



UNIVERSITI PUTRA MALAYSIA

***SWEET POTATO GLUCOSE PRODUCTION BY USING IMMOBILIZED
GLUCOAMYLASE ONTO SODIUM ALGINATE-CELLULOSE
NANOPARTICLE***

NORSHUHADAH BINTI SHAHDAN

**Ip
FK 2018 46**

SWEET POTATO GLUCOSE PRODUCTION BY USING
IMMOBILIZED GLUCOAMYLASE ONTO
SODIUM ALGINATE-CELLULOSE NANOPARTICLE

NORSHUHADAH BINTI SHAHDAN

178701

PROJECT REPORT SUBMITTED IN PARTIALLY FULFILLMENT
OF THE REQUIREMENT FOR THE

BACHELOR OF ENGINEERING (PROCESS AND FOOD)

DEPARTMENT OF PROCESS AND FOOD ENGINEERING

FACULTY OF ENGINEERING

UNIVERSITI PUTRA MALAYSIA

2018

ACKNOWLEDGEMENT

Foremost, I thank the Almighty for providing me the strength and courage to complete this final year project successfully. I wish to give my gratitude to my supervisor, Assoc.Prof. Dr-Ing Mohd Noriznan Mokhtar and my co-supervisor, Dr. Rabitah Zakaria and Dr. Mohd Nazli Naim for their constant support and encouragement during my research work. Their guidance and suggestion was very invaluable for me to complete this final year project. Next, I also want to acknowledge to the department and the Universiti Putra Malaysia for their support.

Moreover, my heartfelt thanks to my final year partner Ho Yik Jeng for her untiring and continued support during my thesis work. Her timely help and friendship will always be remembered.

Finally, this thesis could not have been completed without the endless love and blessing from my parents, family members and friends for their constant encouragement support to go ahead especially during hard times.

ABSTRACT

In this research, glucoamylase was immobilized onto sodium alginate and cellulose nanoparticle which is known as cellulose that have properties as plant cellulose. The commercial glucoamylase enzyme from *Aspergillus sp.* Was immobilized onto sodium alginate and cellulose nanoparticle through modified entrapment technique. Protein loading onto the support was analysed by using UV/VIS Spectrophotometer and the enzymatic activity of producing glucose was analysed by HPLC-Ri. Based on the entrapment method of glucoamylase onto the sodium alginate support only resulted about 33.8% of enzyme loading with 10.8% of enzyme activity. Then, for the immobilization of glucoamylase onto the sodium alginate and cellulose nanoparticle support resulted about 62.10% of enzyme loading with 45.62% of enzyme activity. Next, the reusability of immobilized glucoamylase was also analyzed by using same immobilized glucoamylase for 6 cycles of reaction and the product was tested by using HPLC-Ri. For immobilized enzyme that use sodium alginate only, it resulted in 50% activity retained while for immobilized enzyme that use both support which is sodium alginate and cellulose nanoparticle, it resulted in 70% activity retained. Besides that, the surface morphology study was carried out by using Scanning Electron Microscopy (SEM), the difference in surface morphology of immobilized glucoamylase for with and without cellulose nanoparticle was obtained.

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION.....	14
1.1 Research Background.....	14
1.2 Problem Statement.....	15
1.3 Objectives.....	15
1.4 Scope of Study.....	16
LITERATURE REVIEW.....	17
2.1 Glucoamylase and Catalytic Reactions.....	17
2.2 Immobilized Enzyme and Its Properties.....	18
2.3 Type of Support Matrix.....	19
2.4 Types of Immobilization.....	22
2.4.1 Support Binding.....	22
2.4.2 Entrapment of Enzyme by Inclusion.....	23
2.4.3 Cross-Linking of Enzyme Aggregates or Crystals.....	23
2.5 Techniques of Immobilization.....	23
2.5.1 Adsorption.....	23
2.5.2 Covalent Binding.....	24
2.5.3 Affinity Immobilization.....	25
2.5.4 Entrapment.....	25
2.6 Selected Technique of Immobilization: Modified Entrapment Technique.....	26
CHAPTER 3.....	30
METHODOLOGY.....	30
3.1 Materials.....	30
3.2 Instruments and Apparatus.....	31
3.3 Preparation of Free Enzyme.....	32
3.4 Preparation of Immobilized Enzyme.....	33
3.4.1 Illustration Process of Immobilization Method.....	34
3.5 Glucoamylase Assay.....	35
3.5.1 Glucoamylase Activity.....	35
3.5.2 Protein Loading.....	37
3.6 (SEM) Analysis for Surface Morphology Observation.....	38

3.7 Reusability of the Immobilized Glucoamylase.....	39
RESULTS AND DISCUSSION.....	40
4.1 Glucoamylase Immobilization.....	40
4.1.2 Glucoamylase Activity.....	43
4.1.3 Glucose Production Profile.....	44
4.3 Strength Analysis of Immobilized Bead.....	49
CHAPTER 5.....	52
CONCLUSION AND RECOMMENDATIONS.....	52
5.1 Conclusion.....	52
5.2 Recommendations.....	53
REFERENCES.....	54
APPENDICES.....	56

LIST OF TABLES

Table 1: List of Categories of Supports.....	19
Table 2: Entrapment Immobilization of Glucoamylase Enzyme on Supports.....	41
Table 3: Comparison of Compression Test Results for Different Support.....	49
Table 4: Absorbance Value for Standard BSA	56
Table 5: Absorbance Value for Calibration Curve Of HPLC-Ri	57

LIST OF FIGURES

Figure 1: New Developed Strategy for Entrapment Technique.....	26
Figure 2: Flow Diagram of Preparation of Free Enzyme.....	32
Figure 3: Flow Diagram of Preparation of Immobilized Enzyme.....	33
Figure 4: Illustration Diagram for Immobilization Process.....	34
Figure 5: Flow Diagram of Glucoamylase Activity.....	35
Figure 6: Standard Curve of the Micro Assay Bradford Analysis by Using BSA as standard	42
Figure 7: Glucose Standard Curve for the HPLC-Ri	43
Figure 8: Comparison of Glucose Production Yield for With/without Cellulose Nanoparticle	44
Figure 9: Comparison Immobilized Bead With/Without Presence of Cellulose Nanoparticle at 100X	46
Figure 10: Comparison Immobilized Bead With/Without Presence of Cellulose Nanoparticle at 300X	46
Figure 11: Comparison Immobilized Bead With/Without Presence of Cellulose Nanoparticle at 600X	47

Figure 12: Comparison Immobilized Bead With/Without 47
Presence of Cellulose Nanoparticle at 1000X

Figure 13: Graph of Reusability of Immobilized Glucoamylase50

Figure 14: Compression Test Graph Result for Immobilized58
Bead Without Cellulose Nanoparticle

Figure 15: Compression Test Graph Result for Immobilized Bead58
With Cellulose Nanoparticle

Figure 16: Comparison of Immobilized Bead with Different Support59

Figure 17: HPLC Chromatogram of Glucose Produced with Soluble59
Starch



CHAPTER 1

INTRODUCTION

1.1 Research Background

Enzymes or bio catalyst are mainly being used in food and pharmaceutical industries as their capability to catalyze many chemical and biochemical reactions. Moreover, enzyme has their own special characteristic which is they have high selectivity and specificity, mild reaction temperature and almost natural pH (Malik Jamal J, 2007). This special characteristic makes the enzyme are more environmental friendly. However, the usage of enzyme is expensive and it often need to be restored and reused for economic purpose. Hence, these limitations can be solved by using methods of immobilization of enzyme.

Immobilization is the methods of imprisonment of cell or enzyme in a distinct support or matrix. Next, immobilization of enzymes is one of the inexpensive methods to remove the enzyme from the reaction media. The two main advantages of immobilized enzyme are that it improves the functional efficiency and enhanced reuseability. However, immobilized enzyme also has their disadvantages which are

the limitation of mass transfer, loss of activity upon immobilization and it was also impractical for solid substrates. Despite of that disadvantages, immobilized enzyme is commonly being used in food, chemical and pharmaceutical industries (Malik Jamal J, 2007).

