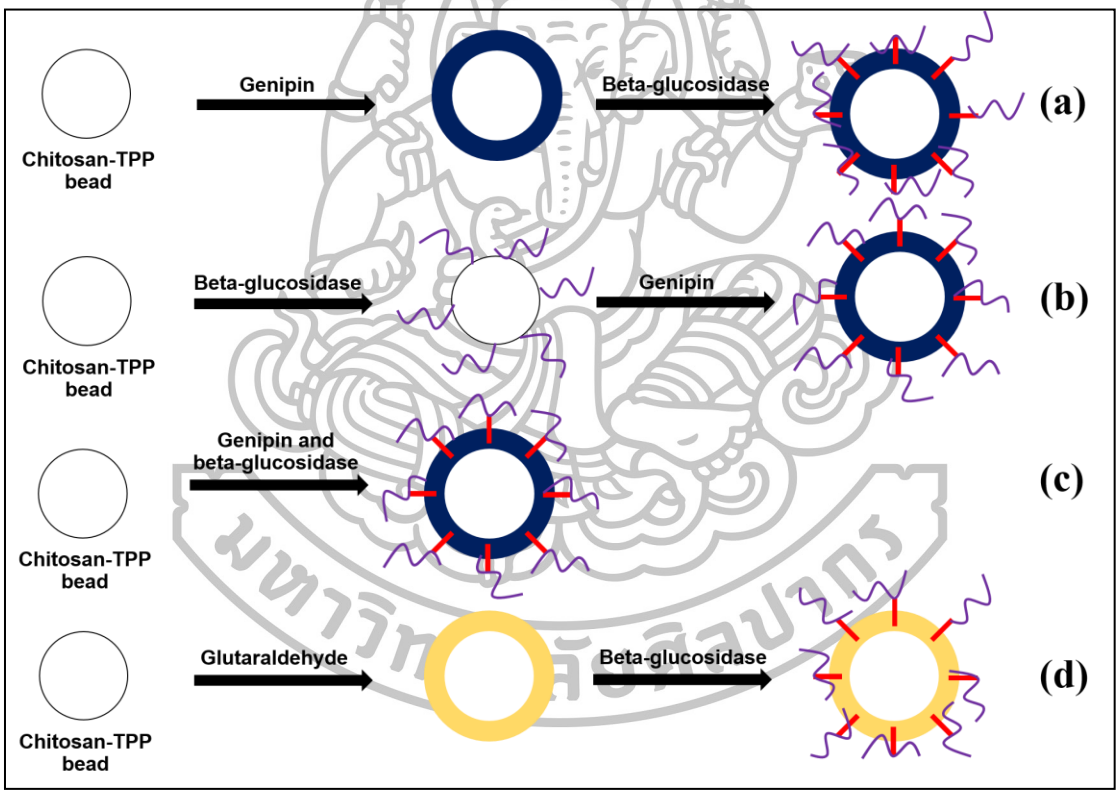

Figure 3.3 The preparation of β-glucosidase immobilized chitosan beads; the first type of beads (a), the second type of beads (b), the third type of beads (c) and the glutaraldehyde cross-linked chitosan beads (d).

3.3.3.3 Assay of β-glucosidase activity on immobilized chitosan beads and protein quantitation

The activity of β-glucosidase on chitosan beads were determined by testing with 2 mM p-NPG. When p-NPG was hydrolyzed by β-glucosidase, it released p-nitrophenol and glucose molecules. The reaction was performed for 5 min at the different temperature (40-80 °C) and pH (4.0-8.0) to investigate the optimal condition, subsequently, 500 µL of resulting solution was collected and the reaction was stopped with 500 µL of 0.5 M Na_2CO_3. It showed the yellow solution which has the maximum absorbance at 410 nm. The standard curve of p-nitrophenol over the concentration ranging from 20-200 µM was prepared. One β-glucosidase activity unit (U) is defined as the amount of enzyme needed to produce one µmol of nitrophenol per minute under the assay conditions. The immobilized protein content was determined by the following equation;

$$\text{Immobilization yield (\%)} = (P_0 - P) / P_0 \times 100 \quad \text{(Eq. 4)}$$

Where; P_0 is the protein content in the solution used for immobilization.
P is the remaining protein in the supernatant at the end of the immobilization procedure).

Bovine serum albumin (BSA) standard solution (5-40 µg/mL) was used as a standard protein and analyzed by Bradford method. The Bradford assay is based on the formation of a complex between the Coomassie Brilliant Blue G-250 dye and protein in solution. The protein dye complex causes a shift in the absorption maximum of the dye from 465 to 595 nm. The amount of absorption was proportional to the protein present.

3.3.3.4 Effect of pH and temperature on activity of immobilized chitosan beads

Generally, the pH and temperature of enzyme might be changed after immobilized on supporter. So, the effect of pH and temperature on activity of β-

glucosidase after immobilization process was investigated by varying the pH and temperature of reaction ranging from 4.0-8.0 and 40-80 °C, respectively.

3.3.3.5 Effect of pH and temperature on stability of immobilized chitosan beads

To investigate the pH stability of free and immobilized enzyme, the residual activity of immobilized chitosan beads was measured after the samples were incubated at different pH (4.0-8.0), and the temperature was fixed at 50 °C for 2 h. For the thermal stability, beads were incubated at various temperatures (40-80 °C) and the pH was fixed at 5.0 for 2 h. After that, the initial activity and residual activity of free and immobilized enzyme were measured at the optimal condition.

3.3.3.6 Physical evaluation of cross-linked and enzyme immobilized chitosan beads

The size of immobilized chitosan beads was determined in triplicate. Moreover, the morphology of chitosan beads was conducted by manual microscopy (DinoCapture) and JMicroVision software.

3.3.3.7 Reusability of immobilized chitosan beads

The immobilized chitosan beads were tested the reusability for 10 cycles by using 2 mM p-NPG as a substrate. In each cycle, the reaction was performed for 5 min at the optimal temperature and pH, subsequently, 500 µL of resulting solution was collected and the reaction was stopped with 500 µL of 0.5 M Na_2CO_3. It showed the yellow solution which has the maximum absorbance at 410 nm. After the reaction in each cycle was completed, the immobilized chitosan beads were washed with deionized water for three times prior submitting to the next cycle.

3.3.3.8 Application of immobilized β-glucosidase for hydrolysis of genistin

The genistin was accurately weighed about 0.010 g into a 100 mL volumetric flask and then dissolved and made up to the volume with the mixture

solution consisting of 5% dimethyl sulfoxide and 50 mM buffer solution at the optimal pH, resulting in the stock solution of genistin 100 µg/mL. In each cycle, 1.0 mL of genistin solution and five immobilized chitosan beads were incubated at the optimal temperature and pH for 2 h. After the reaction was completed, 900 µL of the solution was transferred to a 1.5 mL microcentrifuge tube and 100 µL of 50% trichloroacetic acid was added to stop the reaction. Finally, the resulting solution was filtered through a syringe filter prior analysis the genistin and genistein contents by HPLC.

The immobilized chitosan beads were also tested the reusability for 5 cycles by using 100 µg/mL genistin as a substrate. In each cycle, the reaction was performed for 2 h at the optimal temperature and pH. After the reaction was completed, 900 µL of the solution was transferred to a 1.5 mL microcentrifuge tube and 100 µL of 50% trichloroacetic acid was added to stop the reaction. Finally, the resulting solution was filtered through a syringe filter prior analysis the genistin and genistein contents by HPLC. After the reaction in each cycle was completed, the immobilized chitosan beads were washed with deionized water for three times prior submitting to the next cycle.

3.3.3.9 Analysis of genistin and genistein contents

The HPLC assays for the content of genistin and genistein were carried out by using the system consisting of an Agilent 1100 Serie equipped with a diode array detector and Luna 5u C18 column (250 mm × 4.6 mm, I.D.; 5 µm particle size), The chromatographic conditions followed the methods by Cesar (2006) (Cesar et al., 2006) with slightly change. The column temperature was maintained at 30 °C. UV detection was set at 254 nm and the injection volume was 20 µl. The mobile phase was composed of 0.1% acetic acid and methanol at the ratio of 40:60, at a flow rate of 1 ml/min.

3.3.3.10 Storage stability

The storage stability of immobilized enzyme was evaluated by determining the enzyme activity after storage at 4 °C for 30 days.

3.3.4 Statistical analysis

Data were presented as the means ± standard deviations (SD) for three experiments. The statistical significance was determined using one way analysis of variance (ANOVA). For the comparison of two experiments, student's t-test was used as statistical analysis. P values of < 0.05 were considered significant.

CHAPTER 4

RESULTS ANS DISCUSSION

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4.3.6 Application of immobilized beads for conversion of genistin to genistein

4.3.7 Storage stability of immobilized chitosan beads

4.1 Improvement of genipin production process by using simultaneous enzyme-assisted extraction and cleavage of glucose from glycoside combined with in situ product separation

In this work, enzyme-assisted extraction and in situ product separation were combined to develop a direct route for the production of genipin from dried gardenia fruit. Cellulase is less expensive than β-glucosidase (Zu et al., 2009) and it efficiently catalyzes the removal of sugar moiety from geniposide with satisfactory extent of above 90% in our preliminary experiments. Also, it can break down the plant cell wall. Thus, single cellulase was feasible for use and adequate for dual roles in one-pot reactor. Additionally, the fact that geniposide is likely to be released into the water phase due to the solubility and undergoes hydrolytic reaction which efficiently takes place in aqueous medium whereas genipin formed favorably distributing in the organic phase has offered the possibility for the in situ recovery of product by using two-phase extraction. For this purpose, various parameters such as enzyme concentration, pH of reaction and time of incubation were optimized to achieve the highest yield of genipin in parallel with the satisfactory performance in terms of economic aspect and eco-friendliness.

4.1.1 Optimization of the biocatalysis process

The experimental parameters affecting the enzymatic reaction and the productivity of genipin were optimized in order to obtain the most efficient (highest yield), economical (lowest cost) and time-saving process. These parameters included enzyme concentration, pH of reaction and incubation time. From the literatures, cellulase from *Aspergillus niger* has an optimum temperature at 50 °C (Sowbhagya and Chitra, 2010). Thus, this temperature was used in this study.

4.1.1.1 Effect of enzyme concentration

The effect of cellulase concentration on the extraction yield was examined by adding various amounts of enzyme into the biocatalytic reactions which were then allowed for 24 h and the results are shown in Figure 4.1. It was obvious that the concentrations of geniposide present in the reactions declined with the increasing

enzyme concentrations. It was likely that, albeit the higher release from plant materials upon the breakdown of cell walls, geniposide underwent further enzymatic hydrolysis at the higher extents when larger amounts of enzyme were employed. By this reason, the yields of genipin were thus found to increase when higher concentrations of enzyme were used. Without the aid of cellulase, genipin was extracted into the reaction medium at microgram level per one gram of dried plant. Beyond the enzyme concentration of 10 mg/mL, however, only a slight increment of genipin yield was gained. Considering in terms of yield and economic point of view, cellulase at the concentration of 10 mg/mL was therefore chosen for the subsequent optimization studies for other parameters i.e. pH and time of incubation and used in the standard protocol.

4.1.1.2 Effect of pH of reaction and incubation time

It has been reported that the activity of cellulase depends on pH (Fu, Liu ad Zu, 2008). According to the manufacturer's recommendation, the enzyme preparations are effective between pH 3.0 and 7.0 and the optimum pH generally lies between 4.0 and 5.0. Nevertheless, the most appropriate pH which gave the maximum extraction yield of genipin was needed to be investigated in our case since the reactor was complicated by side reaction occurring between genipin formed and the enzyme at certain pH to produce the unwanted blue products, leading to the loss of yield. Furthermore, genipin itself could be hydrolyzed in acidic condition (Yao, Wu and Wu, 2004). Accordingly, the proper operating pH must be deliberately selected to achieve both the high activity of the enzyme and the low degradation of the product. In this work, the effects of pH and of incubation time were simultaneously studied by keeping the constant enzyme concentration at 10 mg/mL as previously optimized. As shown in Figure 4.2, the high yields of genipin were generally obtained from the reactions having pH 3.5-4.5, depending on the incubation time used. The concentrations of geniposide tended to decrease while those of genipin raised with time for all pH tested due to the ongoing conversion of glycoside to aglycone. Since the maximum yield was achieved at about 35 mg genipin per gram of dried plant in the reaction set at pH 4.0 after 24 h of incubation, they were considered as the optimum pH and reaction time.

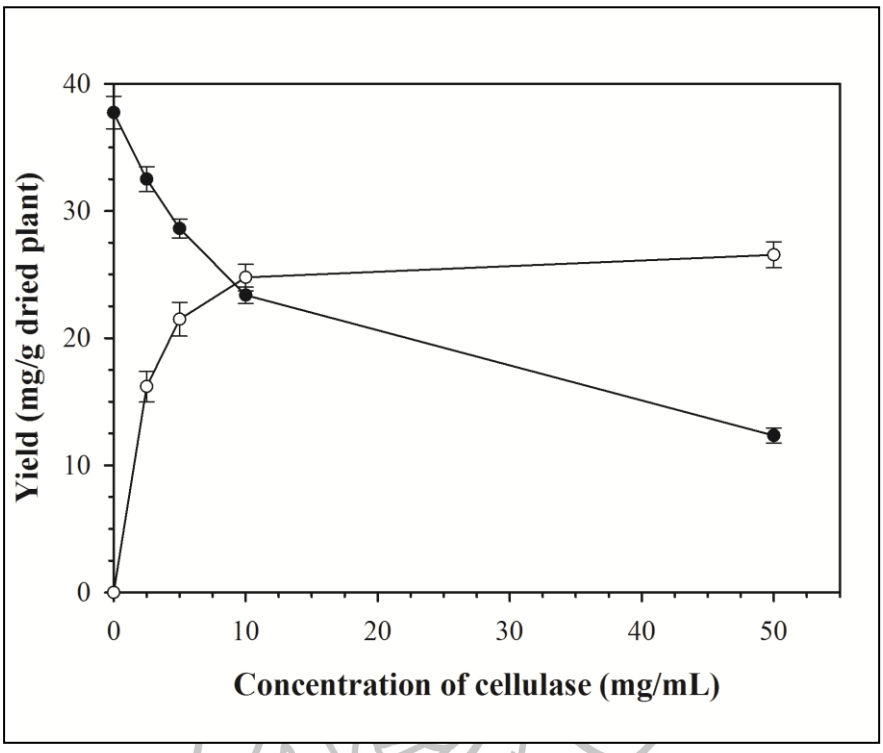

Figure 4.1 Effect of cellulase concentration on the yields of geniposide (●) and genipin (○). In this experiment, pH of reaction was 4.0, incubation time was 24 h and incubation temperature was 50 °C. (n=3).

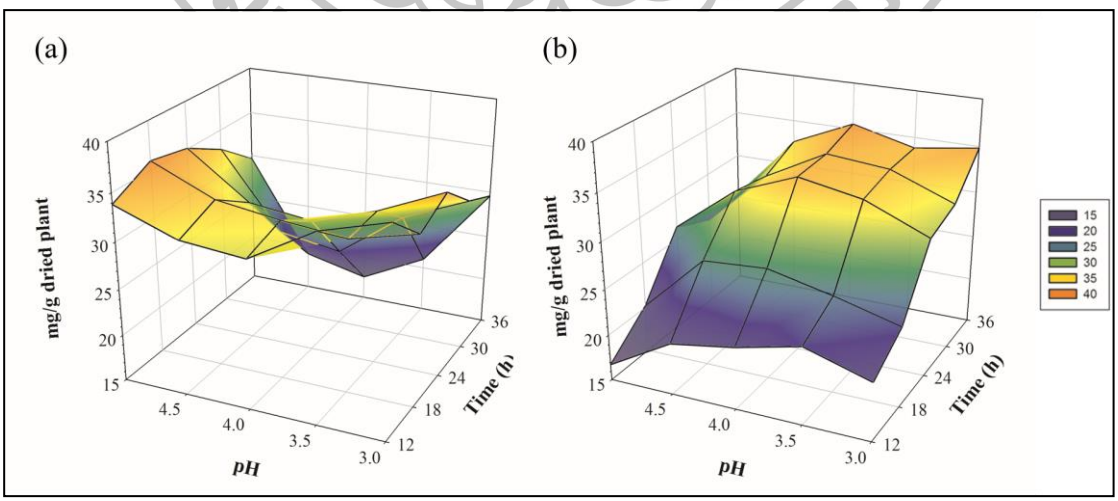

Figure 4.2 Effect of pH and incubation time on the yields of geniposide (a) and genipin (b). In this experiment, concentration of cellulase was 10 mg/mL and incubation temperature was 50 °C (n=3).

