IMMOBILIZATION OF β-GALACTOSIDASE ONTO ACTIVATED CHITOSAN SUPPORT BY USING COVALENT BINDING METHOD

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By

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LIST OF SYMBOLS

Symbol	Description	Unit
A_{410} sample	Absorbance of the reaction sample	nm
A_{410} blank	Absorbance of the blank sample	nm
E_{fin}	Enzyme protein after immobilization	mg/ml
E_{ini}	Enzyme protein before immobilization	mg/ml
DF	Dilution factor	-
IU_{bead}	Unit activity on the support (bead)	IU
IU_{fin}	Unit activity after immobilization	IU
IU_{ini}	Unit activity before immobilization	IU
IU_{loss}	Unit activity loss from the solution	IU
l	Light path length	cm
t	Reaction time	min
$V_{\beta\text{-Gal sol}}$	Volume of β-Gal solution	ml
V_T	Total reaction volume	ml
ε_{ext}	Milimolar extinction coefficient of ONP	$cm^2/\mu mol$

LIST OF ABBREVIATIONS

A.oryzae	*Aspergillus Oryzae*
ANOVA	Analysis of Variance
BCA	Bicinchoninic Acid
β-Gal	β-Galactosidase
CCD	Central Composite Design
E.coli	*Escherichia Coli*
Glut	Glutaraldehyde
HPLC	High Performance Liquid Chromatography
Immo. Yield	Immobilization Yield
IU	International Unit Activity
KH_2PO_4	Potassium Dihydrogen Phosphate
LAB	Lactic Acid Bacteria
Na_2HPO_4	Sodium Hydrogen Phosphate
ONP	O-nitrophenyl
ONPG	O-nitrophenyl-β-galactoside
RNA	Ribonucleic Acid
RSM	Response Surface Methodology
YI	Immobilization Yield
YIU	Activity Yield
[β-Gal]	β-Galactosidase Concentration
[Glut]	Glutaraldehyde Concentration

MENYEKAT GERAK β-GALAKTOSIDASE DENGAN MENGGUNAKAN KAEDAH IKATAN KOVALEN PADA SOKONGAN CITOSAN YANG DIAKTIFKAN

ABSTRAK

Prestasi β-galaktosidase (β-Gal) disekat gerak pada sokongan citosan telah dikaji dalam proses kelompok. Sokongan citosan telah diaktifkan dengan menggunakan ejen kimia iaitu larutan glutaraldehid. Objektif utama kajian ini adalah untuk mengkaji keadaan yang sesuai dalam penyediaan proses menyekat gerak β-Gal untuk mendapatkan nilai aktiviti β-Gal yang tinggi. Semasa penyediaan β-Gal disekat gerak, keputusan keadaan optimum telah diperoleh daripada Kaedah Gerak Balas Permukaan (KGBP). Keadaan optimum yang telah diperoleh untuk kepekatan larutan glutaraldehid dan β-Gal adalah masing-masingnya sebanyak 2% dan 10 mg/ml, yang telah memberi keputusan 65.52% untuk hasilan aktiviti manakala untuk hasilan menyekat gerak adalah sebanyak 94.57%. Keputusan eksperimen untuk mengkaji kesan suhu ke atas aktiviti β-Gal, didapati suhu optimum pada 58℃ telah diperolehi selepas proses menyekat gerak β-Gal. Walau bagaimanapun, ia sangat menarik apabila merujuk kepada julat haba yang lebih luas, β-Gal disekat gerak menunjukkan kestabilan operasi yang lebih baik berbanding dengan β-Gal bebas. Aktiviti β-Gal disekat gerak telah meningkat lebih dari 17% daripada aktiviti awalnya, manakala aktiviti β-Gal bebas berkurang ke 15%. Kestabilan yang tinggi pada β-Gal akan membawa kepada kebolehsaknaan menggunakannya semula yang lebih baik. β-gal disekat gerak boleh digunakan semula sebanyak enam kitaran dan kira-kira 50% daripada aktiviti awalnya dapat dikekalkan.

IMMOBILIZATION OF β-GALACTOSIDASE ONTO ACTIVATED CHITOSAN SUPPORT BY USING COVALENT BINDING METHOD

ABSTRACT

The performance of immobilized β-galactosidase (β-Gal) onto chitosan support was studied in a batch process. The chitosan support was activated by using chemical agent which was glutaraldehyde solution. The main objective of this research was to investigate the suitable conditions in preparation of β-Gal immobilization process that will produce greater β-Gal activity. During the preparation of immobilized β-Gal, the optimum conditions were obtained from Response Surface Methodology (RSM). The optimum conditions were found to be 2% and 10 mg/ml for glutaraldehyde and β-Gal concentrations, respectively, which has resulted in 65.52% for activity yield meanwhile for immobilization yield, is 94.57%. The experimental results for investigation of effect of temperature on β-Gal activity, found that the optimum temperature at 58℃ was obtained after β-Gal immobilization. However, it is so interesting that the immobilized β-Gal showed much better operational stability referring to a broader thermal range as compared to free β-Gal. The activity of immobilized β-Gal increased at about more than 17% of its initial activity, meanwhile the activity of free β-Gal reduced at less than 15%. Higher stability of β-Gal will lead to a better reusability. The immobilized β-Gal can be reused for six cycles and retained approximately 50% of its initial activity.

CHAPTER ONE

INTRODUCTION

The first chapter presents a brief overview of the research background in association to enzyme. Also, the important of immobilized enzyme usage in industry is described in this chapter. Therefore, the enzyme immobilization procedure is proposed in the following subsection. This section also introduces the problem statement, research objectives and the scope of study.