1.2 Problem Statement

Immobilization of enzyme has lead to problems such as mass transfer limitations. The movement of substrate is limited due to the strong support that hold the substrate from converting into a product. Next, poor strength of alginate support also influence the immobilization process. If the alginate support do not hold the substrate molecule strongly, the substrate can easily detached from the support. This will affect the starch conversion process by affecting the glucose production yield at the end of the conversion process.

1.3 Objectives

The major goal of this research is:

- i. To characterize the immobilization of glucoamylase on the surface of calcium alginate with and without cellulose nanoparticle

1.4 Scope of Study

Glucoamylase enzyme is produced by microorganisms such as from genera *Rhizopus* and *Aspergillus* which is used to produce glucose (dextrose) commercially (Abraham et al., 2004). Moreover, these enzyme is not thermally stable and cannot be reused. Hence, to produce a high productivity and stability for repeated of use over an extended period, immobilization of glucoamylase enzyme is introduced. Furthermore, glucoamylase is immobilized onto the support matrix of sodium alginate via modified entrapment methods. In addition, cellulose nanoparticle is also being used to improve the sodium alginate structure. Both of this support matrix is originally from natural polymers. With the aid of both support matrix, the reusability and functionality of the immobilized enzyme can be retained and improved. Moreover, both of this natural polymer are easily available and affordable. Besides, both of this support matrix have strong mechanical properties that is suitable for immobilization in which to retain the enzyme. Thus, immobilized glucoamylase can be used repeatedly with significant losses and will reduced costs of enzyme utilization in producing this glucose syrup. Furthermore, the new innovations that are made during this research project is that the glucoamylase enzyme were entrapped at the outer surface of the immobilized bead. Unlike the usual entrapment method where the enzyme was entrapped inside the immobilized bead. The combination of both support which is sodium alginate and cellulose nanoparticle will produce stronger immobilized bead thus would hold the glucoamylase enzyme strongly and limiting the mass transfer limitation for the starch conversion.

CHAPTER 2

LITERATURE REVIEW

2.1 Glucoamylase and Catalytic Reactions

Glucoamylase is the enzyme which their species are originated from *Aspergillus Niger*. This enzyme is one of the most economically enzyme which are normally being used in the industrial applications. Furthermore, in this project the sweet potato starch is being used as the source of starch the sweet potato starch will act as the substrate in the starch conversion process. The sweet potato starch consists two polysaccharides which is amylose and amylopectin. Next, the action of glucoamylase will take places when the enzyme glucoamylase will attack the sweet potato starch structure by cleaving the α -1,4-glycosidic bond of amylose and α -1,6-glycosidic bond of amylopectin. The glucomylase enzyme will split off the glucose units from the non reducing end of the sweet potato starch.

2.2 Immobilized Enzyme and Its Properties

Immobilization is a technical process which the enzymes are fixed within solid supports that creating immobilized enzyme system. The solid support systems generally stabilize the structure of the enzyme and maintain their activities. Thus, when compared to free enzymes in solution, immobilized enzymes are more robust and more resistant to environmental changes.

On the other hand, commonly immobilized enzyme has lower activity compared with free enzyme. The stability and kinetic properties of enzymes are usually changes due to the micro environment and modifications that imposed by the support. The modifications made will caused changes in the intrinsic activity and by the fact that the interaction between the immobilized enzyme and the substrate takes place in a micro-environment that are different from the bulk solution.

Furthermore, changes in the catalytic properties upon immobilization may also be changes because of the changes in the three-dimensional conformation of the protein provoked by the binding of the enzyme to the matrix. There are many strategies to prevent these problems which is the selection of supports, the selection of enzyme residues involved in the immobilization, and the techniques used in producing the glucose syrup.

Moreover, enzyme immobilization can commonly allow the easy recovery of both enzyme and products, multiple reuse of enzymes, continuous operation of enzymatic processes, rapid termination of reactions, and greater variety of bio reactor designs. Thus, the stabilization should be achieved because it is important for a driving force of immobilizing enzymes (Brena et al., 2013).

2.3 Type of Support Matrix

Support immobilizes the enzyme by holding it permanently or temporarily for a period. It is important in determining the performance of immobilized enzyme. The support could be used as the modifiers of enzyme properties, many carriers of different physical or chemical nature, different hydrophilicity or hydrophobicity, different size or shape. Good support properties include physical resistance to compression, hydrophilicity, inertness towards enzymes, ease of derivation, bio-compatibility, resistance to microbial attack and availability at low cost. The solid support systems generally stabilize the structure of the enzymes and maintain their activities. There are two categories of support which is organic and inorganic. The table 1 below shows the list of organic supports which can be subdivided into natural and synthetic polymers (Zhang et al., 2013).

Table 1: List of Categories of Supports

Type of support	Type of organic support	Example
Organic support	Natural polymers	• Alginate • Chitosan and chitin • Collagen • Gelatin • Starch • pectin

	Synthetic polymers	• Polystyrene • Polyacrylamide • Polyamides • Vinyl • Allyl-Polymers
Inorganic supports		• Zeolites • Ceramics • Diatomaceous Earth • Silica • Glass • Activated Carbon • Charcoal

The support used should be cheap and easily available. The physical characteristic of the supports such as mean particle diameter, swelling behavior, mechanical strength, compression behavior is importance for the performance of the immobilized systems. Furthermore, the hydrophilic character of supports is also one of the most important factors determining the level of activity of an immobilized enzyme (Brena et al., 2013).

Based on all the characteristics that needed for good immobilized system, support that might be suitable for immobilization of sweet potato for syrup production is sodium alginate and cellulose nanoparticle. This is because cellulose has a good property as a support due to the properties such as water absorbing ability, as well as well distributed molecular

orientation and good mechanical strength (Chen, 2014). In addition, cellulose contain functional group that allow derivation of chemical reaction and non-toxic so it is suitable to be apply with glucose syrup. For this research, cellulose nanoparticle is used as the supports.

Cellulose nanoparticle act as a natural nanostructure support in the immobilization of sweet potato to produce glucose. Cellulose nanoparticle has more chance of interacting with enzyme molecules because its provide good mechanical strength which can contribute to stability and reusability. Moreover, the used of nano sized supports in enzyme immobilization is not only to enhance the stability and reusability of immobilized enzyme but also to overcome lower immobilized enzyme activity due to the presence of high surface area per volume ratio. Moreover, nanoparticle support normally has sufficient dimension (diameter and length) in certain range of distribution which provides a wide area for interaction of immobilized enzyme compared with other types of nanostructured supports.

Moreover, cellulose is the main constituent in the abundant organic compound on earth, especially within wood and natural fibers. Almost 65–70 % of cellulose compound is contained in plant fibers and comprised C, H, and O elements. Cellulose is insoluble in water. The poor solubility can attribute to strong intra- and inter-molecular hydrogen bonds within and among individual chains. As a result, the cellulose nanoparticle present a moderately hydrophobic surface at its exterior, and this feature renders it as a promising material for enzyme immobilization.

Next, 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 Na^+) and glutaraldehyde improves the stability of enzymes (Elçin, 1995). Furthermore, sodium alginate

is the sodium salt of alginic acid which is a natural polysaccharide found in brown algae. It is generally used as a stabilizer and thickener in food industry. Sodium alginate may undergo cross-linking in the presence of divalent cations such as Ca^{2+} to form biodegradable stable gels which is commonly used as a material for cell encapsulations and immobilization.

2.4 Types of Immobilization

Basically, there are three types of methods to immobilize an enzyme. The enzyme can be immobilized by using methods of support binding, entrapment of enzyme by inclusion and cross-linking of enzyme aggregates or crystals. In this project, the selected types of methods to immobilize the enzyme is by using entrapment methods.

2.4.1 Support Binding

In this method, the enzyme is bound to a support/carrier material. This can be done by physical, ionic or covalent interactions. However, it is difficult for enzyme to keep fixed to the carrier under industrial conditions of high reactant and product concentrations and high ionic strength if these two are bound by physical interactions (such as hydrophobic and van der Waals interactions). However, ionic interaction is normally stronger than physical binding and covalent binding is even stronger. It has a benefit as it prevents the leakage of enzyme from the surface of support material.