4.1.2 Microscopic observation of the effect of cellulase on plant materials

To demonstrate the effect of cellulase on the structure of plant cells, pulverized dried gardenia fruit powder treated with cellulase under the optimal catalytic condition was compared to that extracted in the similar manner, but without the enzyme. As seen in Figure 4.3(a), the cell walls of enzyme-free extracted plants showed no substantial eruption to the microstructure at 24 h. However, those of plants treated with cellulase for 12 h became thinner and the microstructure began to disorganize (Figure 4.3(b)). After the 24 h treatment, the cell walls were softened, highly disorganized and absolutely deformed as illustrated in Figure 4.3(c). From these figures, it exhibited that cellulase efficiently disrupted the cell walls of plant under the optimal condition, thereby facilitating the release of geniposide and genipin into the extraction medium.

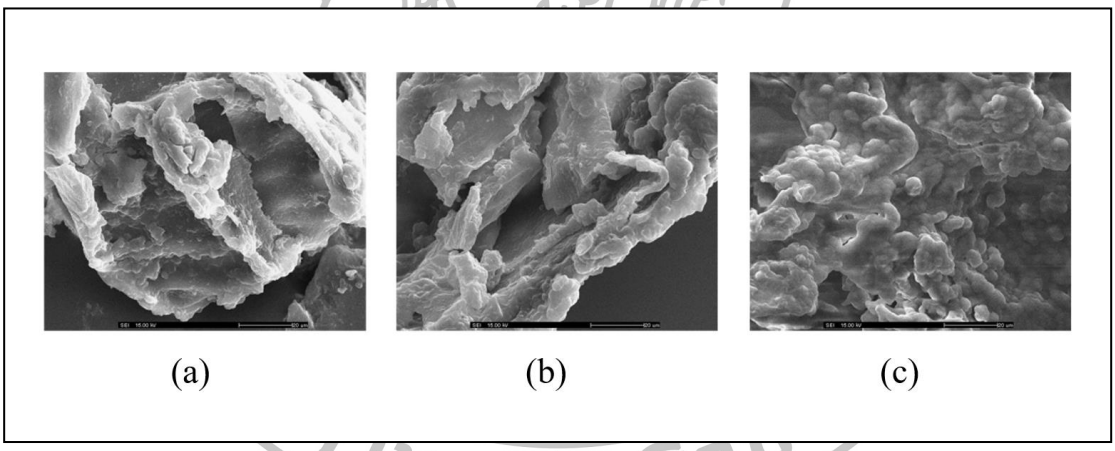

Figure 4.3 SEM images of gardenia fruit powder; without cellulase treatment (a), treated with cellulase for 12 h (b) and treated with cellulase for 24 h (c).

4.1.3 In situ product separation in two-phase system

After the optimized conditions for the enzyme assisted extraction were established, the two-phase in situ product separation was incorporated into the process. This facile but powerful technique offered the advantages in distracting the product from the degradation pathways which might occur via the cross-linking reaction of genipin with the enzyme and the hydrolysis of genipin itself. Furthermore, it helped partially purify the desired product. Although the similar approach has been used by Yang (2011) (Yang et al., 2011), pure geniposide, not crude dried fruit, was

the substrate in that study since the production method did not rely on the direct and simultaneous extraction and transformation. In addition, the enzyme β-glucosidase employed in that work was needed to be immobilized into alginate prior to use in the reactor, because of the incompatibility of enzyme and organic solvent (Yang et al., 2011). To achieve the high extraction efficiency in parallel with the green characteristic of the method, ethyl acetate which is regarded as one of the least toxic of industrial organic solvents and can be currently obtained from green ethanol was chosen in our study as the extraction solvent. The distribution of genipin in this solvent was 2.53 times higher than that in water phase. On the contrary, more hydrophilic geniposide preferred to dissolve in the aqueous medium, serving as the substrate for cellulase until it was converted to genipin. From the experiments, it was also found that the interfacial contact happening between the aqueous phase and ethyl acetate during the agitation of the reactor did not cause significant damage on the soluble enzyme or its activity (data not shown). As clearly seen in Figure 4.4, the yield of genipin (as determined from the total amount found in both phases) produced enzyme-assisted extraction combined with in-situ product separation (T3) was almost twice higher than that carried out by using the only enzyme-assisted extraction (T2). Also, this integrated route produced much better yield of genipin in one pot, in comparion with the procedures employing only the water/ethyl acetate extraction without the aid of cellulase (T1). Under the opimal condition for the combined strategies, a satisfactorily high yield at 58.83 mg of genipin was achieved starting from one gram of the dried plants. In the aspect of purity, the extractive in-situ product separation helped partially purify the product by the selective isolation of genipin into the organic phase. As seen in the HPLC chromatograms (Figure 4.5), the aqueous phase at the end of the reaction contained genipin co-existing with a variety of by-products, whereas the ethyl acetate phase consisted of a predominant amount of genipin with slight contamination of geniposide and other compounds. These results demonstrated the benefit of the in situ extraction in facilitating the subsequent purification process. Looking in a broader way, since many plant aglycones e.g. digitoxigenin, sapogenin and anthocyanidin, have been shown to possess various pharmacological activities and applications, this integrated approach should be an attractive alternative for the preparation of these compounds.

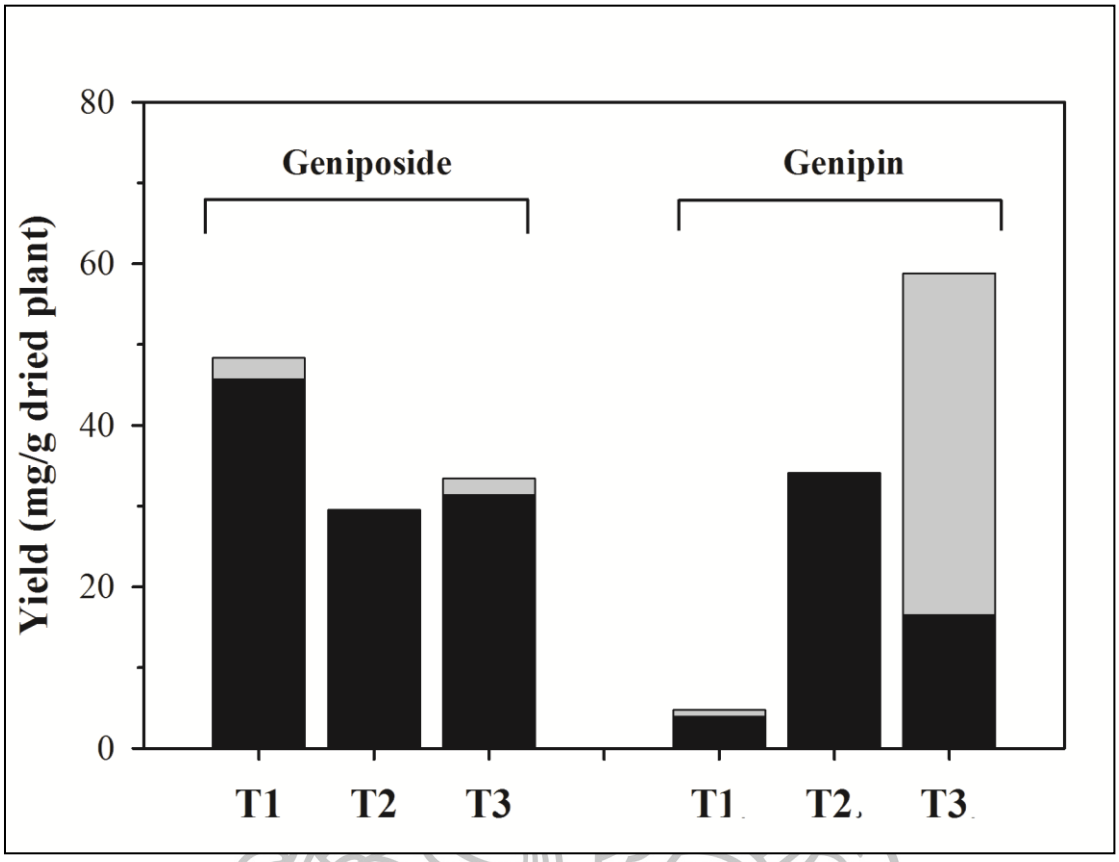

Figure 4.4 The yields of geniposide and genipin present in the aqueous phase (■) and organic phase (▭) from different treatments; in situ product separation without cellulase treatment (T1), cellulase treatment without in situ product separation (T2) and in situ product separation combined with cellulase treatment (T3) (n=3).

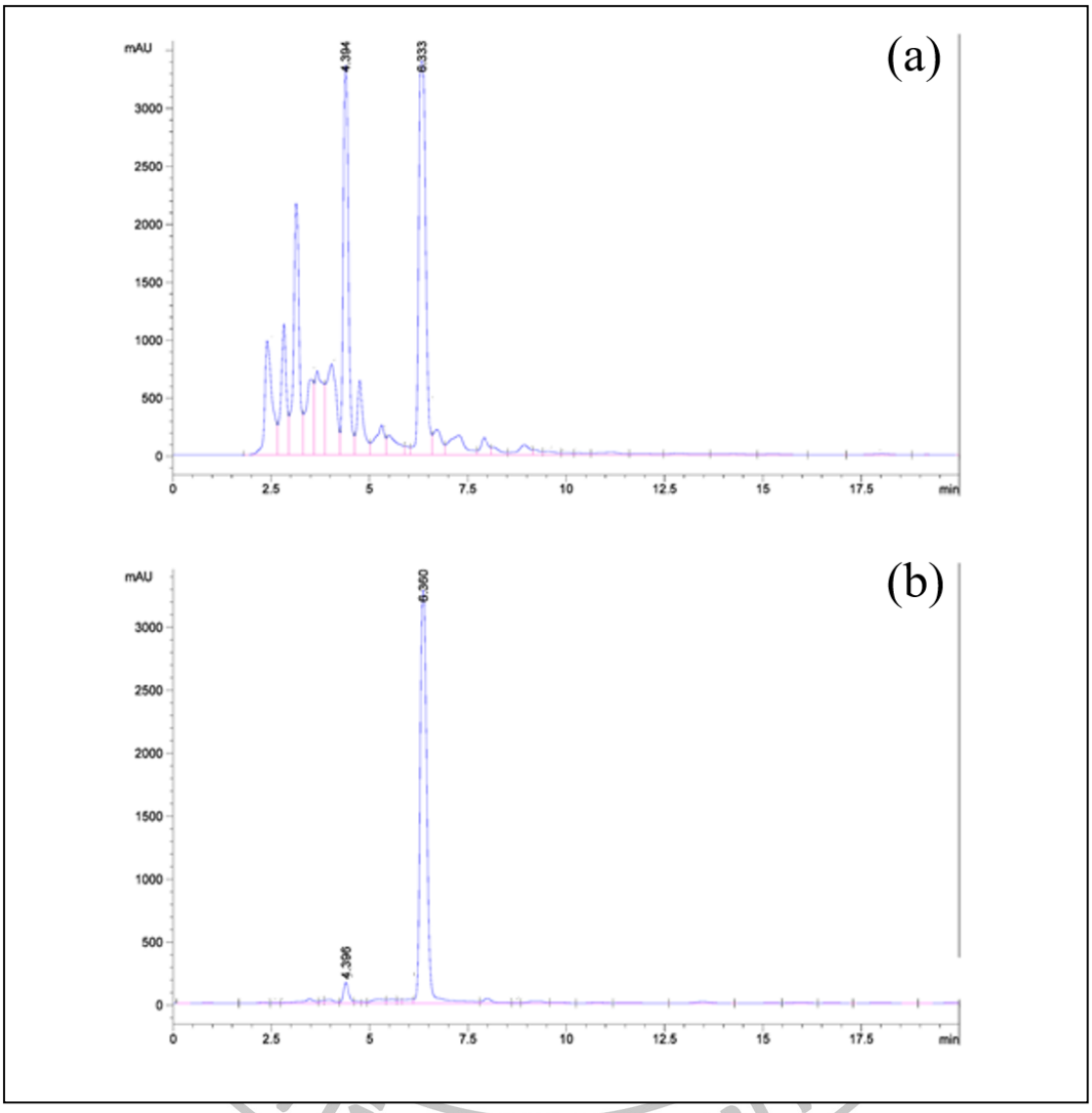

Figure 4.5 HPLC chromatograms representing the peaks of genipin (≈6.3 min), geniposide (≈4.4 min) and other compounds found in the aqueous phase (a) and ethyl acetate (b) after the 24 h-extractive reaction.

4.2 Application of genipin for colorimetric assay of gabapentin bulk drug and capsules

In this work, a novel assay for gabapentin in bulk drug and capsules has been proposed based on the colorimetric reaction with genipin. The development process started from the selection of naturally derived genipin as a colorimetric reagent owing to its ability to react with the primary amino group in gabapentin and form blue colored product (Figure 4.6). Moreover, the absorbance of single compound i.e. standard genipin or gabapentin did not interfere the absorbance of blue product (Figure 4.7). Unlike most other previous works which ended up once the satisfactorily accurate and reproducible methods were obtained; in this study a simple and facile treatment of waste generated from the assay was further proposed and the ecological effects of the treated waste was evaluated to confirm its safety on aquatic organisms. Therefore, this is the first report that not only presents the application of naturally derived genipin to drug analysis, but also fully illustrates the benign-by-design development of the analytical methodologies starting from the safe source of reagents toward the safe sink when the wastes from the assay are released into the environment.

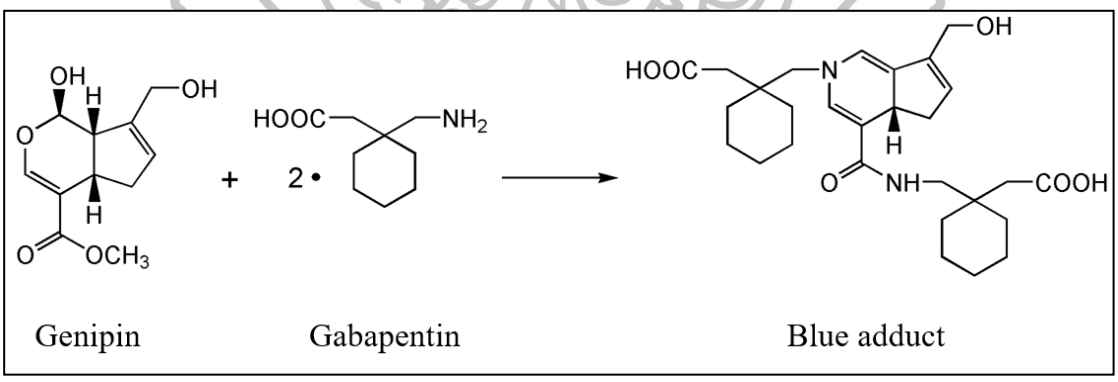

Figure 4.6 Proposed scheme for the formation of blue adduct from the reaction of gabapentin and genipin.

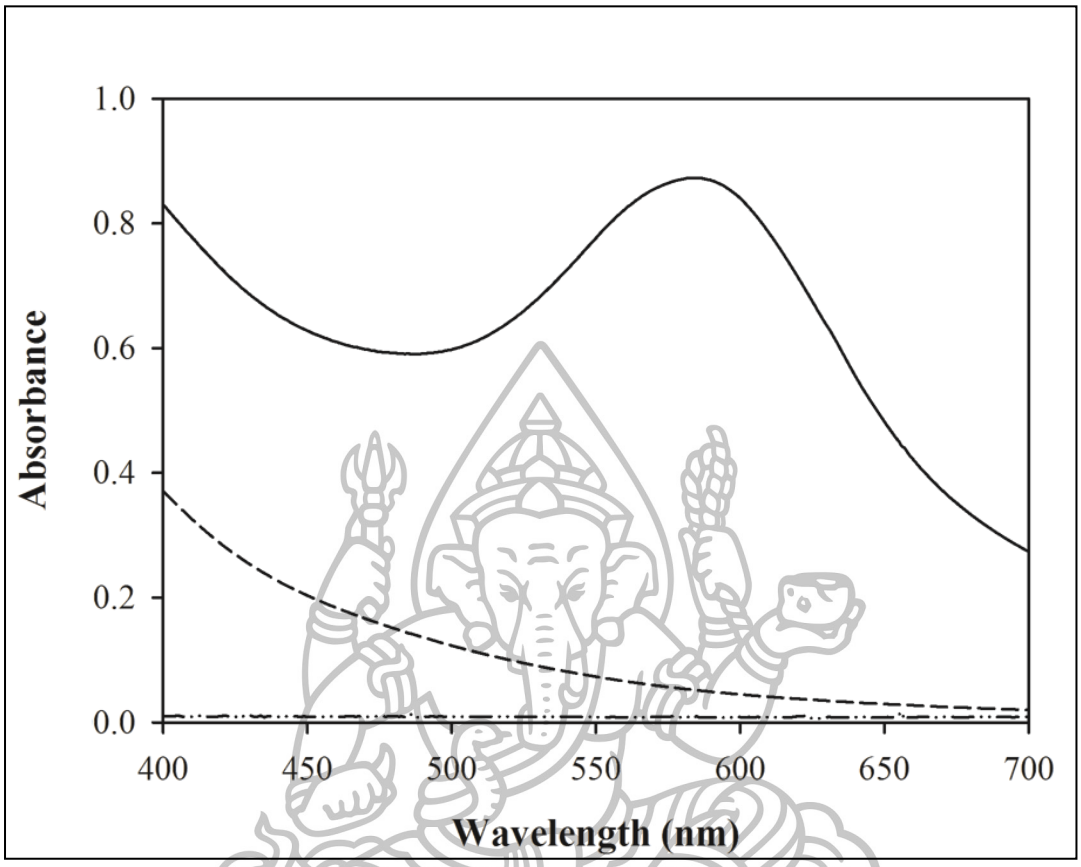

Figure 4.7 Absorption spectrum of blue product (———), 0.175 mM gabapentin (·····), blank (– – – –) and 1 mM genipin (— · · —).