1.1 Enzyme

Generally, enzymes are proteins that act as bio-catalysts in plant cell, animal cell and organisms. Other than that, some ribonucleic acid (RNA) molecules are also have bio-catalytic properties, but the great majority of cellular reactions are mediated by protein catalysts (Bhagavan and Ha, 2015). Enzymes are versatile and very active biological catalysts, lead to greater reaction rates as compared to chemically catalyzed reactions under ambient conditions. They are highly specific and usually used to catalyze one type of reaction and some of them are specified for a single substrate (Liu, 2017). Besides that, some enzymes can be utilized on the same type of reaction but act on several, structurally related substrates.

Bhagavan and Ha (2015) reported that an enzyme-catalyzed reaction is introduced when the enzyme binds its substrate to form an enzyme-substrate complex. Generally, enzyme molecules are significantly larger than the substrate molecules excluding proteinases, nucleases, and amylases that work on macromolecular substrates. Active site is a pocket in the surface of the enzyme that composes only a small portion of the enzyme molecule and at this site, catalytic function occurs after the attachment of

the substrate. The specificity of an enzyme for a substrate can be pictured as reflecting a lock-and-key relationship as illustrated in Figure 1.1. This representation shows that the enzyme has an active site that matches the exact size of the substrate. In fact, the actual structure of an active site of enzyme bounded with substrate is more complex as shown in Figure 1.2.

Figure 1.1: Lock and key model (Bhagavan and Ha, 2015)

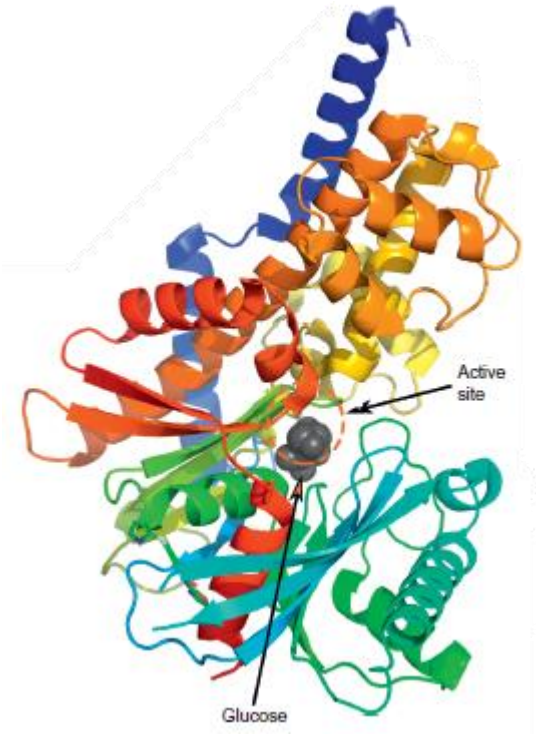

Figure 1.2: Structure of an active site with a bound substrate analog (Bhagavan and Ha, 2015)

Along with several advantages, there exist a number of practical problems in utilizing enzyme in the chemical processes. For example, the high cost of separation and purification of enzymes from the product and their sensitivity both to process conditions

and to substances that known as inhibitor (Krajewska, 2004). Numerous methods have been suggested to overcome these limitations, one of the most favourable being enzyme immobilization. An immobilized enzyme is an enzyme that is physically coupled or trapped to a water-insoluble supporting material, matrix or carrier (Bayindirli, 1995). Enzymes can be immobilized by several methods such as adsorption, covalent binding, entrapment, encapsulation and cross-linking.

Consequently, as compared to soluble enzymes in a solution, the immobilized enzymes are more resistant to environmental changes and more robust. Other than that, the different phase between immobilized enzyme and the product allows easy recovery, can be reused many times, use in a continuous operation of enzymatic processes and can be employed into various types of bioreactor designs (Krajewska, 2004). For the implementation in a commercial process all useful and harmful effects of whether a chemical catalyst or an enzyme is chosen, and whether a free or immobilized enzyme is used, have to be taking into account all relevant aspects, including health, environmental and economic viability (Kennedy et al., 1983).

1.2 Problem Statement

There are principally two different ways to use enzyme in industry which are; soluble enzyme and immobilized enzyme. The soluble enzyme is usually applied for a batch process meanwhile the immobilized enzyme is utilized in a continuous operation (Szczodrak, 2000). Immobilized enzyme has been normally considered as a solution for the problem regarding soluble enzyme, but it has remained inapplicable due to the high cost of support materials which proved technical success in the immobilization procedure (Carrara and Rubiolo, 1994). Regardless the high cost of enzyme attachment, immobilized enzyme remains more economically reasonable than the free enzyme. The

utilization of immobilized enzyme can be performed continuously and provides the possibility of reutilizing the enzyme (Illanes et al., 1990; Axelsson and Zacchi, 1990; Bódalo et al., 1991).

In this research, *A.oryzae* β-Gal is used and chitosan has been chosen as a support in the immobilization of β-Gal. Chitosan is a polysaccharide consisting of 2-amino-2-deoxy-D-glucose units which are combined by β-1, 4-linkages. It is formed by deacetylation with a drastic alkaline treatment of chitin. Chitin is the main component in the exoskeletons of crustaceans, insects and also in the cell walls of some fungi (Carrara and Rubiolo, 1994). In addition, the cost of this material with respect to others used as the support is relatively cheap. The immobilization of enzyme on chitosan can achieved by the glutaraldehyde reaction between the free amino groups of chitosan and the enzymes to form covalent linkages (Carrara and Rubiolo, 1994). This research will focus on the obtaining greater enzymatic activity and stability by manipulating of the glutaraldehyde concentration during support activation and the variation of enzymes used in immobilization procedure.

1.3 Research Objectives

The increase of the enzyme stability is much important in biotechnology and could be achieved by covalent immobilization of enzyme onto the suitable insoluble carriers. With this expectation, the aim of this research is to investigate the most suitable conditions for immobilization process that will produce greater enzymatic activity. The chitosan is used as a support in β-Gal immobilization. The significant objectives include:

i. To determine the optimum process parameters in β-Gal immobilization process.

ii. To study the effect of temperature on β-Gal activity.

iii. To study the reusability of the immobilized β-Gal.