2.4.2 Entrapment of Enzyme by Inclusion

This method involves the entrapment of enzyme in a gel lattice (polymer network) such as organic polymer or gel. The physical barriers are however not too strong to prevent enzyme leakage entirely. So, covalent binding is also done in addition to entrapment. There is no clear-cut difference between entrapment and support binding, however entrapment involves the synthesis of the polymeric mesh work catalyzed by the enzyme, whereas in support binding, the enzyme is attached onto a prefabricated support.

2.4.3 Cross-Linking of Enzyme Aggregates or Crystals

This is relatively an advance method of immobilizing an enzyme. This method involves a strategy in which enzymes are immobilized free of any carrier material such as by cross linking enzyme crystal and cross-linking enzyme aggregates (Sheldon et al., 2006). This method has many advantages such as increased activity of enzyme, high stability and the cost of production and processing is lowered because there is no carrier is required.

2.5 Techniques of Immobilization

2.5.1 Adsorption

Enzyme adsorption can be done by hydrophobic bonding or salt linkages between enzyme and carrier materials. For this purpose, support can be dipped in enzyme solution for enough time to let it physically adsorbed. There is another way to adsorb enzyme by drying it on the surface of an electrode. This protects enzyme against aggregate formation, denaturation and hydrophobic interactions. Adsorption protects enzymes against aggregation, proteolysis and interaction with hydrophobic interfaces (Spahn and Minter, 2008). Coconut

fibers can hold a good amount of water and have a high cation exchange ability and are environmental friendly, so they are used by researchers to immobilize enzyme. Many molecular sieves having silanols on their pore walls are successfully used by scientists for enzyme immobilization. These facilitate us to immobilize an enzyme by hydrogen bonding. Different chemical modifications in currently used support materials can result in even better immobilization. Immobilization of lipase extracted from *Ethical issues* and production costs can be lowered by using environmental friendly support materials for immobilizing enzymes. Use of bio compatible support material such as mesoporous silica nanoparticles (MSNs) for immobilization results in long-term durability and efficiency of enzyme (Popat et al., 2011).

2.5.2 Covalent Binding

Enzymes have different side chain amino acid residues and have reactivity based on different functional groups which is utilized for covalent binding of enzymes to support materials (D'Souza, 1998 & Singh, 2009). Salinized silica gel carriers with removed unreacted aldehyde groups are covalently bound to enzymes resulting in highly stable and hyperactive biocatalysts (Lee et al., 2006). Enzymes when covalently bound to mesoporous silica and chitosan, lead to an increase in the half life and heat endurance of enzymes. Covalent binding of electro spun nanoparticle to enzyme lead to an increase in surface area and porosity and thus residual activity of enzymes is increased tremendously. Alcohol dehydrogenase is cross linked to alginate nanoparticles by the increasing the enzymes thermal stability. Different immobilized enzymes obtained by covalent binding to different supports are used in medicines and drugs manufacturing because of their enhanced stability and reusability.

2.5.3 Affinity Immobilization

In affinity immobilization, specific nature of enzyme to its support under different physiological conditions is utilized. Affinity immobilization can be achieved by two ways which is either by recoupling the template to a ligand to which it has affinity for or conjugating the enzyme to a substance that develops affinity for the template (Sardaret al., 2000). Purification of enzymes is also done by affinity immobilization. Support materials like alkali stable chitosan- coated porous silica beads and agarose-linked multilayered. A harbor larger amounts of enzymes that result in an increase in stability and efficiency of enzyme (Shi et al., 2003).

2.5.4 Entrapment

Entrapment involves the detention of enzymes in gels or fibers by covalent or non-covalent interactions (Singh, 2009). Alginate–gelatin– calcium crossbreed carriers prevent enzyme leakage, provide increased mechanical stability and efficient encapsulation is achieved. Use of nanoparticles such as electro spun nanoparticles as a support material for entrapment have revolutionized the field of enzyme immobilization with their wide-ranging applications in the field of chemistry, bio medicine, bio sensors and bio fuels (Dai & Xia, 2006). It has been reported that lipase of *Candida rugosa* when entrapped in chitosan resulted in prevention of friability and leaching and augmentation of entrapment efficiency and enzyme activity. In addition, this support is also non-toxic, biocompatible and has a great affinity to proteins because of its hydrophilic nature. Use of mesoporous silica entrapment support material has also been reported because of its high surface area, uniform pore distribution and high adsorption capacity. Carrageenan entrapped lipases are considered as highly thermostable and organic solvent tolerant enzymes (Jegannathan et al., 2010).

2.6 Selected Technique of Immobilization: Modified Entrapment Technique

A new strategy of entrapment techniques for immobilization purpose is developed throughout this project. This new developed strategy will entrapped the enzyme mainly at the inside of the immobilized bead and only a small portion of enzyme will be entrapped at the surface of the bead. The new advantages of developing this new strategy is that it will limit the mass transfer of substrate from converting into a product. The new developed strategy will be developed as below:

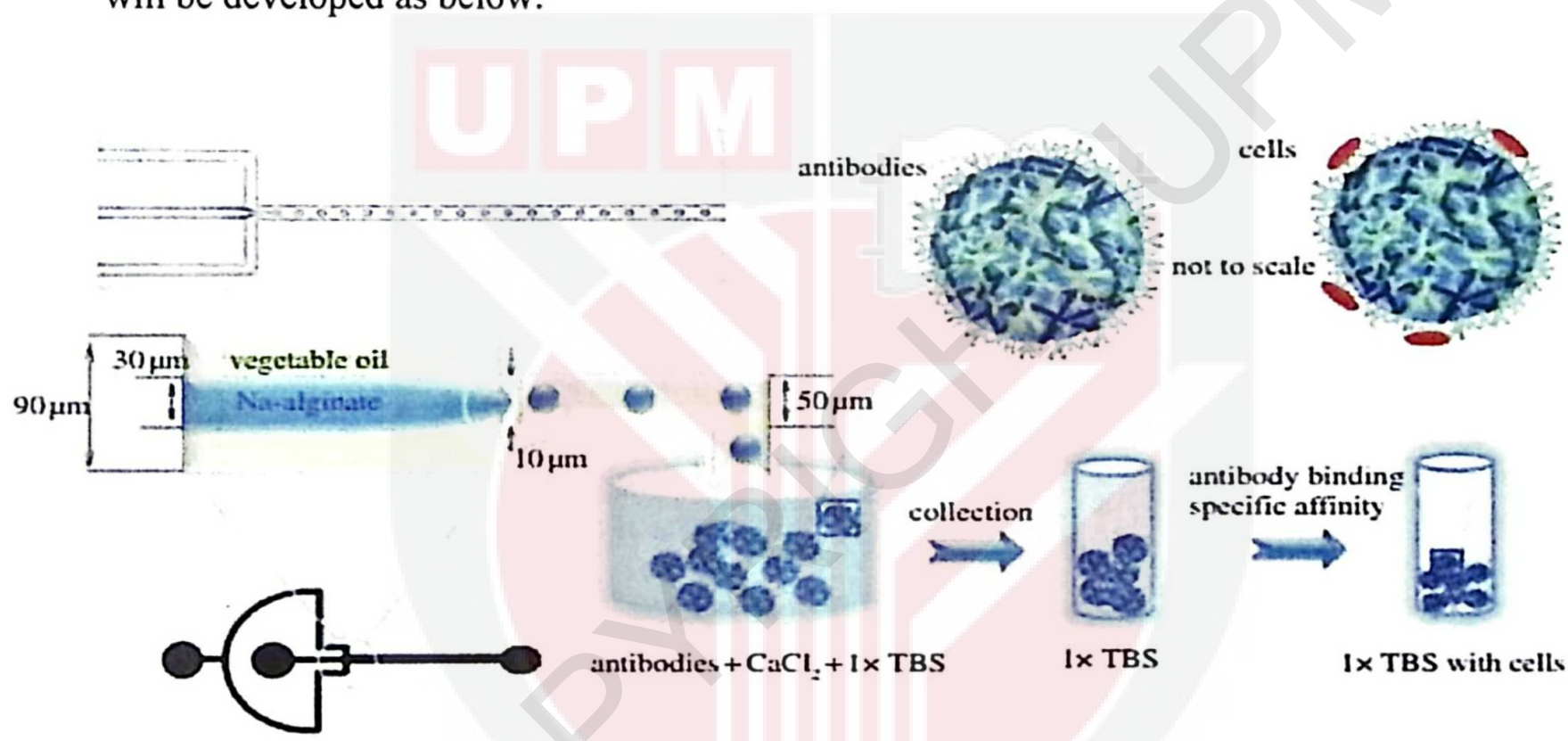


Figure 1: New Developed Strategy for Entrapment Technique (Chen et al., 2013)

As can be shown from figure 1, the enzymes were immobilized primarily on the alginate bead during the gelation process. This is because during the immobilization process, the enzyme were separated from the alginate support. Unlike the conventional method of entrapment technique, the enzyme were mixed together with the alginate support. The separation of alginate support and the enzyme caused the enzyme mainly located inside the immobilized bead and only a few enzyme were found on the surface of the immobilized bead.