4.2.1 Optimization of the analytical procedure

4.2.1.1 Effect of pH

The various pH ranging from 4.0-9.0 were studied to investigate the best condition for the formation of genipin-gabapentin complex (blue product). It was found that the reaction conducted at pH 7 produced the most intense blue color as measured by the absorbance at 590 nm (Figure 4.8). Hence, it was chosen as the optimal pH for the assay.

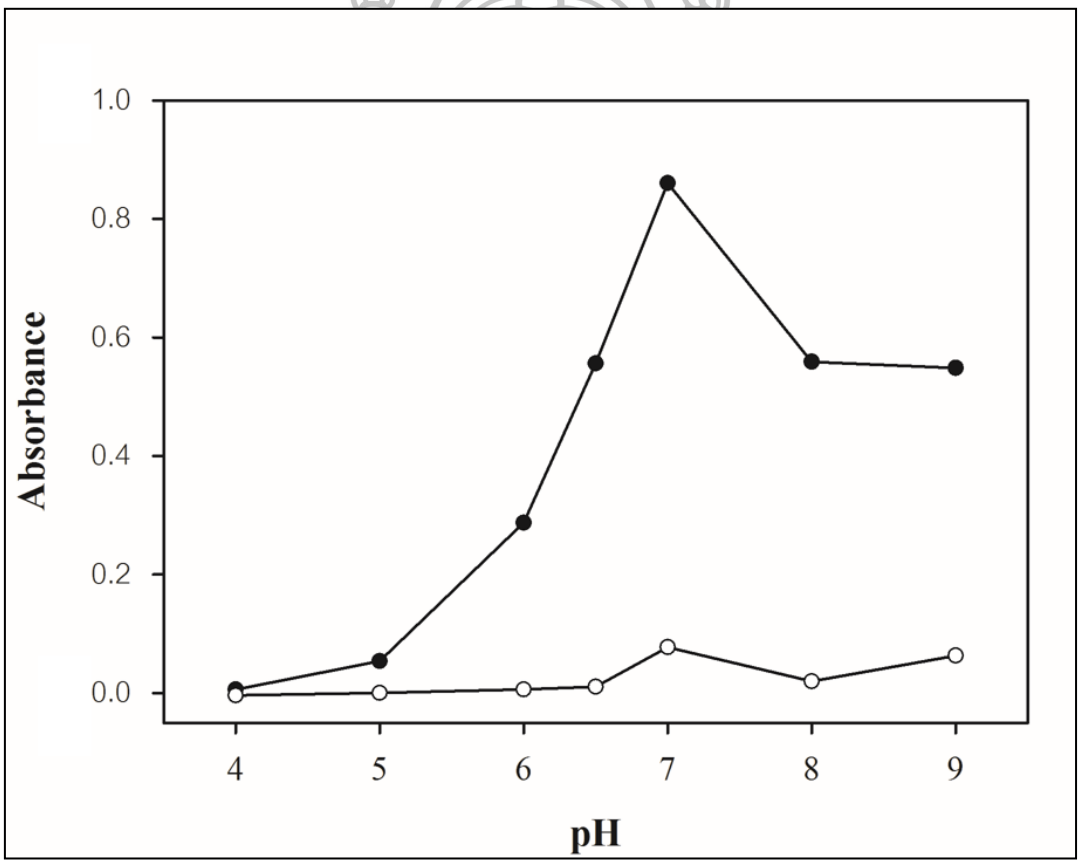

Figure 4.8 Effect of pH on the reaction of genipin with 0.3 mM gabapentin (●) and without gabapentin or blank (○). In this experiment, 600 µL of 2 mM genipin in the buffers with different pH was used as the reaction was heated at 80 °C for 1 h.

4.2.1.2 Effect of genipin concentration

As shown in Figure 4.9, the use of 1 mM genipin solution gave a line with the lowest slope, indicating a poor sensitivity of the method. In contrast, when the concentration of genipin was raised to 2 mM, the steepest slope with the highest absorbance values was obtained. The use of genipin solutions at the higher concentration than 2 mM did not further increase the slope or the sensitivity. Moreover, the plotted lines became lower due to the higher absorbance background which was used for the subtraction as a reagent blank. Thus, 2 mM genipin was the most effective and economical.

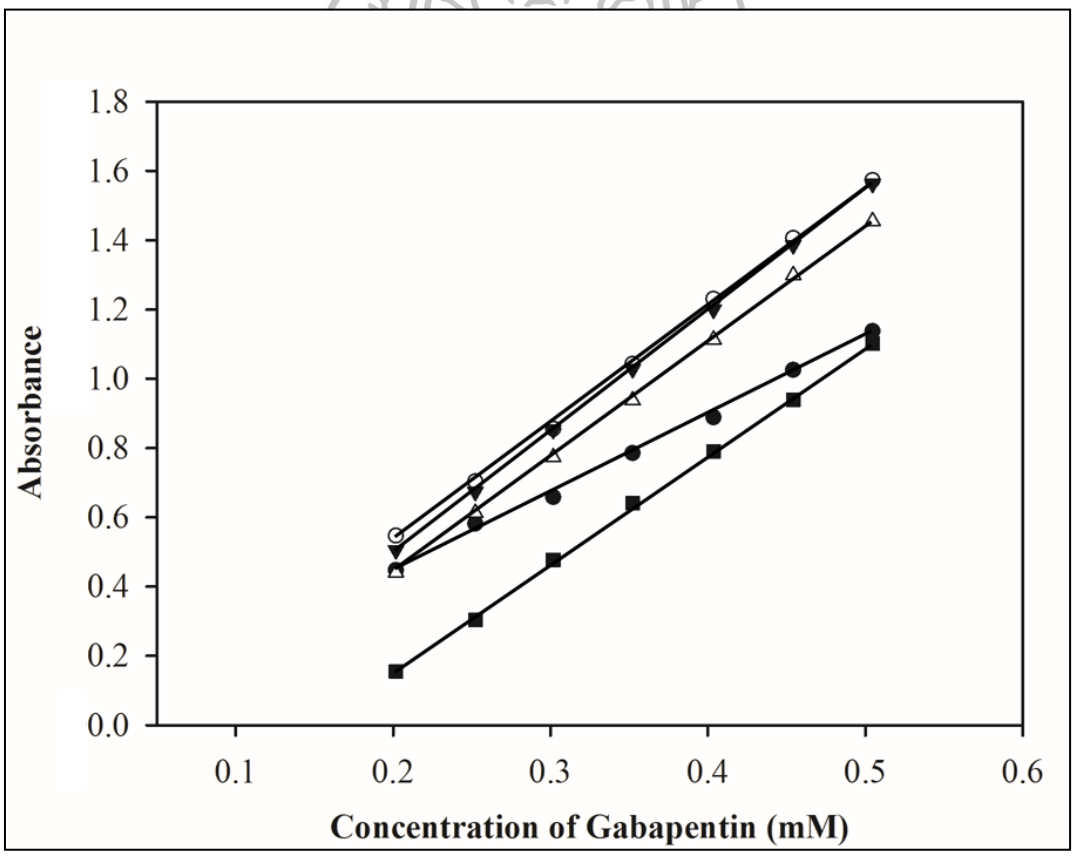

Figure 4.9 Effect of concentration of genipin: 1 mM (●), 2 mM (○), 4 mM (▼) and 6 mM (Δ) and 8 mM (■). In this experiment, genipin solutions were prepared in 50 mM potassium phosphate buffer, pH 7.0 and the reaction was heated at 80 °C for 1 h.

4.2.1.3 Effect of temperature

By varying the heating temperature (60, 70, 80 and 90 °C) and fixing the heating time at 60 min, it was found that the slope of standard curves increased with the increasing temperature (Figure 4.10). Since heating at 80 °C gave aline which was not significantly different from that obtained from heating at 90 °C, it was chosen for the standard protocol.

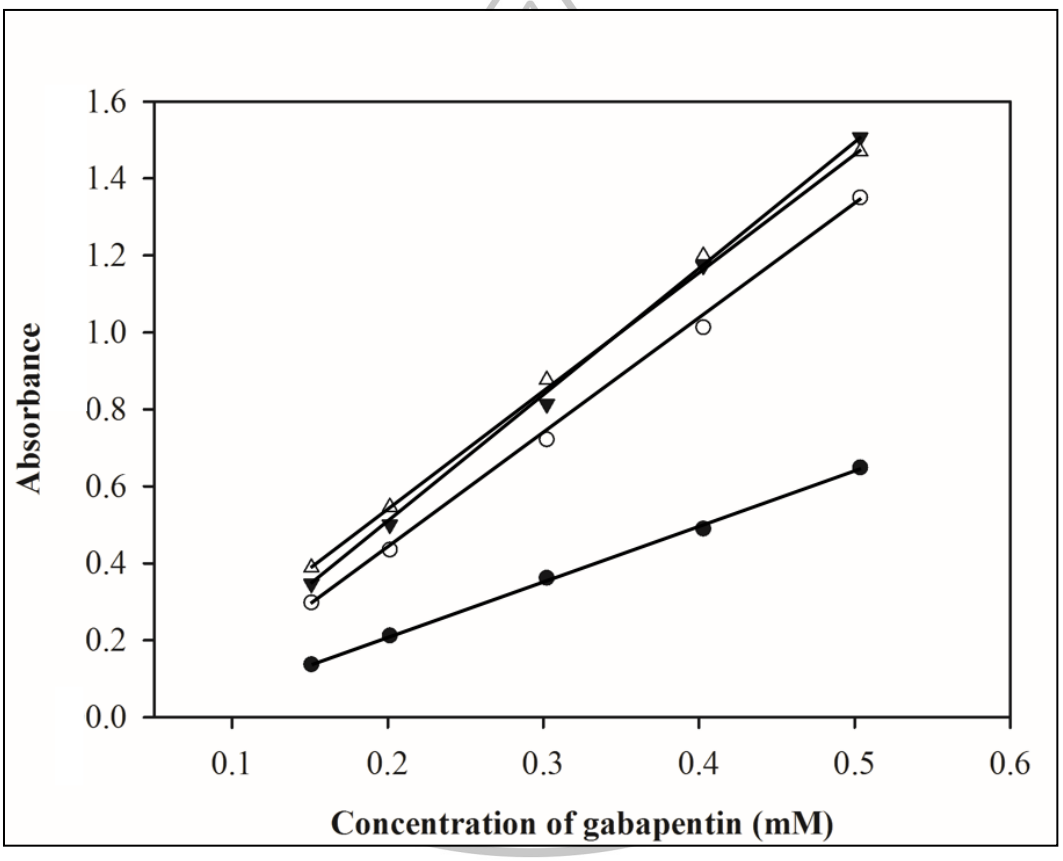

Figure 4.10 Effect of heating temperature: 60 °C (●), 70 °C (○), 80 °C (▼) and 90 °C (Δ). In this experiment, 600 µL of 2 mM genipin in 50 mM potassium phosphate buffer, pH 7.0 was used and the reaction was heated at different temperature for 1 h.

4.2.1.4 Effect of heating time

After 80 °C was selected as the optimal heating temperature, the optimized heating time was subsequently studied at this temperature. As shown in Figure 4.11, the color intensity of the solution gradually increased with time until it reached a constant value after 60 min. Therefore, heating was allowed for 60 min to obtain a complete, energy-minimizing and time-saving reaction.

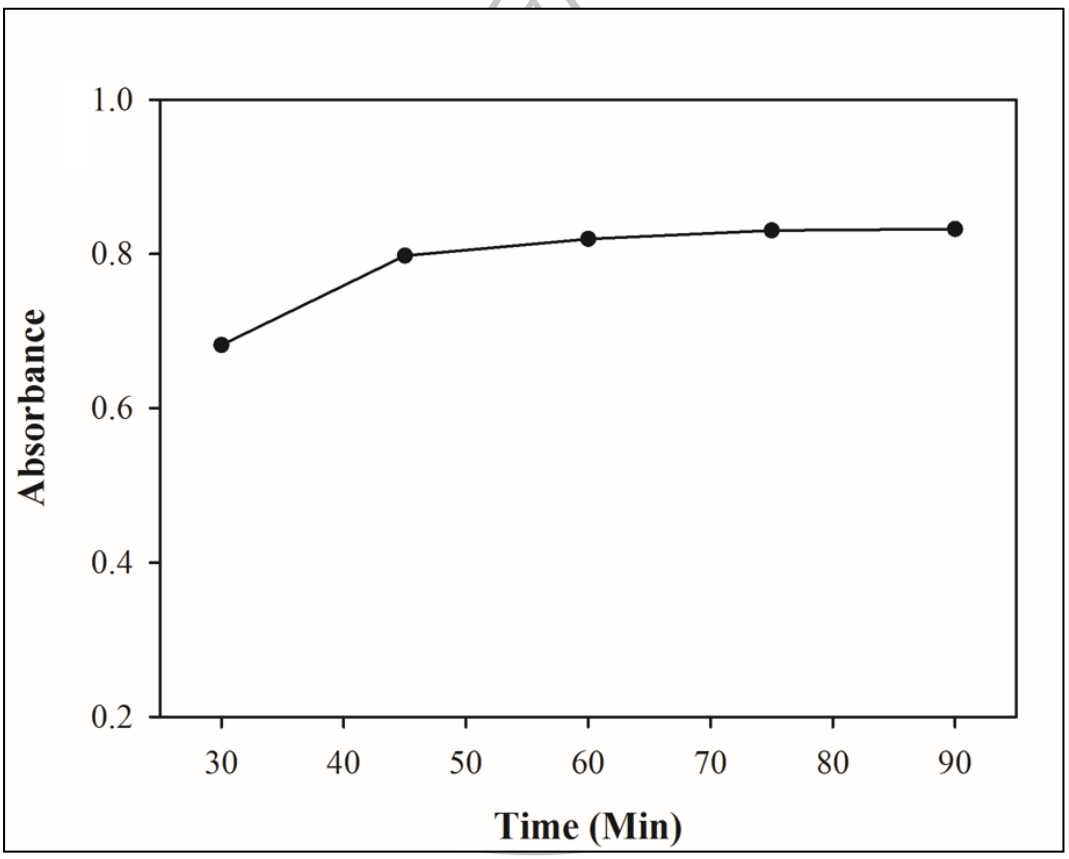

Figure 4.11 Effect of heating time. In this experiment, 600 µL of 0.25 mM gabapentin and 600 µL of 2 mM genipin in 50 mM potassium phosphate buffer, pH 7.0 was used and the heating temperature was set at 80 °C.

4.2.1.5 Stability of blue color products

Since the color intensity of the solution must be measured after the steady blue color was obtained, the stability is inevitably an important issue to be concerned. From the experiment, the color was satisfactorily stable with only slight decrease of the absorbance (less than 1%) within 1 h when the solution was placed under ambient light (Figure 4.12). Furthermore, the color stability significantly improved if the solution was protected from light as evidenced by more than 99% remaining absorbance values over the 6-hr period.