1.4 Scope of Study

In this research, the *A.oryzae* β-Gal was immobilized onto chitosan activated support by covalent binding method. The preparation of chitosan activated support was performed by using chemical agent which is glutaraldehyde.

The concentrations of glutaraldehyde and β-Gal solution were the two parameters being manipulated to obtain the optimum conditions that provide the greater β-Gal activity. In this study, the range of concentrations of glutaraldehyde and β-Gal solution used are 2% to 10% and 10 mg/ml to 50 mg/ml with intervals of 4% and 20 mg/ml, respectively. All of the samples obtained are examined by activity and protein assay. The initial unit activity of *A.oryzae* β-Gal used in this experiment was more than or equal to 8 IU/mg. Generally, the range of temperature and pH applied in this experiment are at room temperature to 37℃ and 5.5 to 6.86, respectively.

The optimal immobilized β-Gal was used further to study the effect of temperature on β-Gal activity and the recyclability of the immobilized β-Gal.

CHAPTER TWO

LITERATURE REVIEW

2.1 General Information of β-Galactosidase

β-galactosidase or lactase (β-D-galactoside galactohydralase) is an enzyme used to catalyze the hydrolysis of β-1,4-D-galactosidic linkages (Anisha, 2017). Lactose is a disaccharide sugar that can be broken down into two monosaccharide sugars, galactose and glucose with the aid of β-Gal as illustrated in Figure 2.1. Galactose and glucose, produced by hydrolysis of lactose are more soluble and sweeter that suitable to be used as a food ingredient and they are easy to consume (Santos et al., 1998). Various microbial sources of β-Gal have been used for the intention of economic production of low lactose in food composition (Roy and Gupta, 2003; Santos et al., 1998).

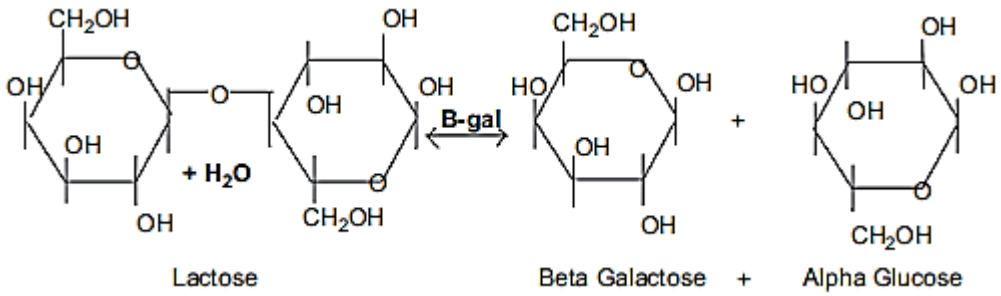

Figure 2.1: Hydrolysis of the disaccharide lactose into two monosaccharides, galactose and glucose by β-galactosidase (Anisha, 2017)

2.1.1 Sources of β-Galactosidase

β-Gal enzyme exists in a wide diversity of organisms including microorganisms, plants and animal tissues. The production of β-Gal from microorganisms offer greater yields compared to plants and animal tissues. β-Gal takes place in a multiplicity of microorganisms including bacteria, fungi and yeast (Rosenberg, 2006).

2.1.1.a Bacterial Sources

Bacterial sources of β-Gal were reviewed by several scientists such as Rosenberg (2006), Chen et al. (2008) etc., and they were summarized in Table 2.1.

Table 2.1: Bacterial sources of β-galactosidase

Sources	Explanation	References
Escherichia coli	Not suitable for food processes due to its possible toxic factors associated with coliforms	(Rosenberg, 2006)
Pyrococcus woesei, Thermus sp., Bacillus stearothermophilus	Produce thermostable β-galactosidase enzyme	(Chen et al., 2008)
Arthobacter psychrolactophilus, Pseudoalteromonas haloplanktis	Produce cold-active and cold-adapted β-galactosidase enzyme	(Nakagawa et al., 2007; Van De Voorde et al., 2014)
Bifidobacterium sp., Lactobacillus sp.	Produce β-galactosidase that acts as probiotics in food	(Husain, 2010)

β-Gal from bacterial sources are generally used for the hydrolysis of lactose because of the ease of fermentation and high enzyme activity compared to β-Gal from fungal sources (Husain, 2010; Picard et al., 2005). Lactic acid bacteria (LAB) from bacterial sources, comprised of variety groups of lactococci, streptococci and lactobacilli which offers the possibility of using the enzyme produced without any further purification (Husain, 2010; Vasiljevic and Jelen, 2002; Vinderola and Reinheimer, 2003).

2.1.1.b Fungal Sources

The β-Gal from fungal sources are commonly extracellular and thermostable, but vulnerable to inhibitor especially galactose (Anisha, 2017). Husain (2010) reported

that there are the two main strategies for the catabolism of lactose by fungi; first is extracellular hydrolysis and subsequent uptake of monomer produced and second is uptake of disaccharides. Fungal sources of β-Gal are summarized in Table 2.2.

Table 2.2: Fungal sources of β-galactosidase

Sources	Explanation	References
Aspergillus niger	Produce thermostable β-galactosidase enzyme	(Chen et al., 2008)
Teratosphaeria acidotherma	Produce acidophilic intracellular β-galactosidase enzyme	(Isobe et al., 2013)
Aspergillus oryzae	Produce of β-galactosidase enzyme	(Bailey and Linko, 1990)

2.1.1.c Yeast Sources

In general, yeast β-Gal is intracellular enzyme. Yeast sources of β-Gal are investigated by the researchers and they were summarized in Table 2.3.