The strong binding of enzyme inside the alginate support have caused a minimum mass transfer that occur during the starch conversion process.

Moreover, entrapment is also one of the common methods that are being used in immobilization industry. Sodium alginate has been an attraction as an immobilization support material. It has been utilized for immobilization of variety of cell types, sub-cellular organelles, multi-component systems, and enzymes. The physicochemical characteristics of this matrix in gel form have an important effect on the reactions of entrapped in selecting a matrix is pore size. The difference between entrapment technique and adsorption and covalent binding is that however the enzyme is restricted in movement by the structure of a gel lattice but it is free in solution. The pore size of a gel lattice is controlled to ensure that the structure become tight enough to prevent loose of enzyme or cells, it also allow free movement of the substrate and product. The support acts as a barrier to mass transfer, and although this have serious reaction kinetics implications, but it can prevent interaction between harmful cell, proteins, and enzymes and immobilized biocatalyst.

There are several major methods of entrapment:

- Ionotropic gelation of macromolecules with multivalent cations (e.g. alginate).
- Temperature-induced gelation (e.g. agarose, gelatin).
- Organic polymerization reaction by chemical/photochemical (e.g. Polyacrylamide).
- Precipitation from an immiscible solvent (e.g. polystyrene).

Entrapment can be accomplished by cross linking the polyionic polymer material with multivalent cations in an ion-exchange reaction after mixing with enzyme to form a structure that traps the enzymes/cells (ionotropic gelation). On the other hand, it is possible to mix the enzyme with material that is then polymerized to form a crosslinked polymeric system, trapping the enzyme in the internal spaces of the lattice. The last method is more widely utilized, and various acrylic materials are available for the formation of hydrophilic copolymers. For example, acrylamide monomer is polymerized to form polyacrylamide and methyl acrylate is polymerized to form polymethacrylate. In addition to the monomer, a crosslinking agent is added during polymerization to form cross linkage between the polymer chains and help to create a three-dimensional network lattice. The formed polymer may be broken up into particles of a desired size, or polymerization can be arranged to form beads of defined size.

Precipitation occurs by phase separation rather than chemical reaction, but does bring the enzymes/cells into contact with a water-miscible organic solvent, and most enzymes/cells are not tolerant of such solvents. Thus, this method is limited to highly stable/previously stabilized enzymes or nonliving cells. This method is depending on localization of an enzyme inside polymer network or membrane lattice. Entrapment has been advanced and broadly utilized for the immobilization of cells more than for enzymes. It is limited for enzymes immobilization as it may be lost during repeatedly using because of the small molecular size of enzyme compared to the cells. Diffusion limitations are also disadvantages for this method. This method may be classified into five categories: lattice, microcapsule, liposome, membrane, and reverse micelle. The most widely one is the lattice method, in this type enzyme is entrapped in the lattice of the different natural or synthetic polymers. Alginate which is naturally occurring polysaccharide that can form gels by ionotropic gelation, is the most popular one.

Another type, microcapsule, involves entrapment to porous polymer. The preparation of micro capsules containing enzyme requires highly controlled conditions. Taqieddin and Amiji developed a new method for encapsulation in which the alginate-chitosan core-shell microcapsules were prepared to immobilize β -galactosidase. The enzyme was confined and protected in the inner core, alginate, while the outer shell, chitosan, manages the transport properties. Utilizing Ca^{2+} ions for alginate crosslinking, microcapsules with liquid core were developed with 60% loading efficiency. And using Ba^{2+} ions, microcapsules with solid core were produced and 100% loading efficiency was obtained. The entrapment in liposome is increasingly recognized as a technique of protecting biocatalysts from inactivation by proteolytic enzymes. Also, this enzyme, liposome, offers a noticeable increment in thermal protection. In the third type, reversed micelle, entrapping within the reversed micelles, it is formed by adding a surfactant with an organic solvent, such as aerosol or isooctane reverse micelles and used to entrap β -galactosidase. While in the membrane type, the enzyme is isolated from the reaction solution microfiltration membrane or a hollow fiber.

CHAPTER 3

METHODOLOGY

3.1 Materials

The materials used in this project were listed as below:

- i. Sweet potato
- ii. Amyloglucosidase from *Aspergillus Niger* in powder and white form (~120 U/mg)
- iii. Cellulose nanoparticle from Kenaf bast fibre was obtained from Lembaga kenaf dan Tembakau Negara (LKTN), Malaysia
- iv. Sodium alginate obtained from Sigma-Aldrich
- v. All chemicals with technical grade such as sodium hydroxide (NaOH), acetic acid (CH_3COOH), Bradford reagent (coomassive blue), glutaraldehyde (GA) were purchased from R&M Chemicals, Malaysia
- vi. Water soluble potato starch were purchased from Sigma, Malaysia

3.2 Instruments and Apparatus

The instruments used in this project were listed as below:

- i. UV/VIS Spectrophotometer of model Amersham Pharmacia Biotech Ultraspec 3100 pro
- ii. Centifuge of Model Eppendorf 5804 R
- iii. Excella E24 Incubator Shaker Series
- iv. Shaking Incubator of model New Brunswick Scientific USA
- v. Scanning Electron Microscopy (SEM)
- vi. HPLC-RI system (LC-10AT with RID 6A), equipped with Shodex Asahipak NH2P-50 column (L:250mm, I.D: 4.6mm, particle size: 5 μ m) operated at 40°C)
- vii. Texture analyzer
- viii. Glassware