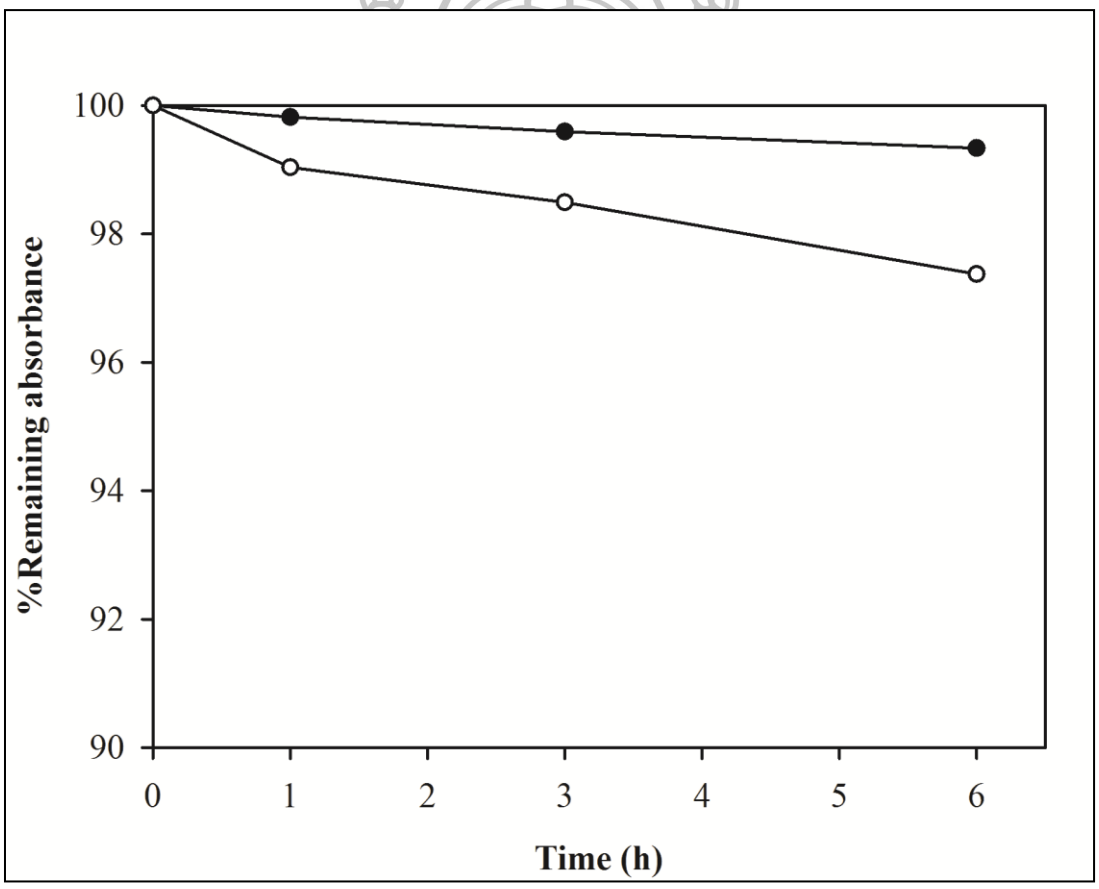

Figure 4.12 Color stability of the blue product kept under light (○) and dark (●) condition. In this experiment, the blue colored solution was prepared by heating 600 mL of 0.35 mM gabapentin and 600 mL of 2 mM genipin in 50 mM potassium phosphate buffer, pH 7.0 at 80 °C for 1 h.

4.2.2 Method validation and comparison to the standard method

After the optimal protocols were established, the method validation was carried out to verify its overall performance. It was found that an excellent linear response of absorbance in relation to the concentration of gabapentin was observed over the range of 0.15-0.50 mM with the regression coefficient of 0.9998. The gabapentin content was calculated by using the following linear equation;

$$A = 3.410\,C + 0.143 \qquad \text{(Eq. 5)}$$

Where; A is absorbance of blue product.
C is gabapentin concentration (mM).

LOD and LOQ as determined from the linear regression were 0.004 and 0.014 mM, respectively. The accuracy of the proposed method was determined by spiking three different levels of standard gabapentin (0.15, 0.20, 0.25 mM) into the pre-analyzed drug solutions (0.15 mM) prepared from the capsules and the added concentration was determined by the proposed method. It was found that the percentage recoveries were in the range of 100.37-100.59% (Table 4.1). Therefore, the method was found to be satisfactorily accurate as the percentage recovery values were in the range of 98-102%. In term of the precision which was studied in bulk drug and capsules, the %relative standard deviation (RSD) was 0.37-0.63% for the repeatability (intra-day precision) and 0.04-0.22% for the intermediate precision (inter-day precision) (Table 4.2). Since both % RSD values were less than 2%, the method was acceptably precise. To investigate the specificity of the proposed method, the interferences from the gabapentin related compounds specified in the USP including Compound A, B, D and E as well as the excipients commonly used in capsule formulations i.e. lactose, microcrystalline cellulose and colloidal silicon dioxide were studied. From the experiments, all substances tested did not react with genipin to form the blue colored product because of the absence of primary amino group in the structures (Figure 3.1). In addition, when they co-existed with the gabapentin at the concentration levels which were limited by the USP monograph (for the related compounds) or commonly used in capsules (for the excipients), the percent

of drug recovery values were in the range of 99.75-100.40% (Table 4.3). This finding indicated the specificity of the method and the absence of interference from the related compounds and excipients. The performance of the proposed method was further tested by applying it to the analysis of gabapentin in bulk and capsule samples in the comparison with the USP chromatographic method. The mean values of percent content or percent labeled amount found with RSD are shown in Table 4.4. In all cases, the samples complied with the official content requirement (98.0-102.0% for bulk drug and 90.0-110.0% for capsules). In addition, the statistical analysis confirmed that there was no significant difference between the proposed method and the pharmacopeial reference method at 95% confidence level.

Table 4.1 Accuracy of the proposed method.

Spiked concentration of standard gabapentin (mM)	**Average recovery* (%)**	**RSD (%)**
0.15	100.37	0.44
0.20	100.59	0.33
0.25	100.49	0.60

* Number of measurement (n) in each concentration level was equal to 3.

Table 4.2 Precision of the proposed method.

Precision	**Bulk drug**		**Capsules**	
	Content found (%)	RSD (%)	Labeled amount (%)	RSD (%)
Intra-day*	100.31	0.37	99.76	0.63
Inter-day**	100.28	0.04	99.75	0.22

* Number of measurement (n) was equal to 6.

** Number of measurement (n) was equal to 3.

Table 4.3 Recovery of gabapentin in the presence of different interferences.

Impurities or excipients	**Added concentration* (%)**	**Recovery of gabapentin** (%)**	**RSD (%)**
Related compound A	0.5	100.31	0.13
Related compound B	0.5	100.40	0.58
Related compound D	0.5	100.40	0.59
Related compound E	0.5	100.26	0.37
Lactose	20	100.11	0.38
Microcrystalline cellulose	20	100.09	0.39
Colloidal silicon dioxide	1	99.75	0.06

* The % values mean the percent of weight of impurities or excipients added by weight of gabapentin in the samples.

** Number of measurements (n) was equal to 3.

Table 4.4 Results of the analysis of gabapentin by the proposed method and USP HPLC method (n=6).

Sample	**Proposed method**	**USP method**
Bulk drug		
Content found (%)	100.31	100.35
RSD (%)	0.37	0.20
Capsules		
Labeled amount (%)	99.76	99.90
RSD (%)	0.63	0.45

4.2.3 Removal of blue adducts from waste solution

Due to the eco-concern and the responsibility on the disposal of colored effluents into the environment, the colored species from either industries or laboratories should be removed from wastewater before they are discharged. In this study, calcium sulfate or gypsum showed the highest ability to remove the blue color (Figure 4.13). So, batch adsorption by using gypsum as an adsorbent was employed for the decontamination of the blue adducts in the assay waste because of the simplicity of the operation as well as the low cost and common availability of materials and equipment. It was found that using the adsorbent for the waste water at the ratio of 1:50 (w/v) incorporation with vigorous shaking could remove more than 95% of the blue colored product from the solution rapidly within 2 min. Thus, the proposed treatment was one of the effective and practical ways to decontaminate and decolorize the waste prior to the release into the environment.

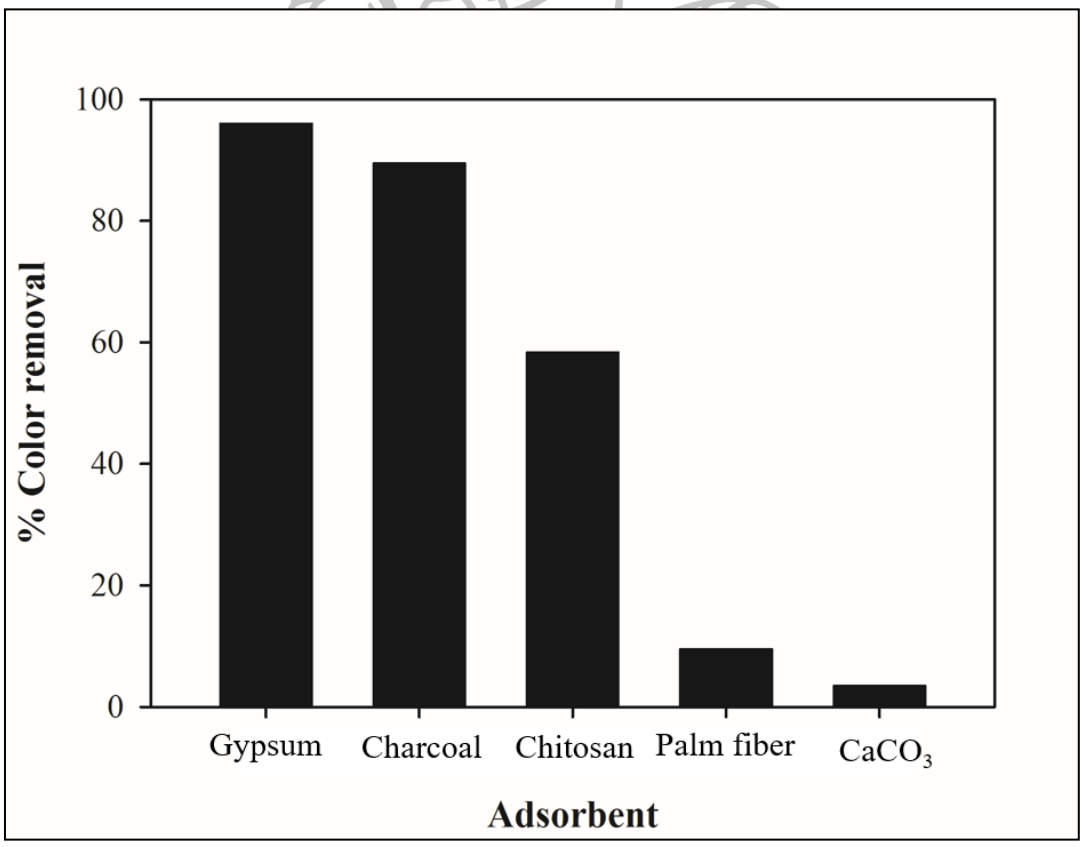

Figure 4.13 The percentage of color removal by using different adsorbents.

4.2.4 Acute toxicity of laboratory waste

The results of the acute toxicity evaluation on non-vertebrate brine shrimp showed that the untreated waste caused 0% mortality of the tested organisms at 50% v/v dilution, the maximum concentration that could be prepared in the experiment, since the untreated waste at the higher concentration than this level was likely to precipitate in 35 ppt saline solution. Nevertheless, 100% of the organisms safely survived in the treated waste even at the undiluted (100%) concentration. In the vertebrate guppy fishes, LC50 was found to be 17% v/v for the waste solution before the treatment (Figure 4.14). This gave the TUa value of 5.8 and thus the waste prior to the treatment was classified as ‘‘slightly toxic’’ substance. However, after the decolorization, LC50 of waste solution was noticeably raised up to 41% and the TUa value was lowered to 2.4 which satisfactorily met the criteria of ‘‘non-toxic’’ effluent. From this result, it clearly demonstrated the performance of the proposed waste treatment method by using gypsum adsorbent to both decolorize and detoxify the assay waste and to produce the resultant cleaner waste which was proven safe for the aquatic life in the environment.

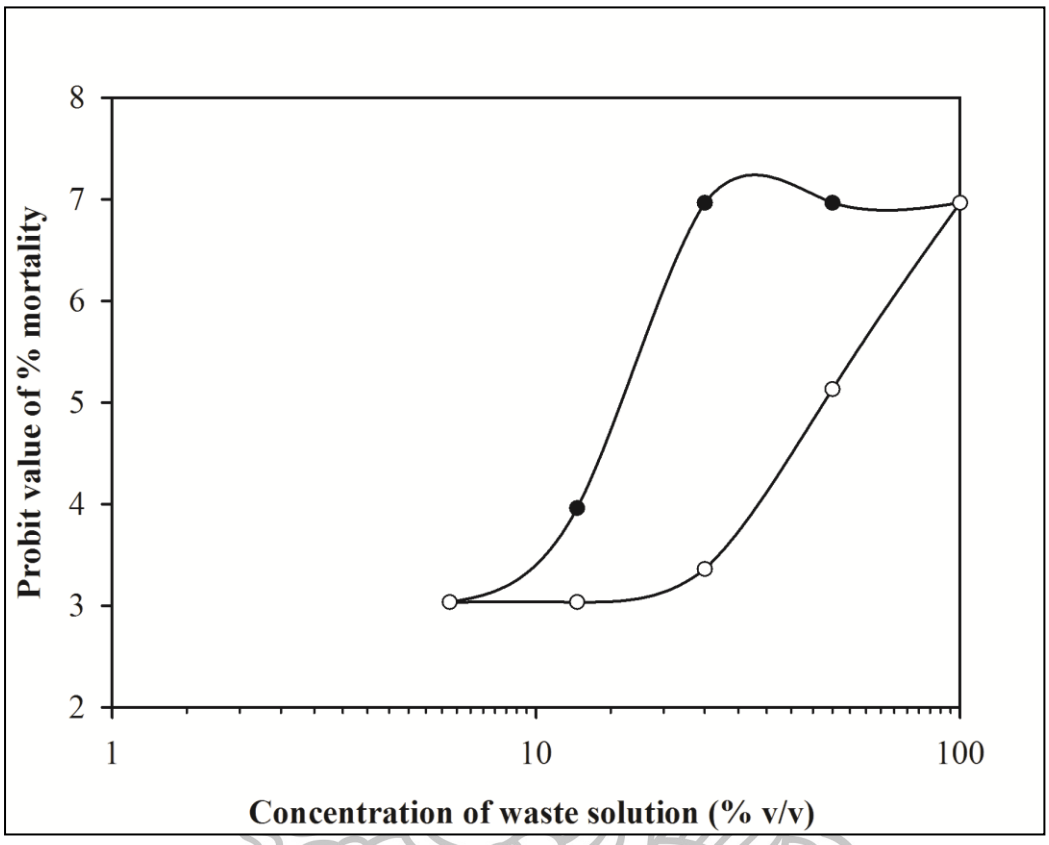

Figure 4.14 The percentage mortality of guppy fishes at different concentrations of waste solutions before (●) and after (○) the gypsum adsorption treatment.

4.3 Application of genipin as cross-linking agent for immobilization of β-glucosidase on chitosan beads

In this study, genipin was used as a natural derived cross-linking agent between primary-amine containing polymers i.e. chitosan and β-glucosidase enzyme, compared with glutaraldehyde. β-glucosidase is an enzyme that hydrolyzes β-glycosidic bonds to release the non-reducing terminal glucosyl residues from glycosides and oligossaccharides. The objective of this study was to investigate the efficiency of genipin which act as a cross-linking agent to increase the stability of β-glucosidase on chitosan beads. The activity of enzyme on chitosan beads was tested by using 4-nitrophenyl β-D-glucopyranoside (p-NPG) as a substrate. After that, the performance of immobilized beads was determined the activity and reusability with genistin, the natural compound which found in a number of dietary plants like

soybean. It can be hydrolyzed by cleavage the sugar moiety from genistin to produce an aglycone compound namely genistein which has various pharmacological activities and can be used for reduction or prevention of several diseases such as cardiovascular disease (Rimbach et al., 2008), osteoporosis, estrogenic and antioxidant activity (Liu, Kanjo and Mizutani, 2010; Ma et al., 2010). In this research, various factors such as type of cross-linking agents, optimal conditions for cross-linking and immobilization procedure were optimized to obtain the highest efficiency of immobilized bead. Furthermore, pH and thermal activity, pH and thermal stability, reusability and storage stability were investigated. To the best of this knowledge, although other β-glucosidase enzymed have been immobilized on various carriers, the use of genipin as cross-linking agent on chitosan beads has not been previously reported.

4.3.1 Optimal conditions for enzyme immobilization

The enzyme activity on immobilized chitosan beads would be greatly influenced by the concentration of crosslinking agent, crosslinking and immobilization time. It was found that the linear response of absorbance in relation to the concentration of p-NPG was observed over the range of 20-200 μM with the regression coefficient of 0.9992. The content of p-nitrophenol was calculated by the following equation;

$$Y = 0.0125X - 0.0367 \quad \text{(Eq. 6)}$$

Where; Y is the absorbance.

X is the concentration of p-nitrophenol (μM).

For the cross-linking procedure using glutaraldehyde as a cross-linking agent, chitosan beads were cross-linked for 3 h prior immobilization with different time points. As shown in Figure 4.15(a), the enzyme activity reached the maximum when the concentration of glutaraldehyde was 1.0%. It was found that as the glutaraldehyde concentration increased, the activity of immobilized β-glucosidase decreased gradually because when the concentration of glutaraldehyde was too high, glutraldehyde can react with several function groups of protein, such as amine, thiol, phenol and imidazole (Habeeb and Hiramoto, 1968) leading to the formation of multi-

point binding between enzyme and carrier. Thereby, the existing of spatial structure leading to inactivation of the enzyme (Bhandari, Gupta Kumar and Singh, 2009; Adriano et al., 2005). Even at low concentration of glutaraldehyde there were enough amount of reactive groups on the surface of chitosan beads to almost immobilize the entire enzyme. In the aspect of time, the enzyme activity was gradually increased with time of immobilization until it reached a constant value after 12 h. For the preparation of glutaraldehyde cross-linked beads, the glutaraldehyde concentration of 1.0% and time of immobilization at 12 h were adopted for the beads preparation procedure.