Table 2.3: Yeast sources of β-galactosidase

Sources	Explanation	References
Yeast *K. lactis*	Its natural habitat being the dairy environment	(Chen et al., 2008; Rech and Ayub, 2007)
Kluyveromyces marxianus	It is capable of growing on various substrates including lactose	(Ribeiro et al., 2007)
Yeast *Guehomyces pullulans*	Produce of cold-active acid β-galactosidase enzyme	(Song et al., 2010)

2.1.2 Assay of β-Galactosidase Activity

The detection of β-Gal enzymatic activity can be carried out by measuring the hydrolysis of the chromogenic substrate of o-nitrophenyl-β-D-galactoside (ONPG) to o-nitrophenol (ONP). The amount of ONP obtained is proportional to the amount of β-Gal

and the time of the reaction. By adding sodium carbonate, the reaction is terminated due to a change of the reaction mixture to pH 11. The fact that, the ONP gives the yellow-colored solution, this is due to the β-Gal is deactivated at high pH (Anisha, 2017). The amount of ONP produced can be analyzed by determining the absorbance at 410 nm. The reaction mechanism for hydrolysis of ONPG by β-Gal is illustrated in Figure 2.2.

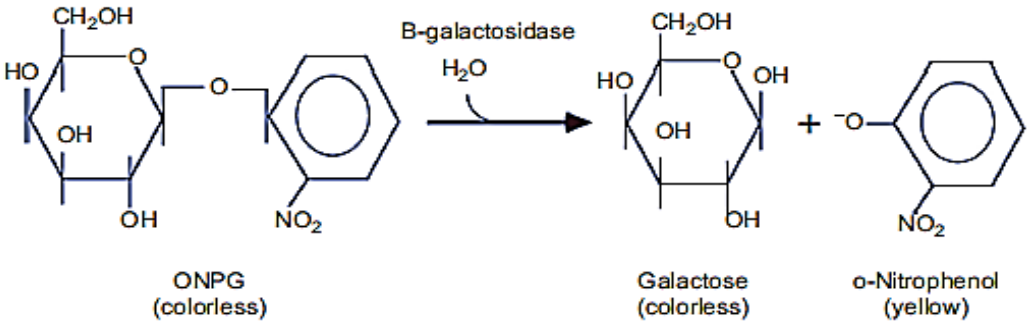

Figure 2.3: Hydrolysis of ONPG by β-galactosidase (Anisha, 2017)

2.1.3 Industrial Scenario

Conventionally, the β-Gal from *Aspergillus* sp. and *Kluyveromyces* sp. are most extensively used in the industry (Husain, 2010; Panesar et al., 2007; Siso, 1996). This is because their productivities are comparatively higher. The optimum conditions for the *Aspergillus* sp. β-Gal are at pH in the acidic range (2.5-5.4) and for temperature, it can withstand high temperature up to 50℃ (Panesar et al., 2007). Therefore, they are suitable applied in the hydrolysis of acid whey, which derives from the synthesis of fresh or soft cheeses (Yang and Silva, 1995).

The β-Gal can be employed in the form of either free or immobilized enzyme in any industrial applications. Nonetheless, the utilization of free enzyme is technically simpler, its main disadvantage is that the enzyme in the reaction solution cannot be reused, thus increases the cost of repeated production of enzyme. The immobilized β-Gal provides the potentiality of repetitive and continuous use of enzyme with excellent operational stability (Husain, 2010).

2.2 Immobilization of Biocatalyst

Nowadays, the immobilized enzymes are the topic of considerable interest. This is because of the anticipated advantages over soluble or free enzyme. According to Tischer and Wedekind (1999), two main targeted advantages from the immobilized enzymes are easy separation of the enzyme from the product and reusability of the enzyme.

Easy separation of the enzyme and the product facilitates enzyme applications and supports a dependable and effective reaction technology, Meanwhile, reusability of enzymes offers cost benefits which is an important requirement for establishing an enzyme catalyzed process (Tischer and Wedekind, 1999).

2.2.1 Support and Method of Immobilization

Theoretically, enzyme immobilization is a technique designed to restrain the movement of an enzymes. The important consideration is to decide on the immobilization method, then the support material by taking into account the intended use and application. Table 2.4 lists some of the points to consider in selecting a support and method of immobilization.

Table 2.4: Fundamental considerations in selecting a support and method of immobilization (Bickerstaff, 1997)

Property	Points for consideration
Physical	Strength, noncompression of particles, available surface area, shape or form, degree of porosity, flow rate and pressure drop
Chemical	Hydrophilicity, inertness toward enzyme, available functional groups for modification and regeneration of support
Stability	Storage, residual enzyme activity, regeneration of enzyme activity and mechanical stability of support material
Resistance	Bacterial of fungal attack, disruption by chemicals, pH, temperature, organic solvents and protease

Safety	Biocompatibility, toxicity of component reagents, health and safety for process workers and end product users
Economic	Availability and cost of support, chemicals, special equipment, reagents, technical skill required, environmental impact, continuous processing and reusable support
Reaction	Flow rate, enzyme loading and catalytic productivity, reaction kinetics, side reactions, multiple enzyme systems, batch, CSTR, PBR, FBR and so on; diffusion limitations on mass transfer of substrates and products

2.2.1.a Adsorption

Immobilization by adsorption is the simplest method and implies reversible surface interactions between enzyme and support material as shows in Figure 2.3 (Woodward, 1985; Messing, 2012). The adsorption of enzyme onto the supports mostly due to Van der Waals forces, ionic and hydrogen bonding interactions. Though these forces are very weak, they are abundantly large in number to enable binding to the surface of support. There is no chemical activation required due to the existing surface chemistry between the enzyme and support is material.