3.3 Preparation of Free Enzyme

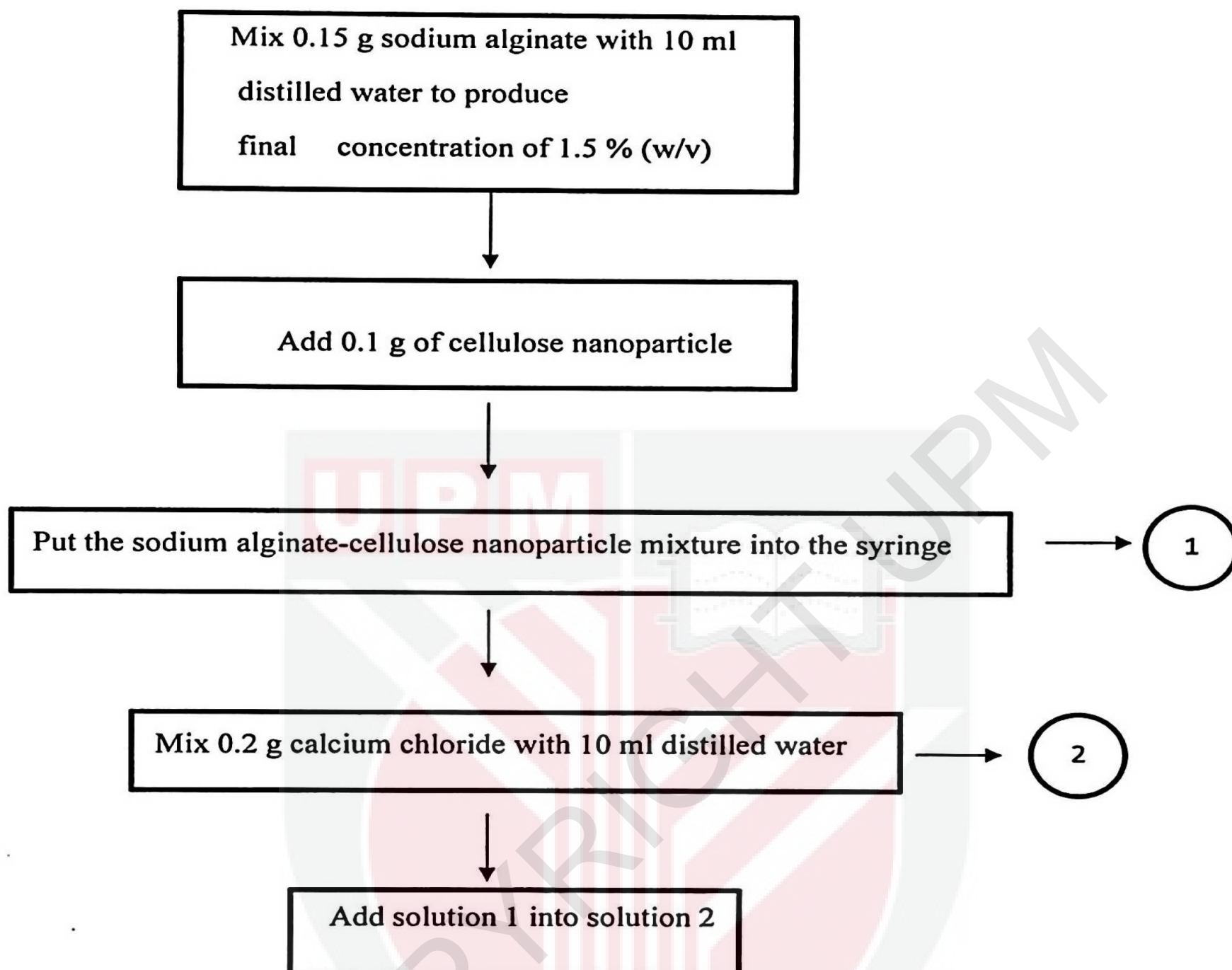


Figure 2: Flow Diagram of Preparation of Free Enzyme

The solution 1 (sodium alginate-cellulose nanoparticle mixture) produced will be mixed with solution 2 (calcium chloride solution) to produce a free enzyme solution.

3.4 Preparation of Immobilized Enzyme

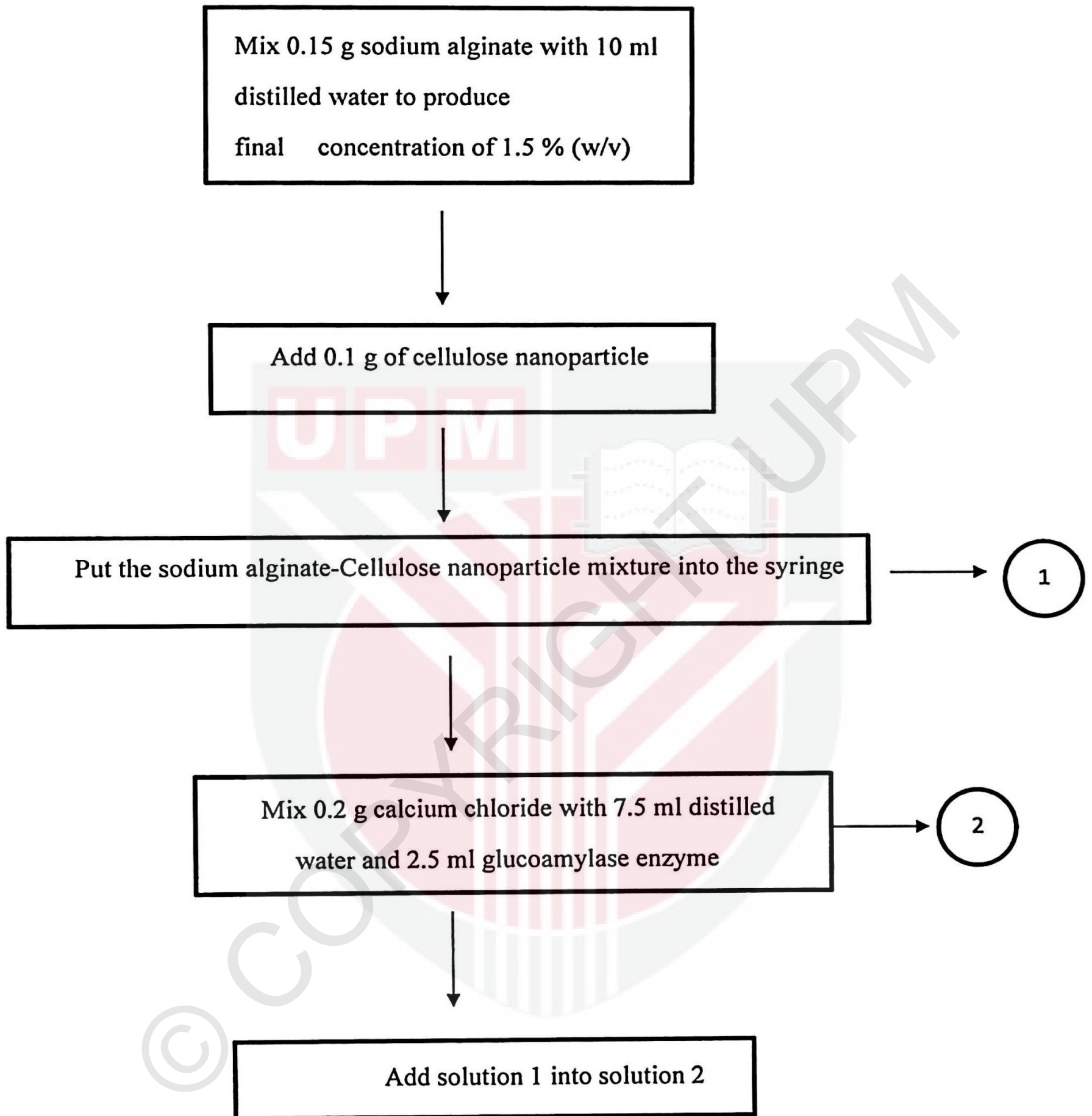


Figure 3: Flow Diagram of Preparation of Immobilized Enzyme

The solution 1 (sodium alginate-cellulose nanoparticle mixture) produced will be mixed with solution 2 (calcium chloride-glucoamylase solution) to produce immobilized enzyme solution.