In this work, genipin was used as an alternative cross-linking agent for immobilization of chitosan beads and it can be prepared by three different ways. For the first type of beads, amino group on chitosan beads was pre-activation with genipin for 3 h to form genipin-chitosan product. Then β-glucosidase was immobilized on genipin cross-linked beads. The results showed that the time of immobilization at 6 h produced the maximum enzyme activity at all of genipin concentrations (Figure 4.15(b)). Furthermore, the using of genipin concentration of 0.1% for cross-linking produced the enzyme activity which was not significantly different from the results from the concentration of 0.5 and 1.0%. Hence, the genipin concentration of 0.1% and time of immobilization at 6 h were selected for the preparation method. For the second type of beads, β-glucosidase was immobilized by adsorption on the surface of chitosan beads prior cross-linking with genipin. So, the formation of β-glucosidase layer on chitosan beads might be occurred, consequently, genipin was not able to cross-link between β-glucosidase and chitosan beads. Therefore, the optimal genipin concentration and time of immobilization were 0.1% and 3 h, respectively (Figure 4.15(c)). For the third type of beads, chitosan beads were cross-linked and immobilized simultaneously. The mixture was cross-linked and immobilized randomly leading to the formation of several products including chitosan-β-glucosidase pruduct, β-glucosidase-β-glucosidase product and chitosan-genipin-β-glucosidase product. In addition, β-glucosidase could be immobilized on chitosan-genipin-β-glucosidase product to form layers of enzyme. The proper conditions for the preparation were 0.1% of genipin concentration and reaction time at 6 h (Figure 4.15(d)). It was obvious that when high concentration of genipin is used, a parallel

reaction between genipin molecules can be formed resulting in losing of enzyme activity.

It can be seen that, the time of immobilization at 6 h for the preparation of adsorption bead produced the highest enzyme activity (Figure 4.15(e)). So, the adsorption beads were prepared by immobilization at 6 h without cross-linking agent.

4.3.2 Assay of β-glucosidase activity on immobilized chitosan beads and protein quantitation

The linear response of absorbance in relation to the concentration of protein was observed over the range of 5-40 µg/mL with the regression coefficient of 0.9976. The protein content was calculated by the following equation;

$$Y = 0.0153X + 0.0326 \quad \text{(Eq. 7)}$$

Where; Y is the absorbance.

X is the protein concentration (µg/mL).

Chitosan beads cross-linked by genipin had higher immobilization yield than those prepared by glutaraldehyde cross-linking agent (method 2). As shown in Table 4.5, the protein contents on chitosan beads were analyzed and compared with the β-glucosidase activity.The results showed that the enzyme contents on chitosan beads were relevant to the enzyme activity.

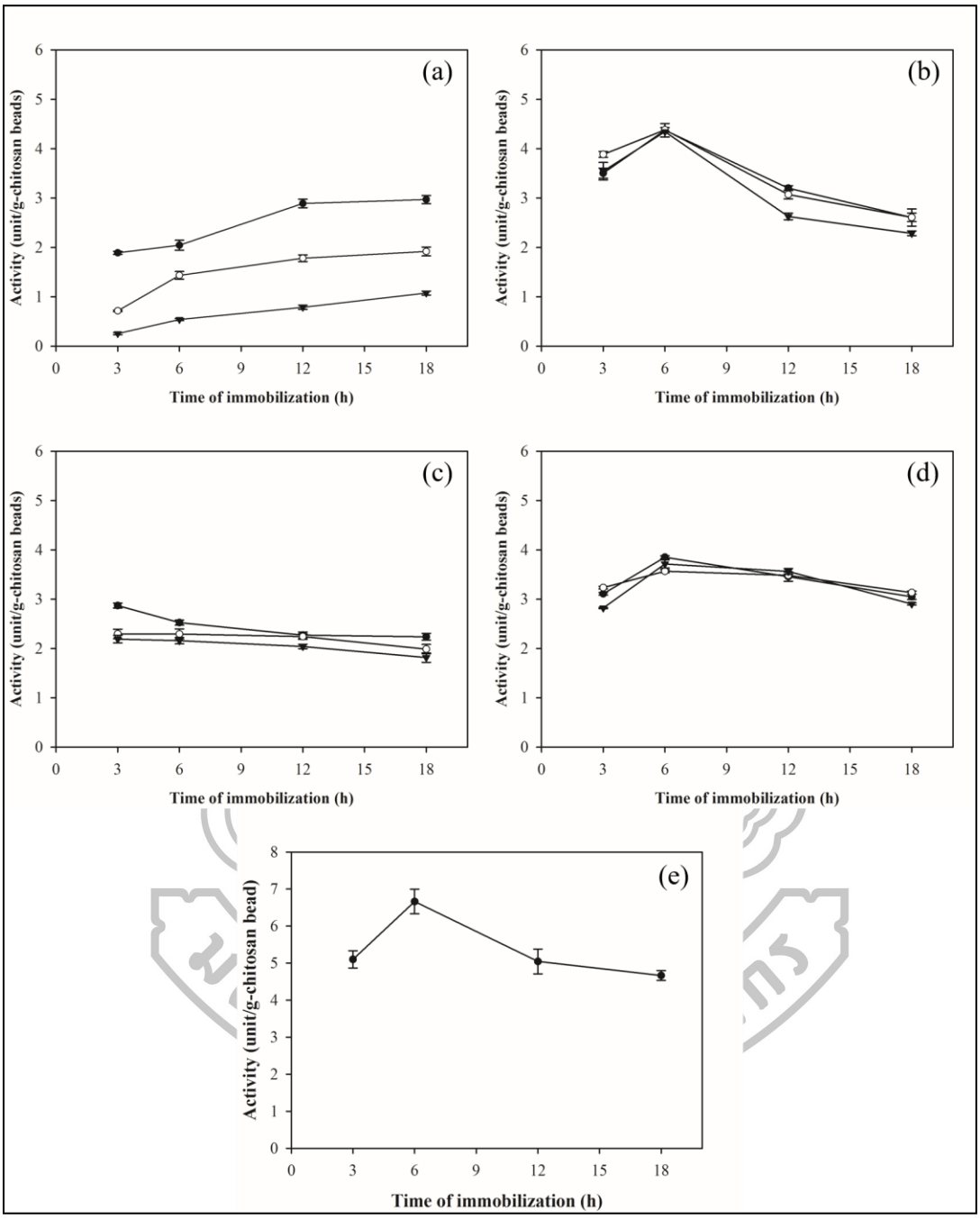

Figure 4.15 Activity of immobilized β-glucosidase on chitosan beads using difference type of cross-linking agents: glutaraldehyde ((a), 1.0% (●), 2.5% (○) and 5.0% (▼)), genipin (method 1) (b), genipin (method 2) (c) and genipin (method 3) ((d), 0.1% (●), 0.5% (○) and 1.0% (▼)) and without cross-linking agent (adsorption bead) (●) (n=3).

Figure 4.5 Immobilization yield and β-glucosidase activity on chitosan beads.

Cross-linking agent	Immobilization yield (%)*	β-glucosidase activity (U/g-chitosan beads)*
Glutaraldehyde	48.6±1.35	2.89±0.24
Genipin (method1)	60.4±1.51	4.37±0.14
Genipin (method 2)	47.4±0.25	2.87±0.05
Genipin (method 3)	54.5±0.44	3.85±0.03
Adsorption	70.6±2.03	6.67±0.33

* The data are shown as average±SD (n=3).

4.3.3 Effect of pH and temperature on β-glucosidase activity after immobilization process

The activity of immobilized enzyme at varying pH (4.0-8.0) and temperature (40-80 °C) was analyzed in order to investigate the optimal pH and temperature after immobilization process by using immobilized chitosan beads from the best preparation cond itions in each type according to Table 4.5.

The properties and behavior of a bound enzyme is affected by its environment i.e. the surface and charges on solid support. From the experiment, the optimal pH of free enzyme were 5.0. After immobilization process, the optimal pH was shifted toward a more basic region, being pH 6.0 considered for the optimal pH for immobilized β-glucosidase (Figure 4.16(a)). Because too acid or too alkaline will cause enzyme denaturation which leads to the enzyme activity reducing (Liang et al., 2006). Figure 4.16(b) shows the effect of reaction temperature on the relative activities for free and immobilized β-glucosidase. The optimal temperature for free enzyme was found at 60 °C. In contrast, the optimal temperature of β-glucosidase was shifted to 70 °C. So, the optimal conditions for determining the enzyme activity β-glucosidase on chitosan beads were pH 6.0 and 70 °C.

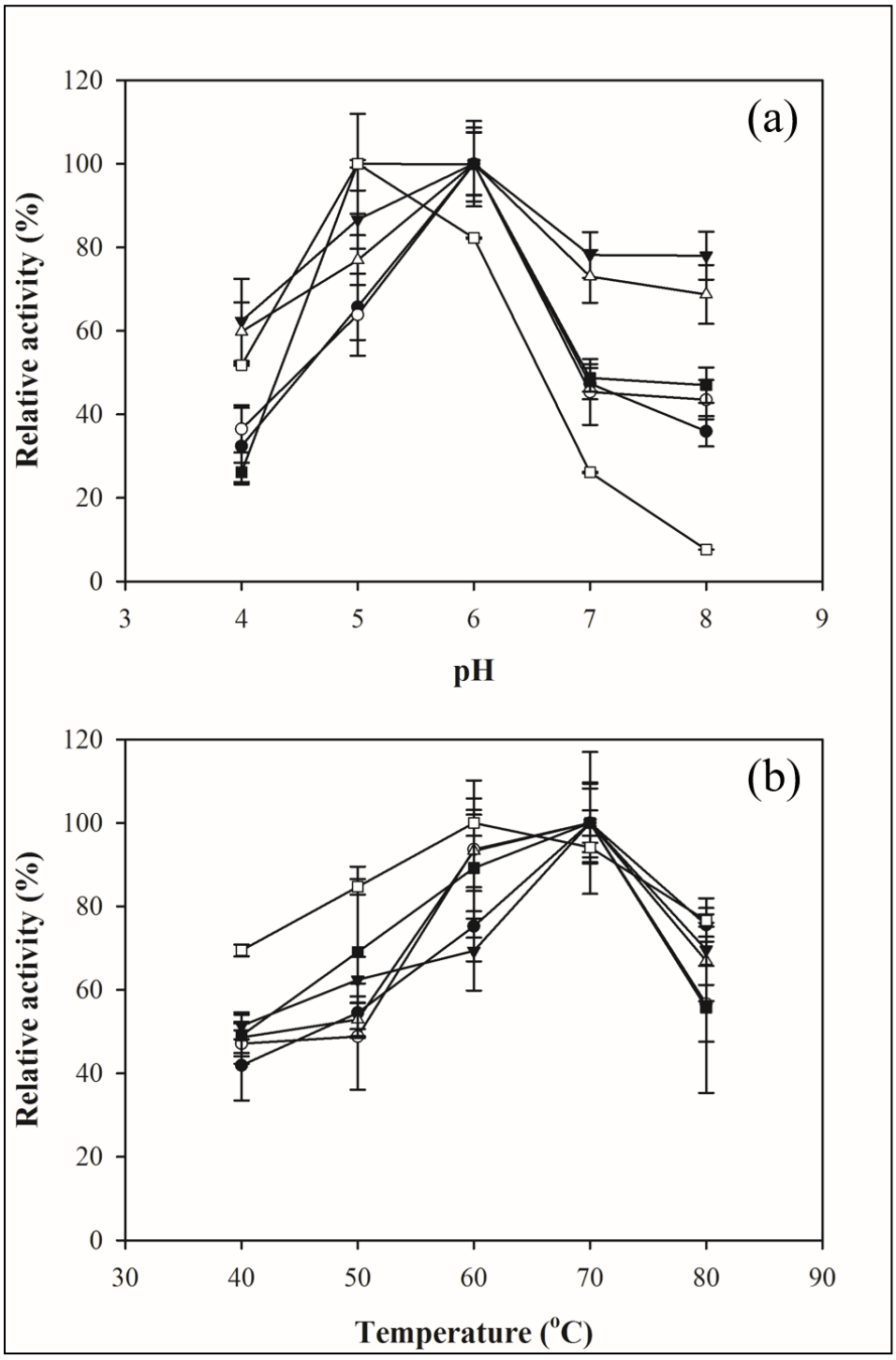

Figure 4.16 Effect of pH (a) and temperature (b) on activity of immobilized β-glucosidase using difference type of cross-linking agents: glutaraldehyde (●), genipin (method 1) (○), genipin (method 2) (▼), genipin (method 3) (Δ), adsorption beads (■) and free enzyme (□) (n=3).

4.3.4 Effect of pH and thermal on stability of immobilized β-glucosidase

Immobilization technique is used primarily to increase the stability of enzyme. Operational stability can be assessed by exposure the immobilized enzyme at different pH and temperature conditions. The extent of stability depends on the immobilization method, the structure of enzyme and the attribute of supporter. Enzymes can be deactivated under a number of conditions such as extremes of pH or temperature. Figure 4.17 shows the residual activity of the different types of immobilized chitosan beads after incubation under various pH (4.0-8.0) by fixing the temperature at 50 °C for 2 h. It was obvious that, the immobilized β-glucosidase had higher stability than the free enzyme over a broad range of pH, especially the immobilized chitosan beads with genipin (method 3), retaining more than 80% of maximal activity at pH 4.0-8.0.

The characteristics of immobilized chitosan beads after incubated at different pH are shown in Figure 4.18. According to many publications, chitosan was dissolved in acid solution, since the pKa value of chitosan is 6.5. Therefore, the swelling of chitosan beads in acid solution could be occurred. The initial diameter of immobilized chitosan beads was 1.58±0.06, 1.72±0.05, 1.62±0.10, 2.00±0.04, 1.78±0.08 and 2.14±0.09 mm for chitosan beads, glutaraldehyde beads, genipin beads (method 1), genipin beads (method 2), genipin beads (method 3) and adsorption beads, respectively. It can be seen that, the appearance of cross-linked beads slightly changed when it was soaked in different pH solutions i.e. the size of beads was slightly increased and the color of beads became darker when the pH was decreased. It was obvious that, the chitosan beads which were cross-linked with glutaraldehyde or genipin had greater stability in acid solution especially at pH 4.0-6.0 than uncross-linked chitosan beads.

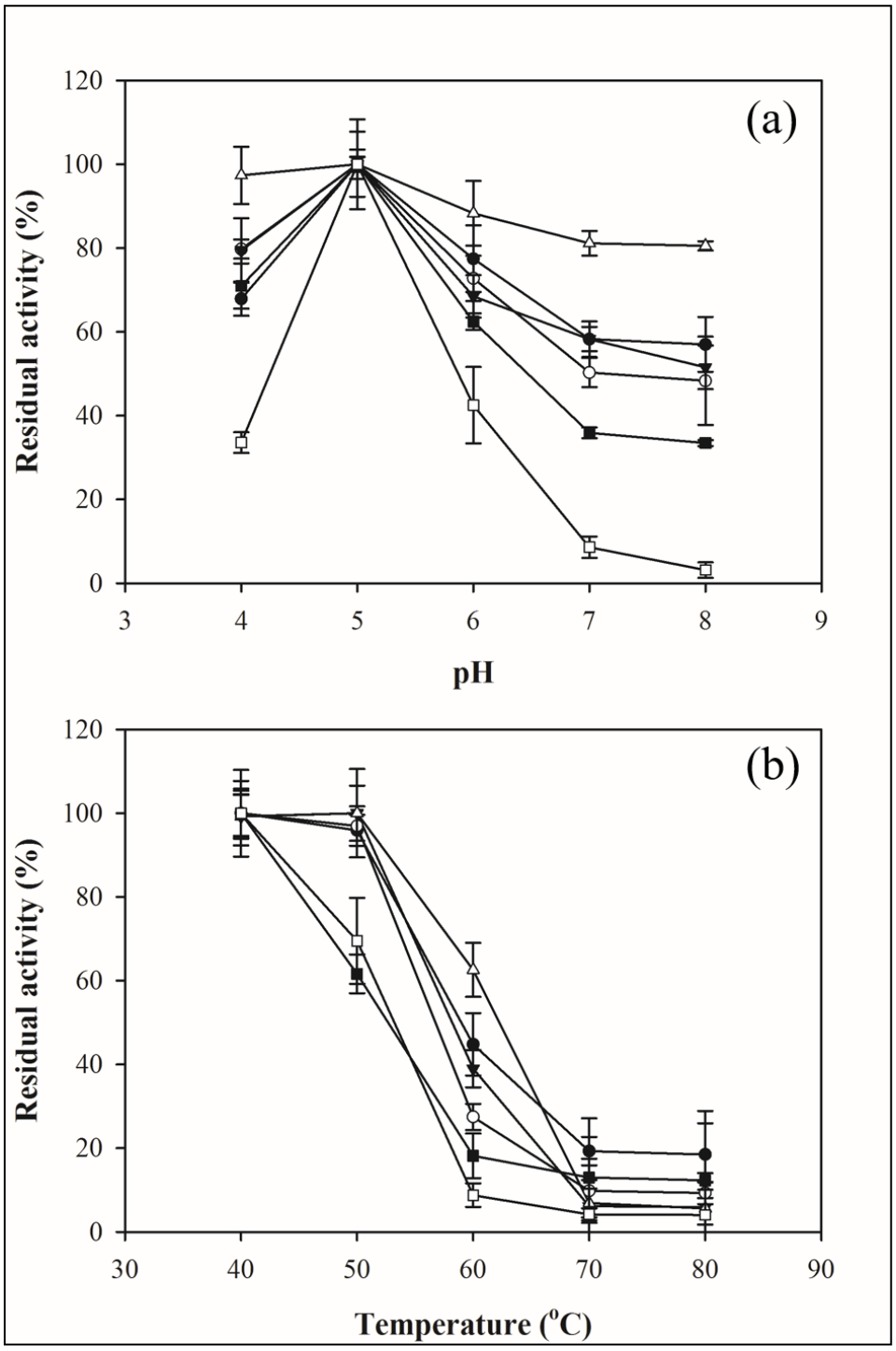

Figure 4.17 Effect of pH (a) and temperature (b) on stability of immobilized β-glucosidase using difference type of cross-linking agents: glutaraldehyde (●), genipin (method 1) (○), genipin (method 2) (▼), genipin (method 3) (Δ), adsorption beads (■) and free enzyme (□) (n=3).