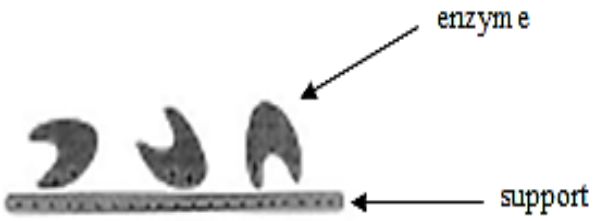

Figure 2.4: Enzyme immobilization by adsorption method (Bickerstaff, 1997)

Bickerstaff (1997) described that the procedures for this method consists of combining together the enzyme and a support with adsorption properties, carried out under suitable conditions of ionic strength, pH, temperature and so on, for a period of incubation, pursued by collection of the immobilized enzyme and immense washing to remove nonbound enzyme.

The advantages of this method are little conformational change of the enzyme or destruction of its active site. Besides it is simple, cheap and quick to obtain immobilized enzymes. Meanwhile, its disadvantages are desorption of the protein resulting from changes in pH, ionic strength and temperature, nonspecific binding and leakage of enzymes from the support (Vuong, 2017; Bickerstaff, 1997).

2.2.1.b Covalent Binding

This kind of immobilization method (see Figure 2.4) implies the formation of a covalent bond between the enzyme and support (Woodward, 1985). Srere and Uyeda (1976) reported that the bond is principally formed between functional groups exist on the surface of the support and functional groups applying to amino acid residues on the surface of the enzyme. The amino groups that usually involved are the amino group (NH_2) of lysine or arginine, the carboxyl group (CO_2H) of aspartic acid or glutamic acid and the hydroxyl group (OH) of serine or threonine.

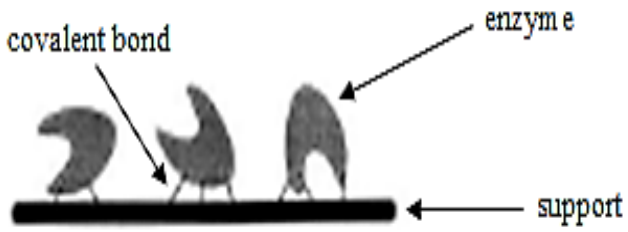

Figure 2.5: Enzyme immobilization by covalent binding method (Bickerstaff, 1997)

According to Bickerstaff (1997)**,** there are many factors may influence the selection of suitable support and hydrophilicity is the most essential factor for maintaining enzyme activity in a support environment. The polysaccharide polymers such as chitosan are very hydrophilic and commonly used as support materials for enzyme immobilization in the bead form. However, they are vulnerable to microbial disintegration

Scouten (1987) reported that there are few reaction procedures for coupling a support and an enzyme in a covalent bond method which are; first is formation of an isourea linkage, formation of diazo linkage, formation of a peptide bond and the last is an alkylation reaction. The most important criteria are to choose an activation method that will not inactivate the enzyme by reacting with amino acids at the active site. Fundamentally, there are two steps are involved in a covalent binding of the enzymes to the support materials. Firstly, the support materials need to be activated by a specific reagent such as glutaraldehyde and subsequently, the enzyme is added in a coupling reaction to form a covalent bond with the support material. The activation process is applied to introduce strong electrophilic functional groups on the support for enzyme binding.

The advantages of this method are preventing elution of protein into the production stream and offer a great of flexibility in designing an immobilized enzyme with specific chemical and physical properties such as charge distribution. Meanwhile, its disadvantages are expensive and involve complicated procedures. Other than that, the activity yield also may be low due to exposure to the harsh environment or harmful reagent (Vuong, 2017).

2.2.1.c Entrapment

Besides of the adsorption and covalent binding, enzyme can be immobilized by entrapment where its movement is restricted by the lattice structure of a gel (Bickerstaff, 1995; O'driscoll, 1976). Figure 2.5 shows the entrapment method of enzyme. The porosity of the gel lattice must be controlled to assure that the structure is fit enough to prevent leakage of enzymes and at the same time all owing the free movement of the substrate and the product. Literally, the support acts as selective

barrier to substrate mass transfer and it can be designed to protect the immobilized enzyme from the toxic chemicals.

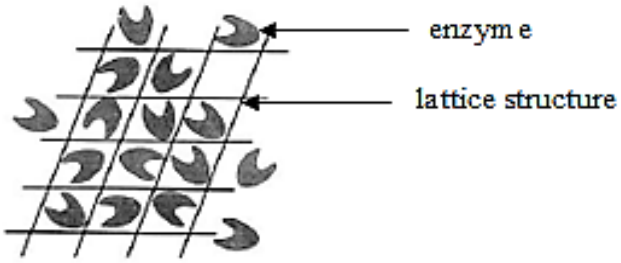

Figure 2.6: Enzyme immobilization by entrapment method (Bickerstaff, 1997)

Bickerstaff (1997) elaborated that entrapment of enzyme can be achieved by mixing the free enzyme solution with a polyionic polymer material and then crosslinking the polymer with multivalent cations in an ion exchange reaction to form a lattice structure that traps the enzyme. This approach is recommended to produce a highly stable immobilized enzyme.

The advantages of this method are simple and can be combined with different method easily, while the advantages are easy leakage and lower stability compared to other methods (Vuong, 2017).

2.2.1.d Encapsulation

The encapsulation of enzymes is similar to entrapment method in which the enzymes are free in solution but restricted in a space. This method usually carried out by enclosing the enzymes within various semipermeable membranes as illustrated in Figure 2.6 (Al-Muftah and Abu-Reesh, 2005; Groboillot et al., 1994; Kierstan and Coughlan, 1991; Nilsson, 1987). Enzyme cannot pass through the membrane, but small substrate and products can pass freely across the semipermeable membrane.

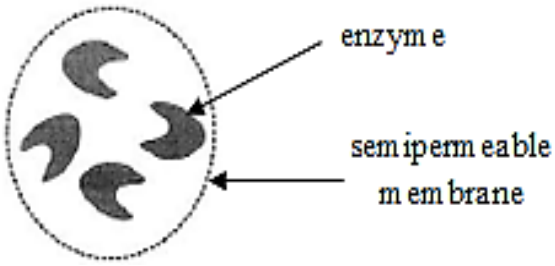

Figure 2.7: Enzyme immobilization by encapsulation method (Bickerstaff, 1997)

2.2.1.e Cross Linking

This approach does not use support to immobilize the enzyme. The enzymes are joined together to form a large three dimensional complex structure. This method can be achieved by the chemical or physical methods (Broun, 1976). Figure 2.7 shows an illustration of cross-linked enzyme.