3.4.1 Illustration Process of Immobilization Method

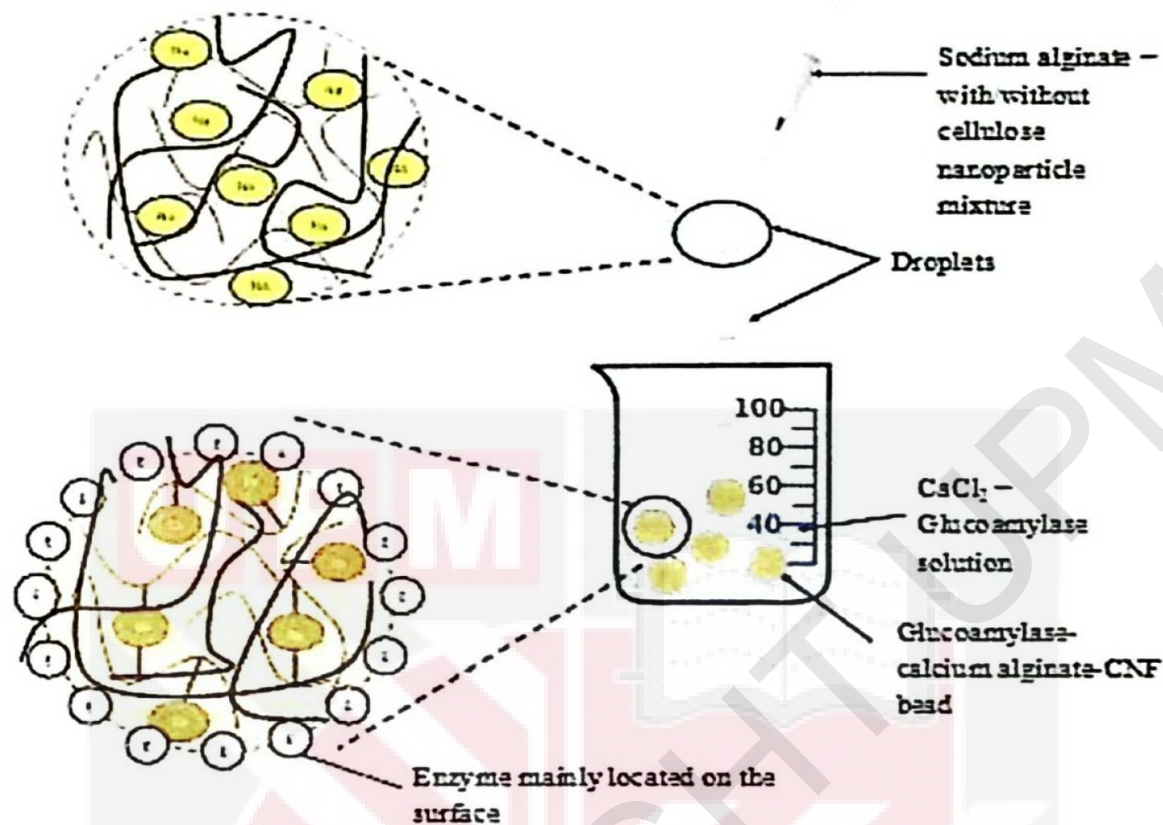


Figure 4: Illustration Diagram for Immobilization Process

Figure 4 above shows the illustration process during immobilization. Inside the syringe, it consists of sodium alginate and with/without cellulose nanoparticle solution. While in the beaker, a calcium chloride and glucoamylase solution were formed. The droplet produced in the syringe will be dropped into the beaker. The droplet produced will be mixed together with the solution inside the beaker and immobilized bead were produced.

3.5 Glucoamylase Assay

3.5.1 Glucoamylase Activity

The glucoamylase activity for both free enzyme and immobilized enzyme was assayed as follows:

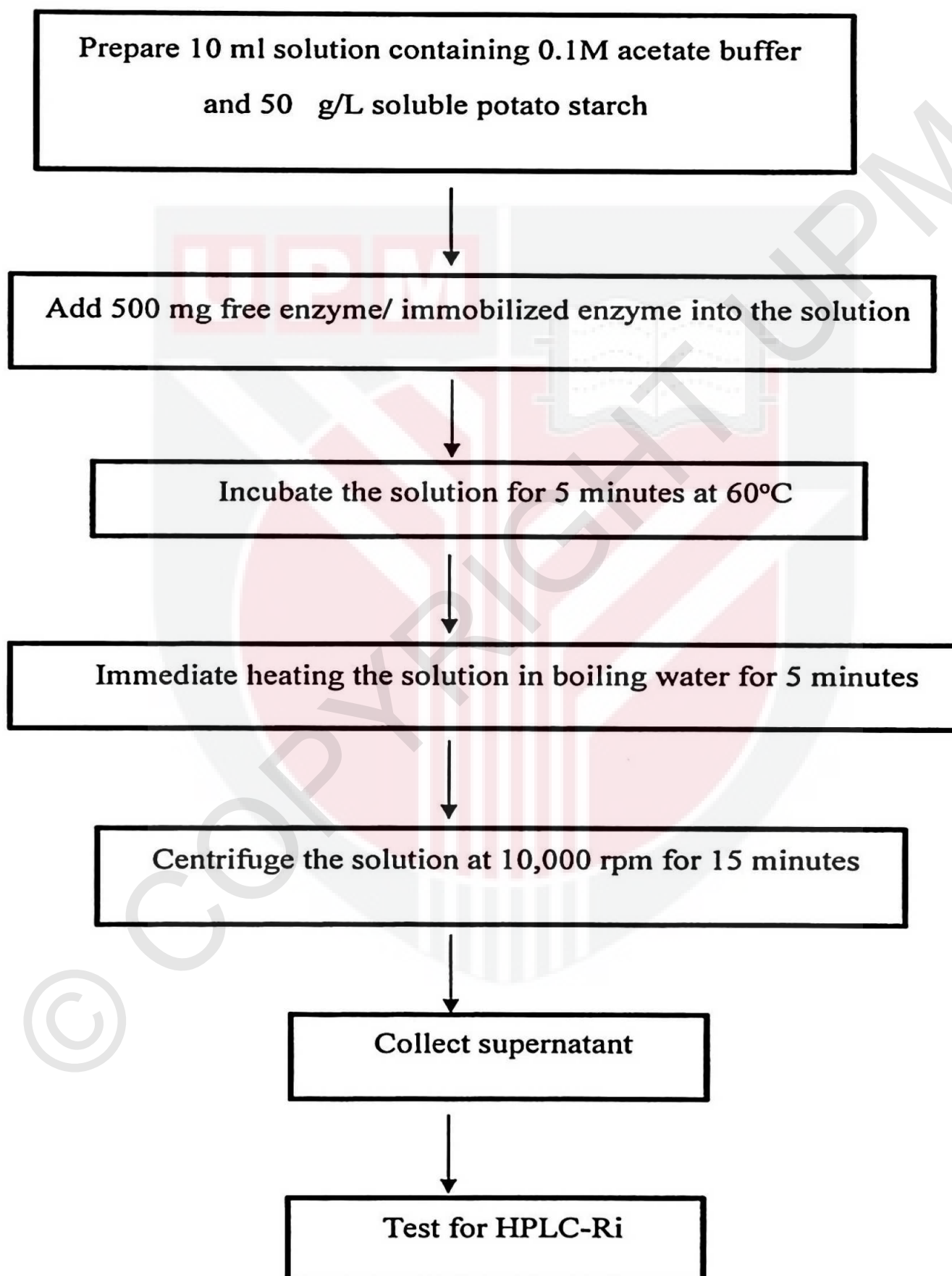


Figure 5: Flow Diagram of Glucoamylase Activity

The enzyme activity of free and immobilized enzyme was determined based on amount of glucose (1 µg) generated for 1 min and it will be defined as enzyme activity (U).

The percentage of glucose production yield was calculated as shown in equation (1):

$$\text{Glucose production yield} = \frac{C}{C_i} \times 100 \%$$

Equation (1)

Where;

C= concentration of glucose that obtained from the analysis (g/L)

C_i=initial starch concentration (g/L)

3.5.2 Protein Loading

Protein loading determination was done by using Bradford method using Bovine Serum Albumin as the standard. The standard curve is based on (Chieh, 2012). Bradford reagent was prepared by using 100 mg of Coomassie Blue G250 dissolved in 50 ml of 95% ethanol. The solution is then mixed with 100 ml of 85% phosphoric acid and made up to 1L with distilled water. When Bradford reagent is added to the protein solution, the maximum absorbance of Coomassie Blue will shift from 465 nm to 595 nm and brings a visible color changes. The change in absorbance in 595 nm is directly proportional to the concentration of protein in the solution. The measurement will be done by using UV/VIS spectrophotometer at wavelength of 595 nm.

The amount of protein loaded on cellulose nanofiber was obtained by analyzed the concentration before and after immobilization. This was done by taking 100 μ l of the protein sample and mixed well with 5 ml of Bradford reagent by gentle vortexing. The mixture was left for 5 minutes at room temperature and then 1 ml of the solution was used for measuring the absorbance by using the spectrophotometer at the wavelength of 595 nm. The absorbance obtained was used to calculate the protein concentration of samples from the standard curve. All the measurements were done three times.