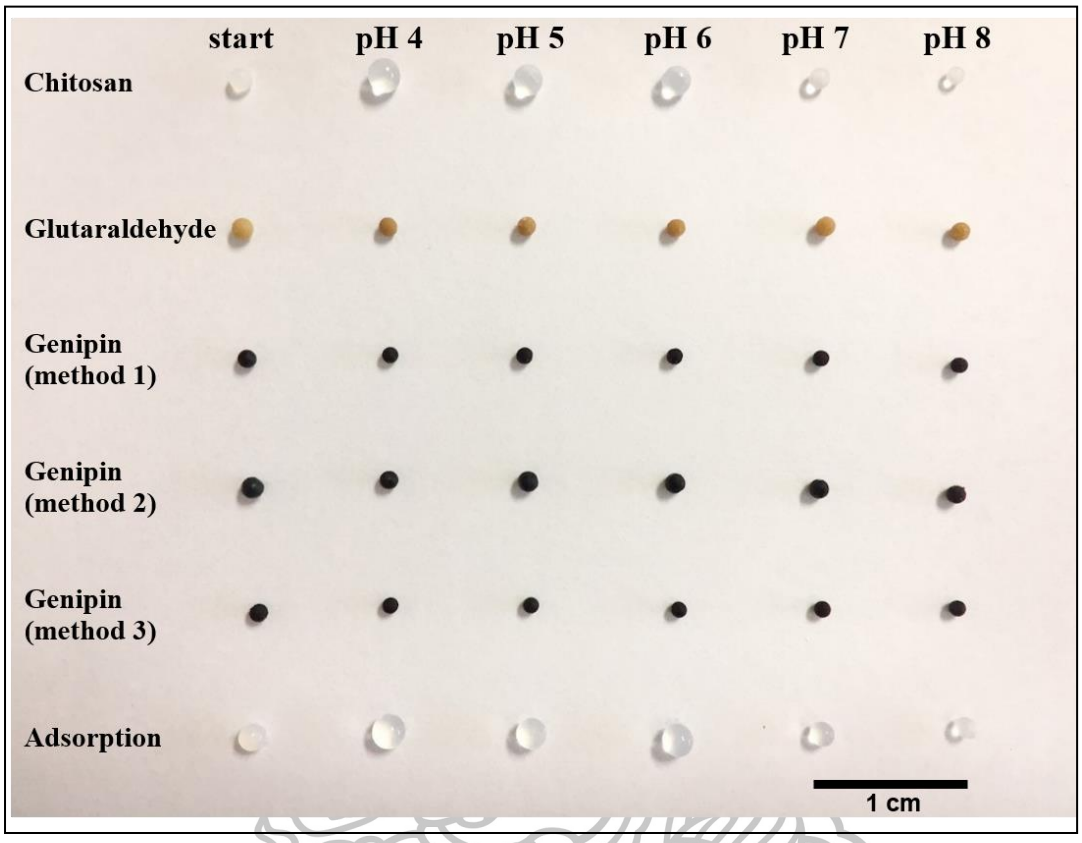

Figure 4.18 Different types of immobilized chitosan beads at various pH, compared with original chitosan beads.

In the aspect of temperature stability profile, the immobilized β-glucosidase was incubated at pH 5.0 at different temperature (40-80 °C) for 2 h before determining the enzyme activity at the optimal condition. The result showed that, the thermal stability of immobilized β-glucosidase was greatly increased at 50 and 60 °C compared with free enzyme (Figure 4.17(b)). At 50 °C, the residual activity of free enzyme was 68%, while the immobilized enzyme which was cross-linked with genipin (method 3) exhibited 95% of residual activity. In addition, it showed 6 times higher stability over free enzyme at 60 °C. Although, the enzyme activity was significantly decreased at the temperature above 60 °C, but it still had greater thermal stability than free enzyme.

4.3.5 Reusability of immobilized chitosan beads

The reusability of enzyme is one of the major advantages of immobilization. The immobilized chitosan beads were evaluated for the reusability using p-NPG. As seen in Figure 4.19, β-glucosidase could be used repeatedly over an extended period of time. The maximum activity was occurred in the preparation method which used genipin (method 3) as a cross-linking agent, it retained 80% of its activity after 10 cycles with significantly different from the other preparation methods including glutaraldehyde method. Therefore, the immobilized β-glucosidase has the advantage over the free enzyme that it could be used repeatedly during the industrial process.

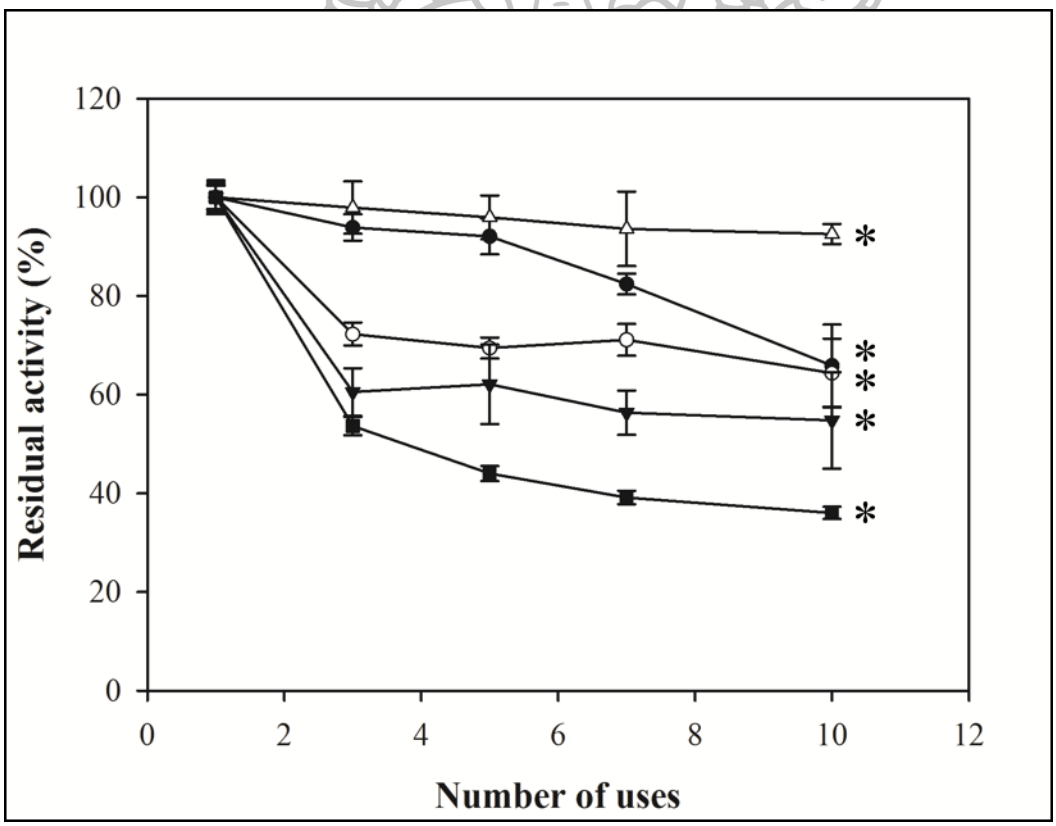

Figure 4.19 Reusability of immobilized chitosan beads by different preparation methods: glutaraldehyde (●), genipin (method 1) (○), genipin (method 2) (▼), genipin (method 3) (Δ) and adsorption beads (■) (n=3), * statistically significant between glutaraldehyde, genipin (method 1), genipin (method 2), genipin (method 3) and adsorption beads

4.3.6 Application of immobilized beads for conversion of genistin to genistein

The catalytic conversion of isoflavone glycosides into aglycones was performed using standard genistin at the concentration of 100 µg/mL as a substrate. The optimal pH and temperature for the catalytic hydrolysis process were 5.0 and 50 °C, respectively. It was found that, the linear response of absorbance in relation to the concentration of genistin and genistein was observed over the range of 2.0-30 µg/mL with the regression coefficient of 0.9994 and 0.9995, respectively. The content of genistin and genistein was calculated by the following equation;

$$Y = 72.31X - 10.086 \text{ (genistin)} \quad \text{(Eq. 8)}$$

$$Y = 96.097X + 32.525 \text{ (genistein)} \quad \text{(Eq. 9)}$$

Where; Y is peak area.
X is concentration (µg/mL).

The immobilized β-glucosidase was added to the standard genistin solution and the content of isoflavone genistein in each cycle was quantified (Figure 4.20). After 5 cycles of enzymetic reaction, the immobilized β-glucosidase which cross-linked by genipin (method 3) showed the highest percent conversion of approximately 70%. For the glutaraldehyde cross-linking chitosan beads, the percent conversion was about 60% at the first cycle. After the five cycles of reaction the percent conversion was decreased to approximately 40%. Although, the adsorption beads showed the high percent conversion at the first cycle, but the percent activity was dramatically decreased in the next cycles. Therefore, the immobilized β-glucosidase on genipin cross-linked chitosan beads could be applied to be utilized for the conversion of genistn to genistein. So, the developed method could be applied for the further applications including the production of isoflavone content in the commercial soy drink as well as other glycoside compounds.

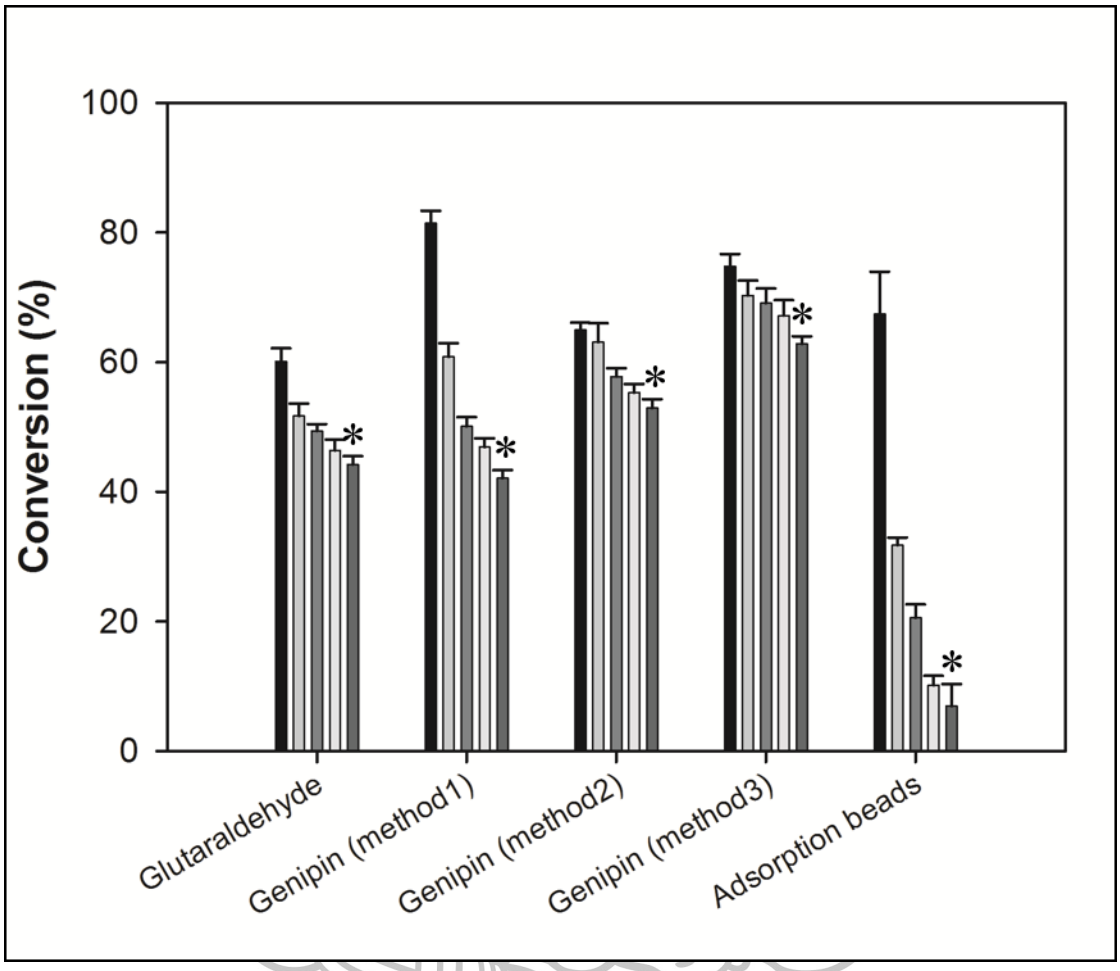

Figure 4.20 Percent conversion of genistin to genistein by immobilized chitosan beads at different preparation methods: cycle 1 (■), cycle 2 (▭), cycle 3 (▭), cycle 4 (▭) and cycle 5 (▭) (n=3), * statistically significant between glutaraldehyde, genipin (method 1), genipin (method 2), genipin (method 3) and adsorption beads

4.3.7 Storage stability of immobilized chitosan beads

The storage stability of immobilized β-glucosidase was evaluated determining the enzyme activity after storage at 4 °C for 30 days. The residual activity of immobilized β-glucosidase was 80% of its initial activity at day 0, exception the chitosan preparation using genipin (method 1) and adsoption beads (Figure 4.21).

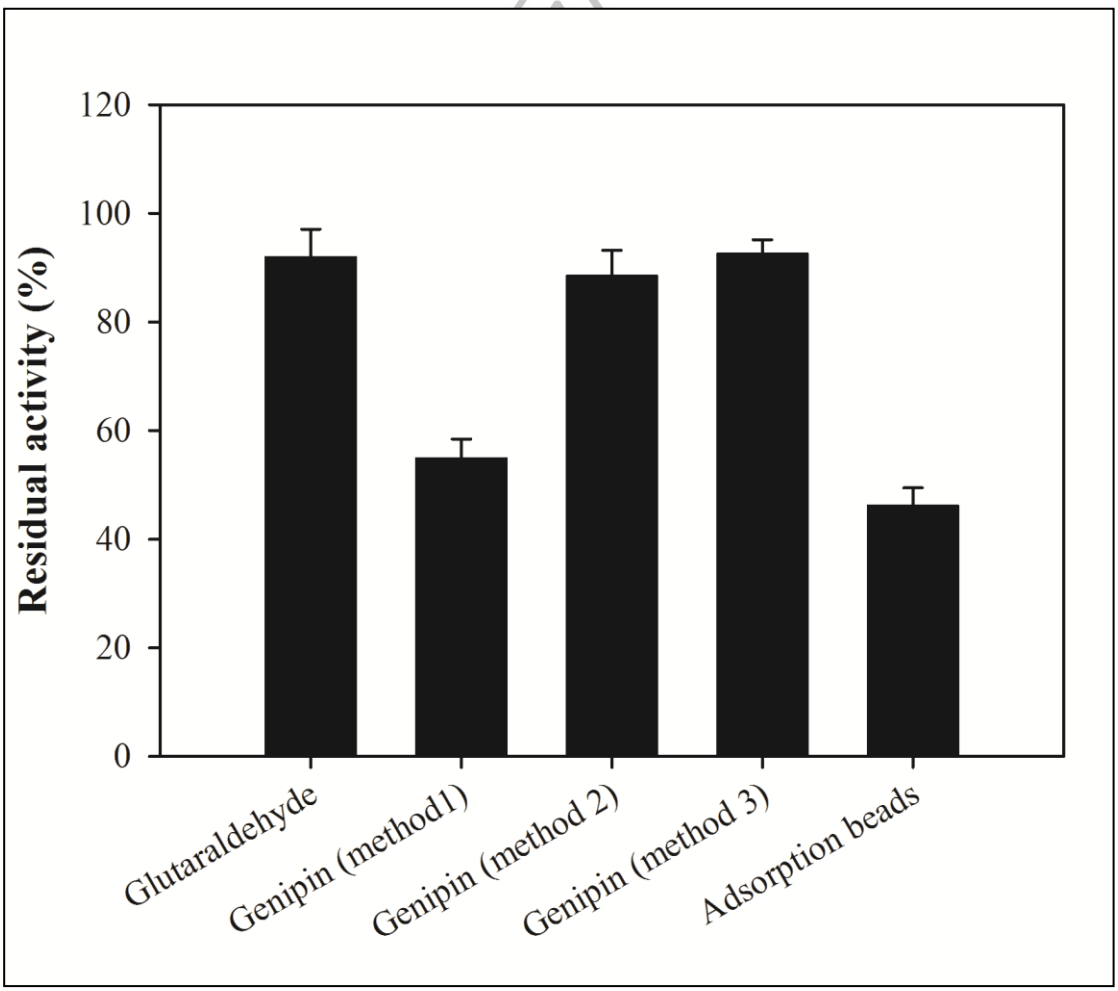

Figure 4.21 The residual activity of immobilized β-glucosidase by different preparation methods after storage at 4 °C for 30 days (n=3).