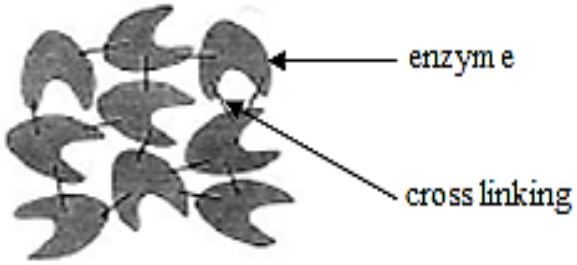

Figure 2.8: Enzyme immobilization by cross linking method (Bickerstaff, 1997)

The cross linking by physical method can be achieved through flocculation which leads to high density of enzyme. There are some flocculating agents have been used widely such as polyamines, polystyrene sulfonate and various phosphates. The chemical cross linking involves the covalent bond formation between the enzymes assistance by multifunctional agent such as glutaraldehyde and toluene diisocyanate (Bickerstaff, 1997).

The advantages of this method are very little desorption and the enzyme is strongly bounded. However, this method may change the chemical structure of the active site of enzyme and thus reduced activity yield (Vuong, 2017).

2.2.2 Materials Utilized as Support

There are many materials have been used as a support or carrier to immobilize the enzyme. Generally, the supporting materials are categorized as organic, inorganic, and organic synthetic supports.

2.2.2.a Organic Support

Most enzymes have been easily immobilized by adsorption, entrapment, covalent binding or cross linking onto organic support from carbohydrates source due to its strong hydrophilicity which offer suitable conditions for immobilization (Datta et al., 2013; Tischer and Wedekind, 1999; Chakraborty et al., 2016). The organic support from carbohydrates source such as chitosan, chitin, cellulose, carrageenan, gelatin, alginate and starch are widely used to immobilize an enzyme. In addition, proteins source including albumin, collagen and ceratin also can be used as the organic support. Usually, the organic supports are relatively cheap and available in abundant amount. However, they tend to be affected by factors such as pH and microbial contamination (Bayindirli, 1995).

Vorlop and Klein (1987) suggested that the chitosan or chitin is perfect material to be used as a support since it is susceptible to enzyme immobilization reactions and widely available on the market. Besides, it is simple and can be easily activated by chemical agent. Recently, application of chitosan into magnetic nanoparticles for immobilization of enzymes and encapsulation drawn more attention (Waifalkar et al., 2016).

2.2.2.b Inorganic Support

The inorganic support including alumina, nickel oxide, silica gel, activated carbon, glass (solid and porous) and ceramic are most commonly used as a support material (Tischer and Wedekind, 1999). They are very stable which lead to a better

diffusion and flowing characteristics compared to the organic support. Moreover, they are not susceptible to microbial contamination attacks, good thermal stability and have suitable mechanical quality. However, inorganic supports are less reactive than organic ones and some metals contain little binding surface due to their nonporous characteristics (Hettiarachchy et al., 2018).

Generally, silica gel is used to entrap enzymes for maintaining natural properties of the carried enzymes to protect their catalytic efficiency and stability (Ronda et al., 2015). For the inorganic supports such as silica gel, its required to be modified and activated, but before activation, the supports need to be treated with reagents with the aim to give amino groups to the support since they are not reactive chemically (Hettiarachchy et al., 2018).

2.2.2.c Organic Synthetic Support

Polypropylene, polyamide, polyvinyl, polystyrene and nylon are the examples for organic synthetic support material for enzyme immobilization (Çetinus and Öztop, 2003; Costa et al., 2005). Similar to inorganic support, they are not receptive to be harmed by microbial contamination.

According to Tischer and Wedekind (1999), enzymatic binding to the organic synthetic polymers is better achieved at a reduced level of salt or reduced concentration and occurred mainly by adsorptive forces. Normally, reduction of enzyme activity after binding is achieved due to the water-repellent property on the support.

2.3 Effect of Temperature and pH on β-Galactosidase Activity

The important factors that will give effect on the soluble and immobilized enzymes are temperature and pH (Brena and Batista-Viera, 2006; Tischer and Wedekind, 1999).

2.3.1 Temperature

Generally, in many chemical reactions, as the temperature increases, the reaction rate also will be increased up to a certain point. In enzymatic reaction, the rate of reaction in a free diffusion environment rises up exponentially as suggested by the Arrhenius equation (Tischer and Wedekind, 1999). The enzyme stability will be affected and the protein structure of enzyme denatured at high temperature, resulting in decreasing enzyme activity (Brena and Batista-Viera, 2006).

Meanwhile, for immobilized enzyme, Tischer and Wedekind (1999) reported that if the immobilized enzyme carried out in diffusion reaction, the rate of reaction that affected by temperature is measured by the factor of efficiency. The efficiency factor is calculated by the ratio of substrate to product formed by the immobilized enzyme divided by the ratio of substrate to product formed by the free enzyme.

Eventually, the immobilized enzyme is more resistant to temperature denaturation (Brena and Batista-Viera, 2006). This is due to the high thermal stability of immobilized enzyme and its can be used in a high temperature condition.

2.3.2 pH

Each enzyme exhibits high activity at limited range of pH. Theoretically, the relationship between the enzyme and pH is matches to shape of the bell; the middle point of the bell is depicts the optimum point and symmetrical progression of activity reducing is located around that point (Brena and Batista-Viera, 2006).