The percentage of protein loaded onto cellulose nanofiber can be determined by subtracting the amount of protein added before immobilization and the amount of protein measured in supernatant and all the washings after immobilization by using below equation (2) (Beulah et al., 2015).

$$P_{pl} (\%) = \frac{P_o - P_e}{P_o} \times 100\%$$

Equation (2)

Where;

P_{pl} = Percentage of protein loaded (mg)

P_o = Amount of protein added before immobilization (mg)

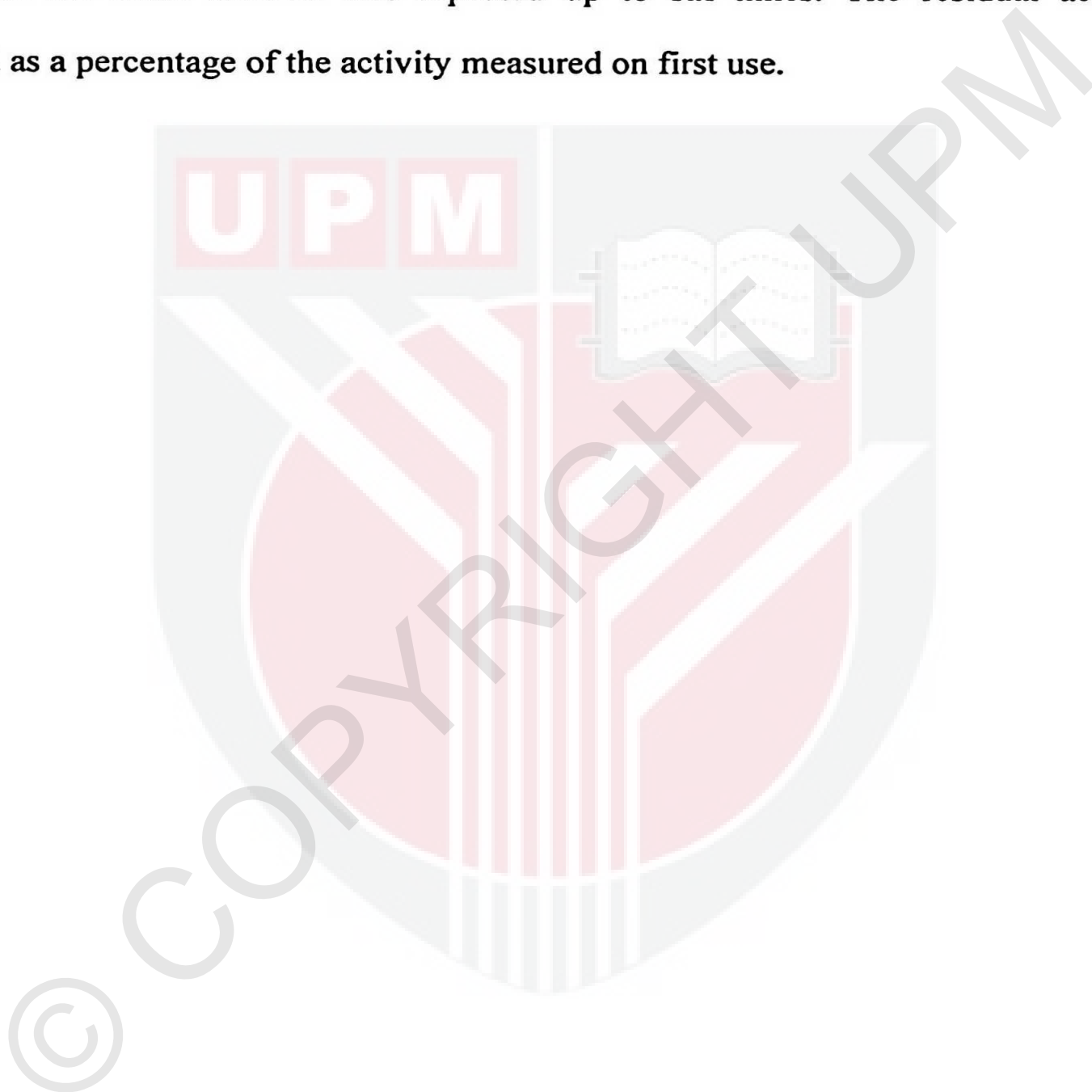
P_e = Amount of protein in all washing after immobilization (mg)

3.6 (SEM) Analysis for Surface Morphology Observation

The surface morphology of glucoamylase-alginate bead and glucoamylase-alginate-nanofiber bead will be observed using Scanning Electron Microscopy (SEM S-3400N, Hitachi, Japan). The suspension of each sample will be freeze-dried overnight. The sample will be treated with solvent exchange technique during freeze-drying (from water to acetone). The acceleration was set up in range of 5-20 Kv and the samples were sputter-coated with gold to avoid charging effect during SEM observations. Both of the immobilized bead with/without cellulose nanoparticle were examined under SEM analysis.

3.7 Reusability of the Immobilized Glucoamylase

To investigate the reusability of immobilized glucoamylase, they were repeatedly used for the determination of their activity. After each activity assay, the immobilized glucoamylase were taken out from the reaction system and washed thoroughly with deionized water. Then, the activity of the immobilized glucoamylase was determined in a new reaction system and the same method was repeated up to six times. The residual activity was calculated as a percentage of the activity measured on first use.



CHAPTER 4

RESULTS AND DISCUSSION

4.1 Glucoamylase Immobilization

In this study, glucoamylase was immobilized onto support using entrapment technique. There are two types of support that are being used in this study which are sodium alginate and cellulose nanoparticle. Both of this support were used either by using only sodium alginate alone or using both sodium alginate and cellulose nanoparticle to immobilize the glucoamylase. The addition of cellulose nanoparticle support onto the immobilized glucoamylase will provide more strength to the immobilized enzyme bead and the immobilized enzyme can retain its activity up to 7 cycles (Safwan et al., 2015).

Table 2: Entrapment Immobilization of Glucoamylase Enzyme on Supports

Type of support	Enzyme added		Unbound enzyme		Immobilized enzyme		Immobilization yield	
	Protein (mg/g support)	Activity (U/g support)	Protein (mg/g support)	Activity (U/g support)	Protein (mg/g support)	Activity (U/g support)	Binding (%)	Activity (%)
Without cellulose nanoparticle	10.0	360.0	6.62	297.67	3.38	6.73	33.80	10.80
With cellulose nanoparticle	10.0	360.0	3.79	106.89	6.21	115.48	62.10	45.62

4.1.1 Bound Pectinase Determination

The absorbance value was determined by UV/VIS spectrophotometer and protein concentration by standard curve of Bradford Micro Assay by using Bovine Serum Albumin as standard from (Cieh.L, 2012) as shown in figure 6. Based on the graph, if the absorbance value of a sample is determined as shown in Appendix B and the protein concentration can in that sample can be estimated by using the equation from the standard curve which is $y = 0.0081x$. The value of R^2 shows in graph is equal to 0.995, thus the estimation of protein concentration has high accuracy which is 99.5%

From table 2, the percentage loading of enzyme glucoamylase onto sodium alginate is 33.80% which is the percentage is less than half and it is quite low for immobilization of enzyme. In contrast, the percentage loading of enzyme glucoamylase onto sodium alginate and cellulose nanoparticle is 62.10% which is more than half and it is moderately high for immobilization of enzyme.

Furthermore, the size of the cellulose nanoparticle used as a support for immobilization also play an important role of percentage loading of glucoamylase. The size of the wet cellulose nanoparticle used is between 0.5 to 3.0 μm (Chi Yu and Lia, 2007). The smaller size provides high surface area of volume ratio, thus more enzyme can be immobilized. In other words, high surface area of the support provides a high number of functional group on the surface support.