CHAPTER 5

CONCLUSION

This dissertation was based on the green chemistry concept starting from the improvement of genipin extraction process by using enzyme-assisted extraction combined with in situ product separation. Genipin was adopted to be used as a natural colorimetric agent for the determination of gabapentin drug. Moreover, it can be used as an alternative cross-linking agent instead of the conventional one namely glutaraldehyde to increase the stability of carrier and to immobilize β-glucosidase onto the supporter.

5.1 Improvement of genipin production process by using simultaneous enzyme-assisted extraction and cleavage of glucose from glycoside combined with in situ product separation

The merging of enzyme-assisted extraction and in situ product separation technique had created a new route to the direct preparation of genipin from crude gardenia fruit. Under the optimal conditions, the extraction method was conducted at pH 4.0 for 24 h by using the cellulase concentration of 10 mg/mL. Since the method could be performed in one pot without any purification between steps and required only cellulase as biocatalyst and environmentally friendly ethyl acetate as extracting solvent, it was a facile, economical, green and efficient alternative suitable for the production of not only genipin, but also other valuable plant aglycones.

5.2 Application of genipin for colorimetric assay of gabapentin bulk drug and capsules

In the present study, a novel assay for gabapentin in pharmaceutical samples has been successfully developed based on the colorimetric reaction with naturally derived genipin. The optimal conditions for the analysis as following; genipin concentration was 2 mM, pH of reaction was 7.0, the temperature of reaction was 80 °C and the

heating time was 60 min. The philosophy of the method design relied on the so-called ‘‘from safe source to safe sink’’ concept in which all the reagents used as well as the procedures performed were delicately chosen and optimized to obtain the efficient, safe and environmentally friendly assay. Furthermore, a practical procedure for the treatment of waste after the analysis was also proposed and the treated waste was finally guaranteed for its eco-safety prior to the release into the environment. Therefore, this work not only presents a new route for the assay of gabapentin which is applicable to the pharmaceutical industries, but also calls for the environmental awareness and responsibility from all the analytical chemistry communities to carefully develop the analytical methodologies through this fully green concept for our world’s sustainability.

5.3 Application of genipin as cross-linking agent for immobilization of β-glucosidase on chitosan beads

A naturally derived genipin can be used as an alternative cross-linking agent instead of a toxic and harmful cross-linking agent, glutaraldehyde. The beads preparation method with using genipin was done by one-step preparation including cross-linking of chitosan molecules to increase the strength of beads and immobilization of β-glucosidase on the surface of chitosan. The immobilized β-glucosidase performed the satisfactory activity and stability that can be applied to be used at the wide range of pH and temperature. It has the potential for the conversion of isoflavonoid glycosides to isoflavonoid aglycone which retained 70% conversion after 5 cycles used. The immobilized β-glucosidase presented the residual activity of 90% remaining after storage at 4 °C for 30 days. Moreover, the replacement of glutaraldehyde with genipin was the totally green method with safe for operator and food applications.

It can be concluded that the developed extraction method was the effective mean to increase the yield of genipin. In addition, it was promising to be used as a colorimetric agent for drug analysis and also to be applied for immobilization of enzyme.

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APPENDIX

APPENDIX A

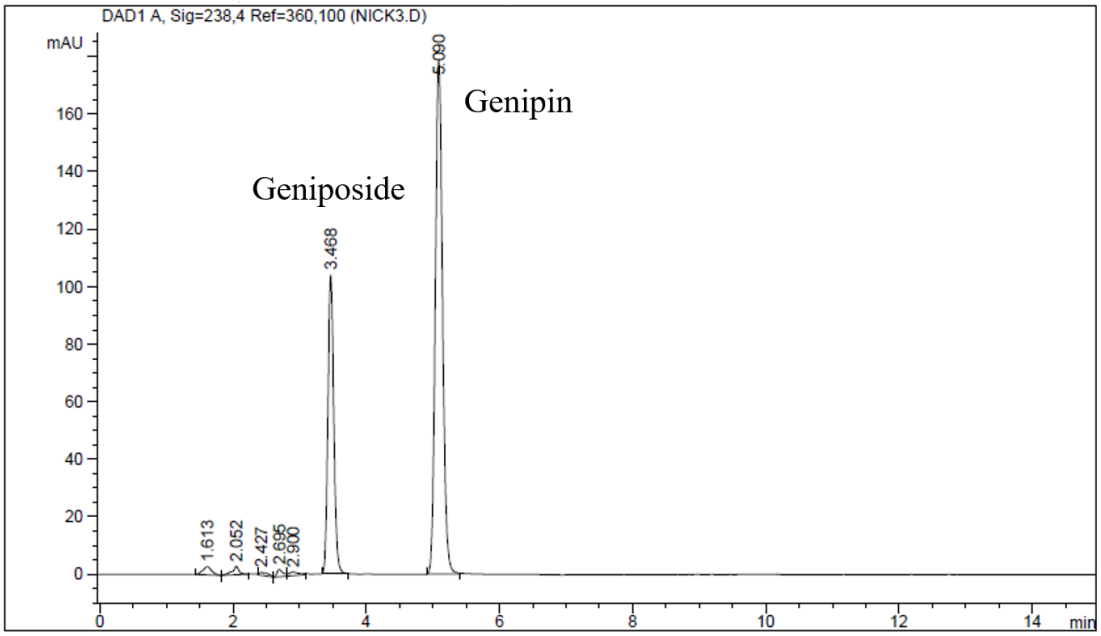

Figure A.1 Chromatogram of geniposide and genipin.

Table A.1 The contents of geniposide at various cellulase concentrations.

Cellulase concentration (mg/mL)	**Sample**	**Geniposide concentration (µg/mL)**	**Geniposide content (mg/g-plant)**	**Average (mg/g-plant)**
0	1	2329.5	38.8	37.7±1.3
	2	2282.4	38.0	
	3	2180.1	36.3	
2.5	1	2015.4	33.6	32.5±1.0
	2	1929.4	32.2	
	3	1903.9	31.7	
5	1	1725.9	28.8	28.6±0.7
	2	1755.8	29.3	
	3	1669.1	27.8	
10	1	1378.5	23.0	23.4±0.7
	2	1381.3	23.0	
	3	1448.0	24.1	
50	1	720.5	12.0	12.3±0.6
	2	719.9	12.0	
	3	780.8	13.0	

Table A.2 The contents of genipin at various cellulase concentrations.

Cellulase concentration (mg/mL)	**Sample**	**Genipin concentration (µg/mL)**	**Genipin content (mg/g-plant)**	**Average (mg/g-plant)**
0	1	-	-	-
	2	-	-	
	3	-	-	
2.5	1	918.8	15.3	16.2±1.2
	2	940.7	15.7	
	3	1054.5	17.6	
5	1	1231.9	20.5	21.5±1.2
	2	1255.1	20.9	
	3	1379.8	23.0	
10	1	1431.9	23.9	24.8±1.1
	2	1471.2	24.5	
	3	1555.7	25.9	
50	1	1548.1	25.8	26.5±1.0
	2	1567.9	26.1	
	3	1662.5	27.7	

Table A.3 The contents of geniposide (mg/g-plant) at different pH and time of incubation.*

Time of incubation (h)	**pH**				
	3.0	**3.5**	**4.0**	**4.5**	**5.0**
12	36.8±6.8	34.8±3.4	30.8±1.9	31.4±0.7	33.8±1.4
18	33.1±2.0	31.4±2.8	32.1±3.7	33.0±3.3	35.9±2.8
24	34.7±0.3	31.7±0.9	29.5±1.4	30.3±0.2	35.1±0.9
30	31.4±0.7	28.5±4.0	23.4±1.3	24.5±1.9	32.8±0.3
36	29.6±1.0	21.0±0.4	17.6±0.2	19.2±0.4	29.6±0.4

* The data are presented as average±SD (n=3).

Table A.4 The contents of genipin (mg/g-plant) at different pH and time of incubation.*

Time of incubation (h)	**pH**				
	3.0	**3.5**	**4.0**	**4.5**	**5.0**
12	21.3±2.8	23.4±2.2	21.7±3.1	20.6±3.3	16.7±3.1
18	23.7±2.6	25.5±1.9	27.0±2.8	26.6±2.6	20.2±2.0
24	30.0±2.2	32.9±0.4	34.1±0.6	31.5±0.4	26.4±0.9
30	31.1±1.0	33.7±2.4	34.3±1.2	30.8±1.7	24.7±0.9
36	34.8±4.8	33.8±0.9	35.5±1.3	32.6±0.4	25.0±1.7

* The data are presented as average±SD (n=3).

Table A.5 The genipin and geniposide content from the enzyme-assisted extraction combined with in situ product separation experiment.*

Treatment	**Geniposide (mg/g-plant)**		**Genipin (mg/g-plant)**	
	Water phase	Organic phase	Water phase	Organic phase
T1	45.72±1.31	2.62±0.15	4.03±0.27	0.72±0.11
T2	29.50±0.42	0.00±0.00	34.10±0.12	0.00±0.00
T3	31.38±0.46	2.02±0.20	16.66±0.27	42.17±0.99

* The data are presented as average±SD (n=3).

T1 is in situ product separation without cellulase treatment

T2 is cellulase treatment without in situ product separation

T3 is in situ product separation combined with cellulase treatment

APPENDIX B

Table B.1 Percent reduction of blue color after treatment with various adsorbent.*

Adsorbent	**% reduction of blue color**		Average
	sample 1	**sample 2**	
Calcium sulphate	96.15	96.00	96.06±0.07
Activated charcoal	89.52	89.51	89.52±0.01
Chitosan	58.42	58.38	58.40±0.02
Palm fiber	9.60	9.55	9.58±0.03
Calcium carbonate	3.53	3.50	3.52±0.01

* The data are presented as average±SD (n=2).

APPENDIX C

Standard curve for the determination of enzyme activity

Determination of p-nitrophenol in the sample

Standard	:	p-nitrophenol
Method	:	UV-visible spectrophotometry
UV-visible detector	:	410 nm
Concentration (µg/mL)	:	20, 40, 60, 80, 100, 120, 140, 160, 180 and 200

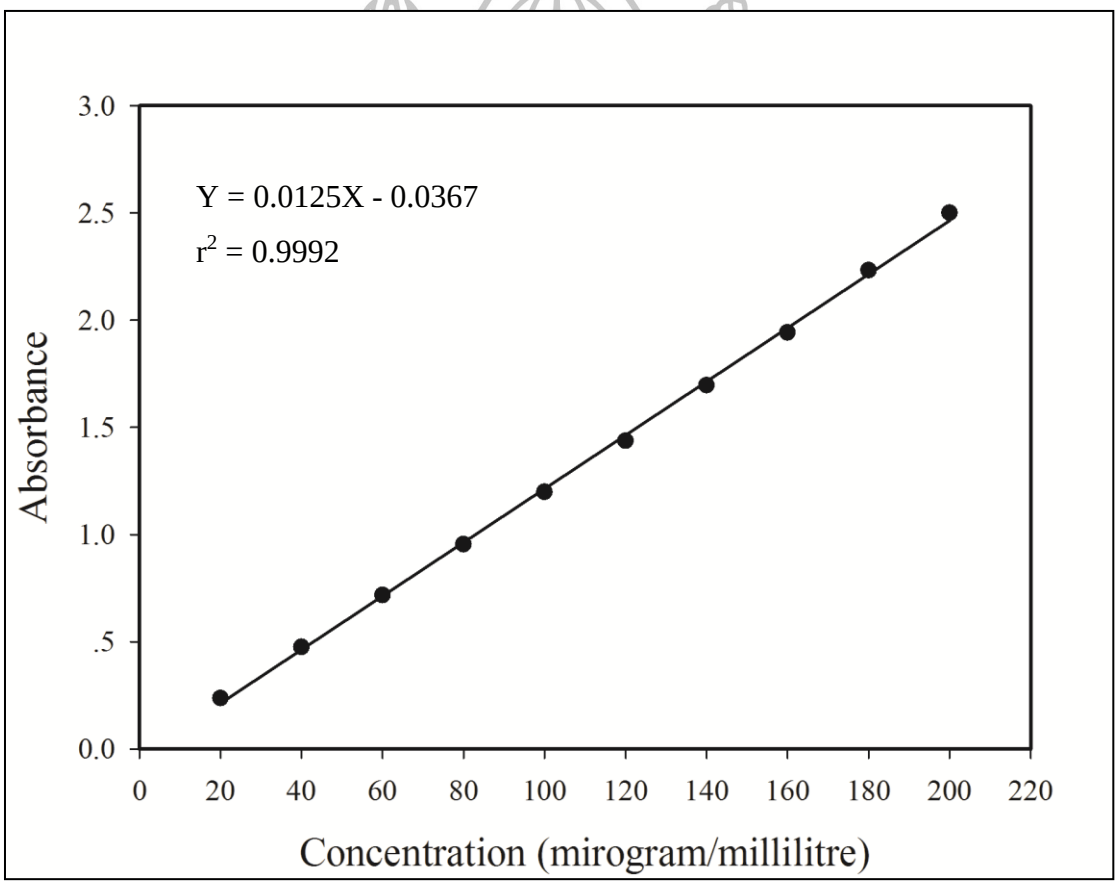

Figure C.1 Standard curve of p-nitrophenol.

Standard curve for the determination of protein content

Determination of protein in the sample

Standard	:	Bovine serem albumin
Method	:	UV-visible spectrophotometry
UV-visible detector	:	595 nm
Concentration (µg/mL)	:	5, 10, 20, 30 and 40

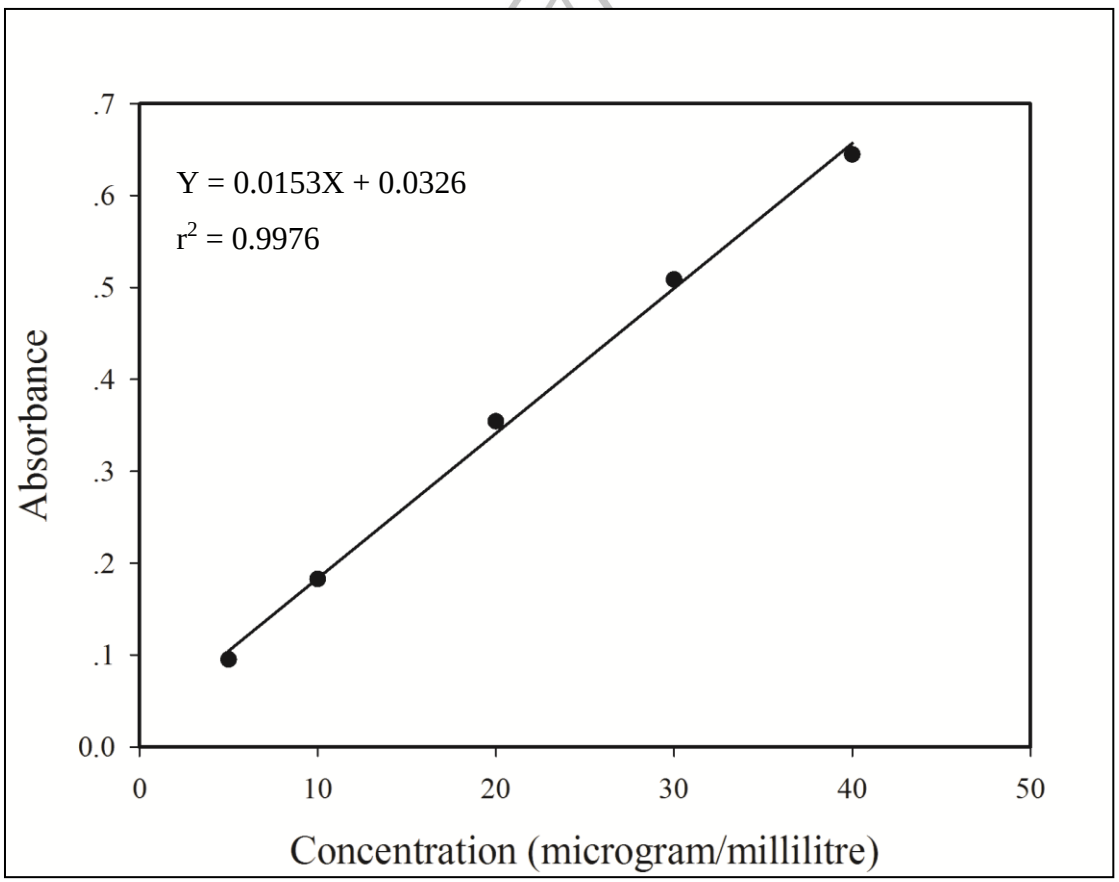

Figure C.2 Standard curve of bovine serum albumin.