Busto et al. (2007) had carried out a research related to the effect of pH on free and immobilized *Aspergillus niger* β-gal. The determination of activity for the free and immobilized enzyme are performed at a pH range between 5.2 to 12.6 and the result shows the highest activity for both free and immobilized enzyme at pH 4.5. Conclusively, the optimal pH for both free and immobilized enzyme is similar.

CHAPTER THREE

RESEARCH METHODOLOGY

In general, the research methodology is divided into several sections. First, the acceptable conditions of immobilization process were studied. The study mostly highlights on the effect of concentration of glutaraldehyde during support activation and the β-Gal concentration during immobilization. The significant effect of the temperature on the β-Gal activity was carried out by the assay of β-Gal activities and the reusability of the immobilized β-Gal was studied.

3.1 Research Flowchart

The research methodology is outlined according to the following flowchart in Figure 3.1. The research was started with preliminaries study of the immobilization process. An appropriate immobilization was carried out and optimum conditions were obtained. The effect of temperature on the activity of immobilized β-Gal and its recyclability were also investigated.

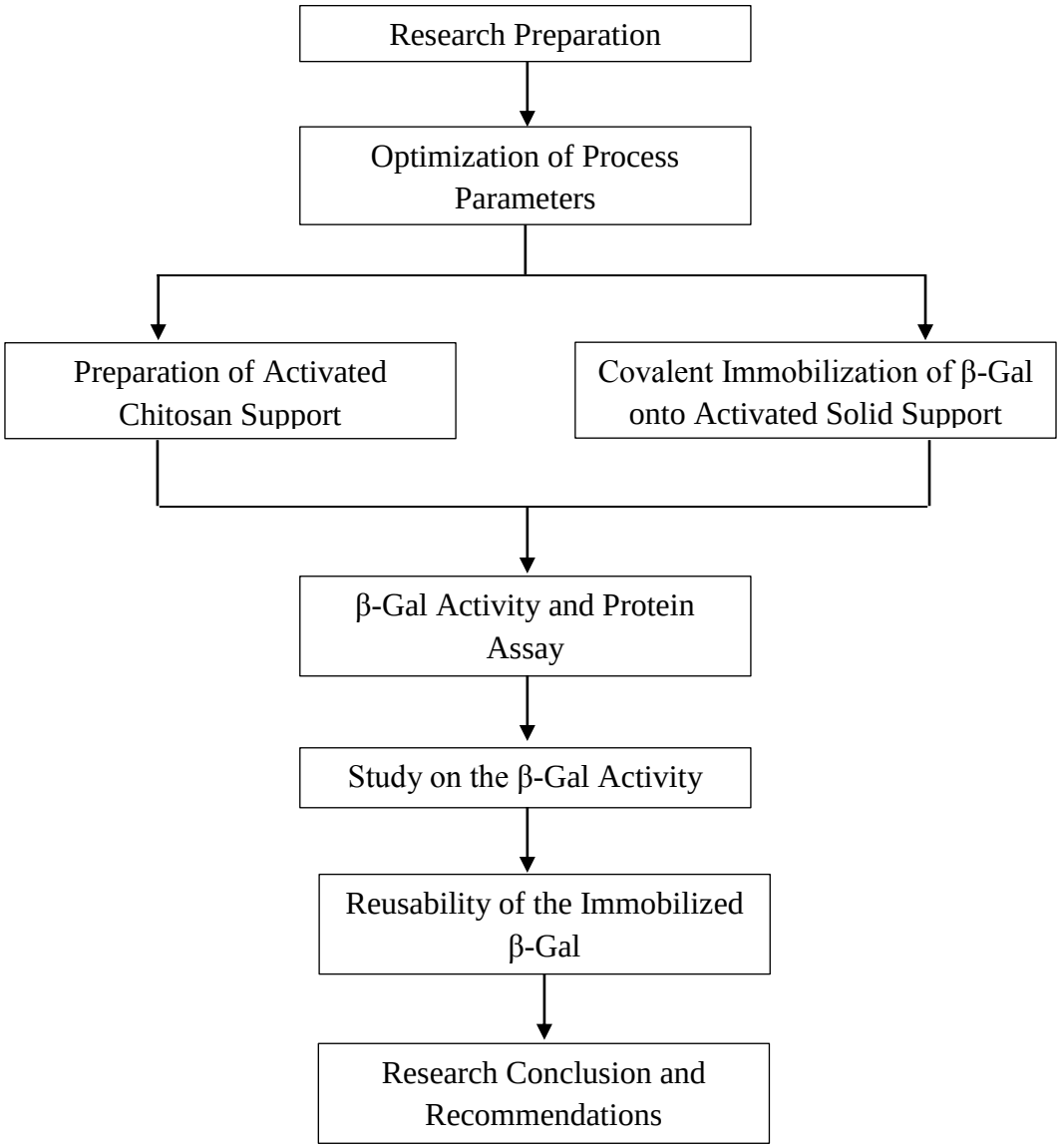

Figure 3.1: Research flowchart

3.2 Materials and Chemicals

The materials and chemicals used in this study are listed in Table 3.1.

Table 3.1: List of materials and chemicals used

Materials or chemicals	Brand	Country
β-Gal *A. oryzae* ≥8 IU/mg	Sigma-aldrich	United States
Chitosan – Poly(D-glucosamine)* Deacetylated chitin	Sigma-aldrich	United States
Bicinchoninic acid (BCA) protein assay kit	Thermo Fisher	United States
4% Culpric sulfate	Thermo Fisher	United States
Ortho-nitrophenyl-β-galactoside (ONPG)	Merck	Germany
Glutaraldehyde (25% (v/v))	Sigma-aldrich	United States
Potassium dihydrogen phosphate (KH_2PO_4)	Fisher Scientific	United Kingdom
Sodium hydrogen phosphate (Na_2HPO_4)	Fisher Scientific	United Kingdom
Sodium carbonate	Merck	Germany
Penta-sodium triphosphate	Merck	Germany
Acetic acid (glacial)	Fisher Scientific	United Kingdom

3.3 Preparation of Activated Chitosan Support

Chitosan is used as a support for immobilization of β-Gal *A. oryzae.* Chitosan support was prepared by following the method of Carrara and Rubiolo (1994) with some modifications. The 1.5 g of chitosan was dissolved in 100 ml of 1.2% acetic acid aqueous solution. The resulted mixture was stirred for 2 h at room temperature. By using hypodermic needle of 2.2 mm diameter, the beads of the chitosan were added dropwise to a gently stirred 150 ml of 1.5% (w/v) sodium triphosphate solution. After that, the recovered beads were washed until neutrality and suspended in 0.025 M KH_2PO_4 and 0.025 M Na_2HPO_4 buffer solution of pH 6.86.