Standard curve for the determination of genistin content

Determination of genistin in the sample

Standard	:	Genistin
Method	:	HPLC analysis
Analytical column	:	Luna 5u C18 column (250 mm × 4.6 mm, I.D.; 5 µm particle size)
Mobile phase	:	0.1% acetic acid/methanol (40:60, v/v)
Flow rate	:	1.0 mL/min
UV-visible detector	:	254 nm
Concentration (µg/mL)	:	2.5, 5, 10, 20 and 30

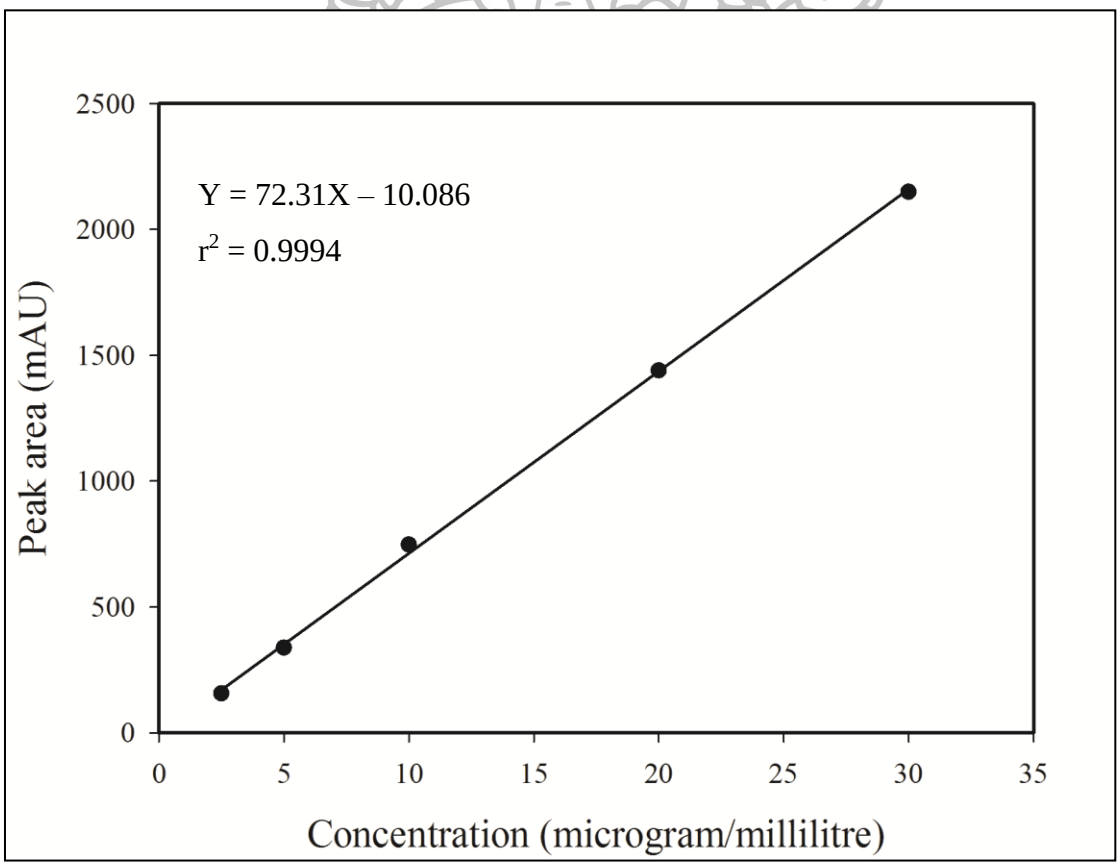

Figure C.3 Standard curve of genistin.

Standard curve for the determination of genistein content

Determination of genistein in the sample

Standard	:	Genistein
Method	:	HPLC analysis
Analytical column	:	Luna 5u C18 column (250 mm × 4.6 mm, I.D.; 5 µm particle size)
Mobile phase	:	0.1% acetic acid/methanol (40:60, v/v)
Flow rate	:	1.0 mL/min
UV-visible detector	:	254 nm
Concentration (µg/mL)	:	2.5, 5, 10, 20 and 30

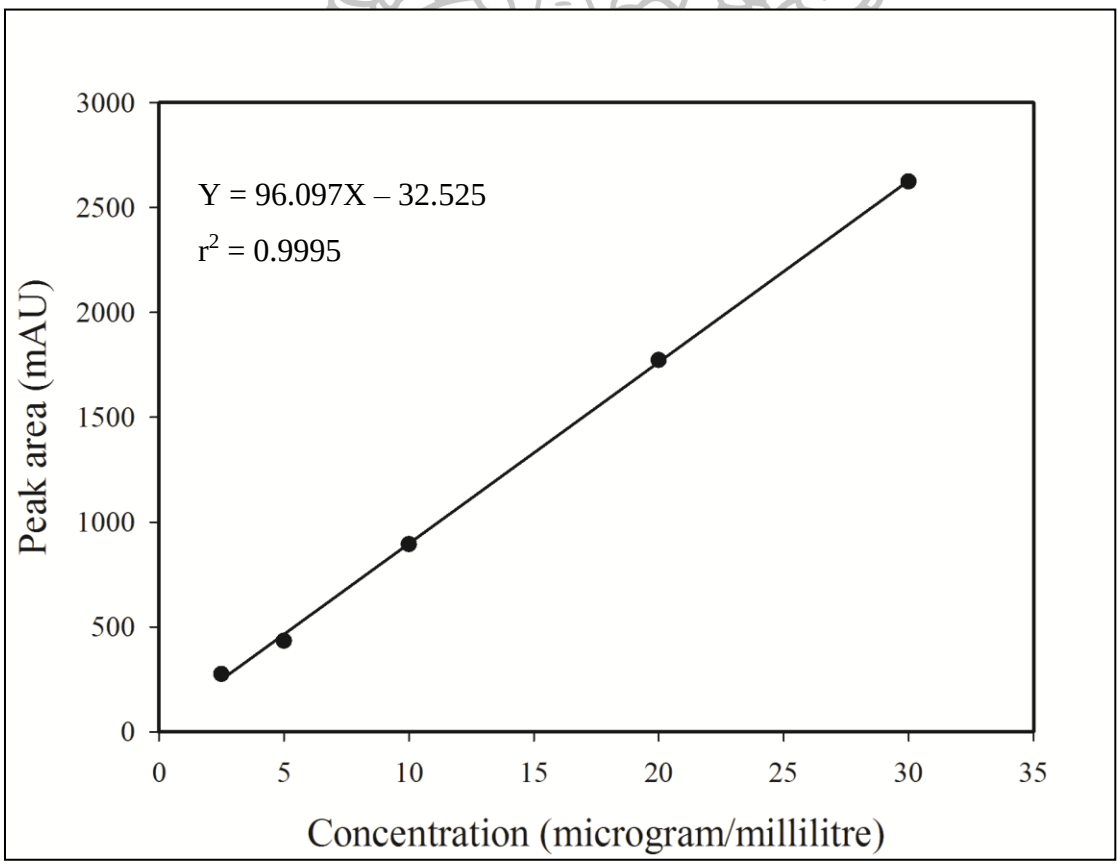

Figure C.4 Standard curve of genistin.

Table C.1 The enzyme activity of immobilized bead after cross-linking with glutaraldehyde.*

Concentration (%)	**Time of immobilization (h)**			
	3	**6**	**12**	**18**
1.0	1.89±0.03	2.04±0.10	2.89±0.09	2.97±0.08
2.5	0.71±0.02	1.43±0.08	1.78±0.07	1.92±0.09
5.0	0.25±0.02	0.54±0.02	0.79±0.04	1.08±0.04

* The data are presented as average±SD (n=3).

Table C.2 The enzyme activity of immobilized bead after cross-linking with genipin method 1.*

Concentration (%)	**Time of immobilization (h)**			
	3	**6**	**12**	**18**
0.1	3.50±0.10	4.37±0.14	3.20±0.05	2.61±0.17
0.5	3.88±0.06	4.38±0.05	3.07±0.09	2.61±0.09
1.0	3.54±0.18	4.34±0.01	2.63±0.07	2.28±0.05

* The data are presented as average±SD (n=3).

Table C.3 The enzyme activity of immobilized bead after cross-linking with genipin method 2.*

Concentration (%)	**Time of immobilization (h)**			
	3	**6**	**12**	**18**
0.1	2.87±0.05	2.52±0.05	2.27±0.07	2.24±0.07
0.5	2.29±0.10	2.29±0.10	2.24±0.06	1.99±0.10
1.0	2.19±0.08	2.16±0.06	2.04±0.04	1.81±0.10

* The data are presented as average±SD (n=3).

Table C.4 The enzyme activity of immobilized bead after cross-linking with genipin method 3.*

Concentration (%)	**Time of immobilization (h)**			
	3	**6**	**12**	**18**
0.1	3.11±0.03	3.85±0.03	3.46±0.09	3.05±0.05
0.5	3.23±0.03	3.57±0.03	3.48±0.03	3.13±0.03
1.0	2.82±0.01	3.71±0.08	3.56±0.06	2.90±0.02

* The data are presented as average±SD (n=3).

Table C.5 The enzyme activity of immobilized bead without cross-linking process.*

Concentration (%)	**Time of immobilization (h)**			
	3	**6**	**12**	**18**
-	5.10±0.23	6.67±0.33	5.05±0.34	4.67±0.13

* The data are presented as average±SD (n=3).

Table C.6 The enzyme activity from the experiment of effect of pH on activity of immobilized enzyme.*

Preparation method	**pH**				
	4.0	**5.0**	**6.0**	**7.0**	**8.0**
GLU	0.97±0.09	1.97±0.34	3.00±0.37	1.42±0.15	1.08±0.04
GEN1	2.00±0.21	3.51±1.08	5.49±0.33	2.49±0.34	2.39±0.24
GEN2	2.01±0.14	2.79±0.28	3.22±0.34	2.52±0.22	2.51±0.24
GEN3	2.53±0.34	3.25±0.26	4.22±0.10	3.08±0.30	2.90±0.34
ADS	1.15±0.02	4.38±0.12	4.38±0.09	2.14±0.03	2.06±0.04
Soluble enzyme	5.42±0.14	10.46±0.89	8.60±0.22	2.73±0.16	0.79±0.01

*The data are presented as average±SD (n=3).

Table C.7 The relative reduction of enzyme activity from the experiment of effect of pH on activity of immobilized enzyme.*

Preparation method	**pH**				
	4.0	**5.0**	**6.0**	**7.0**	**8.0**
GLU	0.32±0.09	0.66±0.08	1.00±0.00	0.47±0.04	0.36±0.04
GEN1	0.36±0.06	0.64±0.10	1.00±0.00	0.45±0.08	0.44±0.05
GEN2	0.62±0.10	0.87±0.07	1.00±0.00	0.78±0.05	0.78±0.06
GEN3	0.60±0.07	0.77±0.06	1.00±0.00	0.73±0.06	0.69±0.07
ADS	0.26±0.03	1.00±0.00	0.99±0.02	0.49±0.01	0.47±0.02
Soluble enzyme	0.52±0.01	1.00±0.00	0.82±0.02	0.26±0.02	0.08±0.01

*The data are presented as average±SD (n=3).

Table C.8 The enzyme activity from the experiment of effect of temperature on activity of immobilized enzyme.*

Preparation method	**Temperature (°C)**				
	40	**50**	**60**	**70**	**80**
GLU	1.00±0.08	1.30±0.18	1.79±0.41	2.38±0.15	1.80±0.20
GEN1	2.94±0.21	3.04±0.69	5.84±0.84	6.23±1.68	3.53±0.49
GEN2	2.28±0.19	2.77±0.28	3.08±0.10	4.44±1.14	3.09±0.63
GEN3	2.13±0.13	2.31±0.15	4.08±0.49	4.37±0.47	2.93±0.16
ADS	2.68±0.05	3.76±0.31	4.86±0.27	5.45±0.27	3.04±0.20
Soluble enzyme	14.08±0.01	17.17±0.02	20.27±0.03	19.08±0.01	15.53±0.01

*The data are presented as average±SD (n=3).

Table C.9 The relative reduction of enzyme activity from the experiment of effect of temperature on activity of immobilized enzyme.*

Preparation method	**Temperature (°C)**				
	40	**50**	**60**	**70**	**80**
GLU	0.42±0.08	0.55±0.04	0.75±0.08	1.00±0.00	0.76±0.04
GEN1	0.47±0.05	0.49±0.13	0.94±0.17	1.00±0.00	0.57±0.09
GEN2	0.51±0.03	0.62±0.05	0.69±0.10	1.00±0.00	0.70±0.12
GEN3	0.49±0.04	0.53±0.04	0.93±0.09	1.00±0.00	0.67±0.06
ADS	0.49±0.04	0.69±0.07	0.89±0.03	1.00±0.00	0.56±0.05
Soluble enzyme	0.69±0.01	0.85±0.02	1.00±0.00	0.94±0.01	0.77±0.01

*The data are presented as average±SD (n=3).

Table C.10 The percent of residual activity the experiment of effect of pH on stability of immobilized enzyme.*

Preparation method	**pH**				
	4.0	**5.0**	**6.0**	**7.0**	**8.0**
GLU	67.89±4.07	100.00±0.00	77.44±7.97	58.29±2.90	56.99±6.55
GEN1	79.74±2.28	100.00±0.00	72.76±5.40	50.28±3.35	48.31±1.53
GEN2	79.42±7.71	100.00±0.00	68.45±5.05	58.22±4.25	51.54±5.21
GEN3	97.35±6.83	100.00±0.00	88.3±7.69	81.14±2.94	80.51±7.28
ADS	70.93±5.39	100.00±0.00	62.44±01.95	35.88±1.29	33.45±1.71
Soluble enzyme	33.55±2.37	100.00±0.00	42.49±9.02	8.60±1.48	3.12±1.05

*The data are presented as average±SD (n=3).

Table C.11 The percent of residual activity the experiment of effect of temperature on stability of immobilized enzyme.*

Preparation method	**Temperature (°C)**				
	40	**50**	**60**	**70**	**80**
GLU	100.00±0.00	95.91±3.68	44.80±7.45	19.27±3.38	18.47±1.39
GEN1	100.00±0.00	96.95±4.73	27.44±3.12	9.81±1.76	9.29±2.65
GEN2	99.23±5.31	100.00±0.00	38.99±4.46	6.15±1.62	5.91±1.41
GEN3	99.25±5.12	100.00±0.00	62.59±6.46	6.89±1.34	5.61±1.62
ADS	70.93±5.39	100.00±0.00	62.44±1.95	35.88±1.29	33.45±1.71
Soluble enzyme	100.00±0.00	69.50±1.63	8.76±1.83	4.18±1.46	4.06±0.99

*The data are presented as average±SD (n=3).

Table C.12 The percent of residual activity of immobilized chitosan beads by using 2 mM p-NPG as a substrate.*

Preparation method	**Number of used**				
	1	**3**	**5**	**7**	**10**
GLU	100.00±0.00	93.86±2.68	92.06±3.65	82.42±2.10	65.90±8.33
GEN1	100.00±0.00	72.27±2.32	69.45±2.12	71.11±3.21	64.36±6.99
GEN2	100.00±0.00	60.54±4.83	62.11±8.06	56.34±4.47	54.78±9.77
GEN3	100.00±0.00	97.91±2.35	95.94±5.31	93.59±7.55	92.55±2.04
ADS	100.00±0.00	53.64±1.86	44.01±1.52	39.15±1.36	36.03±1.25

* The data are presented as average±SD (n=3).

BIOGRAPHY

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2012	Outstanding research "Kawaguchi award" The 9th National Kasetsart University Kamphaeng Saen Conference.

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2016	Green Chemistry Centre of Excellent, Department of Chemistry, University of York, United Kingdom

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2. **Weerapath Winotapun**, Praneet Opanasopit, Tanasait Ngawhirunpat, Theerasak Rojanarata. “Increment of neuroprotective activity against 6-hydroxydopamine and hydrogen peroxide in NG108-15 cells by genipin conjugated with gabapentin.” The JSPS-NRCT Follow-Up Seminar 2015 and 31st International Annual Meeting in Phamaceutical Science, 22-23 Janurary 2015, Chulalongkorn University, Bangkok, Thailand.

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