On the next step, the chitosan beads were activated with glutaraldehyde cross-linking (Huerta et al., 2011). Activation of the supports was performed with 2, 6, and

10% (v/v) glutaraldehyde solutions dissolved in 0.025 M KH_2PO_4 and 0.025 M Na_2HPO_4 buffer of pH 6.86 for 2 h at room temperature. Different concentration of glutaraldehyde used, in order to determine the concentration of glutaraldehyde that offers most enzyme activity in the support. The glutaraldehyde reaction with amino groups of chitosan or enzyme is fast in aqueous solution at room temperature (Carrara and Rubiolo, 1994). After that, the supports were recovered by filtration and completely washed with the same buffer (pH 6.86).

3.4 Immobilization of β-Galactosidase onto Activated Chitosan Support

Immobilization of β-Gal onto activated support was conducted with 10, 30, and 50 mg/ml of enzyme solution. An enzyme solution is prepared by dissolving 0.01, 0.03, and 0.05 g of β-Gal in 1 ml of 0.025 M KH_2PO_4 and 0.025 M Na_2HPO_4 buffer solution of pH 6.86 in 2 ml centrifuge tube. Subsequently, the 0.35 g of the activated support was added to the enzyme solution. The mixture was left at room temperature for 48 h to complete the covalent binding of the enzyme onto the surface of activated chitosan support.

Later, the immobilized enzyme was collected and washed with the same buffer (pH 6.86) solution. Also, the β-Gal activity and protein concentration in the supernatant will be measured to determine the yield and the activity of immobilized enzyme which further described in the subsequence sections.

3.5 Yield of β-Galactosidase Immobilization

Theoretically, the yield of β-Gal immobilization can be determined in term of expressed activity or protein content. Here, the immobilization yield (YI) was determined in term of expressed protein according to the Equation (3.1), E_{ini} and E_{fin}

were enzyme protein (mg/ml) in the supernatant (soluble enzyme) before and after immobilization, respectively.

$$\mathrm{YI} = \frac{\mathrm{E_{ini} - E_{fin}}}{\mathrm{E_{ini}}} \times 100 \qquad (3.1)$$

3.6 Activity Yield of Immobilized β-Galactosidase

Activity yield (YIU) of immobilized β-Gal was determined by the Equation (3.2), IU_{bead} and IU_{loss} were unit activity on the support (bead) and the amount of unit activity loss from the solution, respectively:

$$\mathrm{YIU} = \frac{\mathrm{IU_{bead}}}{\mathrm{IU_{loss}}} \times 100 \qquad (3.2)$$

The IU_{loss} can be determined from the Equation (3.3), where IU_{ini} and IU_{fin} were unit activity in the supernatant (soluble enzyme) before and after immobilization, respectively.

$$\mathrm{IU_{loss}} = \mathrm{IU_{ini}} - \mathrm{IU_{fin}} \qquad (3.3)$$

3.7 Optimization of β-Galactosidase Immobilization

The Central Composite Design (CCD) was utilized for optimization of β-Gal immobilization. The series of 13 experimental runs, involving two independent variables including glutaraldehyde and β-Gal concentrations. The reproducibility of the data was determined using center points. The two variables were coded to the (-1, +1) interval, where the low and high level were coded as -1 and +1 respectively. The axial points of the variables were located at $(\pm\alpha, 0, 0), (0, \pm\alpha, 0)$ and $(0, 0, \pm\alpha)$. The α value was fixed at 1.4142, represents the distance from the axial point from center and allows the design rotatable. Table 3.2 presents the ranges and levels of the variables studied and Table 3.3 demonstrates the complete design matrix of the experiments.

Table 3.2: Independent variables and their coded levels for the CCD

Variables (factors)	Coded variables level				
	-α	-1	0	+1	+α
Glutaraldehyde conc. [Glut]	0.34	2.00	6.00	10.00	11.66
β-Gal conc. [Enzyme]	1.72	10.00	30.00	50.00	58.28

Table 3.3: Experimental design matrix

Run	Enzyme immobilization variables	
	[Glut], %	[β-Gal], mg/ml
1	10.00 (+1)	50.00 (+1)
2	6.00 (0)	30.00 (0)
3	11.66 (+α)	30.00 (0)
4	2.00 (-1)	50.00 (+1)
5	0.34 (-α)	30.00 (0)
6	6.00 (0)	58.28 (+α)
7	6.00 (0)	30.00 (0)
8	6.00 (0)	30.00 (0)
9	6.00 (0)	30.00 (0)
10	10.00 (+1)	10.00 (-1)
11	6.00 (0)	30.00 (0)
12	6.00 (0)	1.72 (-α)
13	2.00 (-1)	10.00 (-1)

3.8 Effect of Temperature on β-Galactosidase Activity

From the previous analysis, the immobilized β-Gal at optimum conditions was obtained. That immobilized β-Gal was further used to study the enzyme activity. The effect of temperature on the activity of the immobilized β-Gal was studied and it was determined with the conditions detailed in Section 3.10.1, at the temperature between 30℃ to 58℃ by 7℃ of a step. As comparison, the soluble enzyme also tested in this experiment.