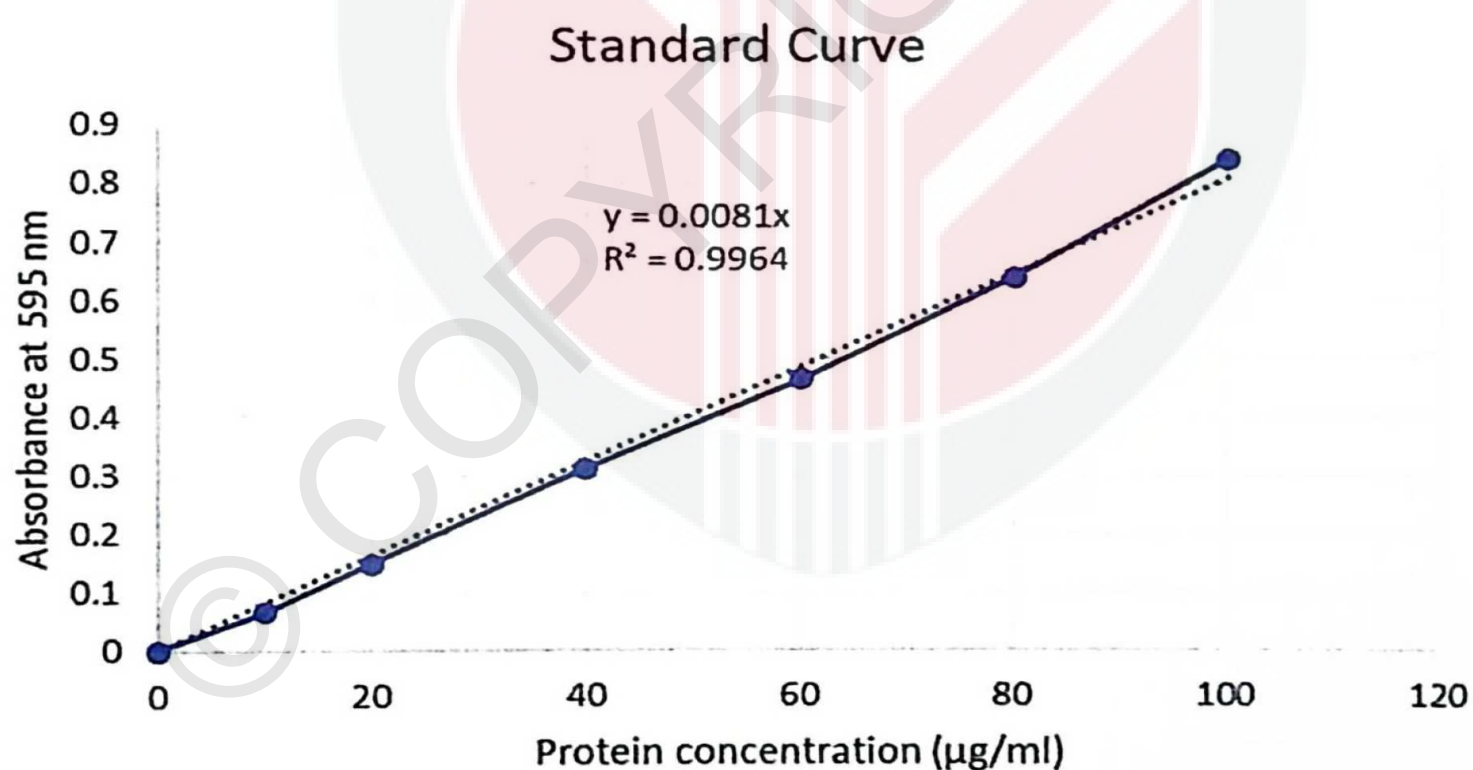


Figure 6: Standard Curve of the Micro Assay Bradford Analysis by Using BSA as standard

4.1.2 Glucoamylase Activity

Glucoamylase activity were measured by using HPLC-Ri. From table 2, for sodium alginate support it shows that the immobilization yield for protein binding is 33.80% and 10.80% retained enzyme activity were obtained. In contrast, for the sodium alginate and cellulose nanoparticle support it shows that the immobilization yield for protein binding is 62.10% and 45.62% retained enzyme activity were obtained. Based on these comparison, the immobilized enzyme that has sodium alginate and cellulose nanoparticle as the support has more higher percentage as compared to sodium alginate alone. The higher the immobilization yield, the higher the enzymatic reaction of glucoamylase that is converted into sugar.

Based on the graph on figure 7, if the absorbance value of a sample is determined as shown in Appendix B and the glucose concentration in that sample can be estimated by using the equation from the standard curve which is $y = 0.0004x$. The value of R^2 shows in graph is equal to 0.9998, thus the estimation of glucose concentration has high accuracy which is 99.9%

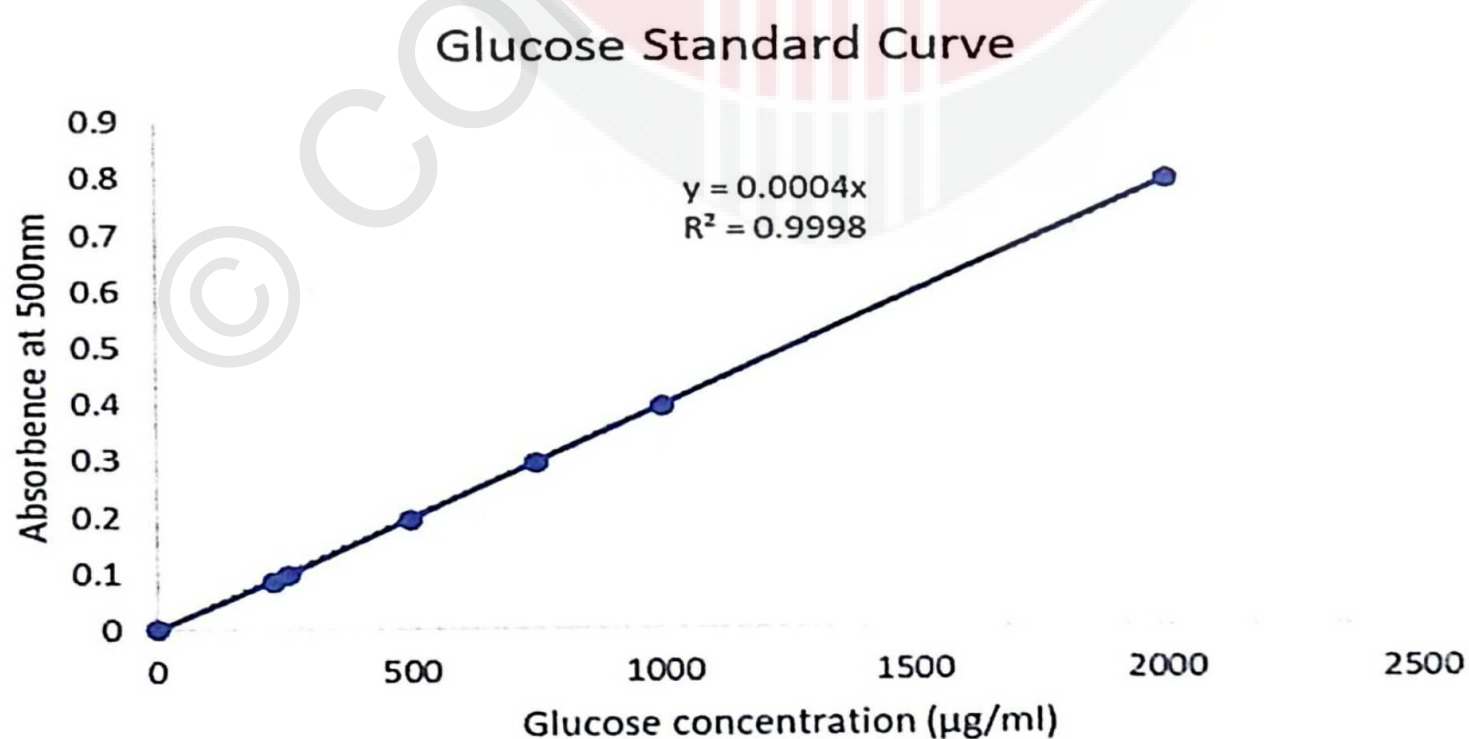


Figure 7: Glucose Standard Curve for the HPLC-Ri

4.1.3 Glucose Production Profile

Glucose is produced from the starch conversion process of sweet potato. The conversion of starch that is present in the sweet potato which is amylose and amylopectin were converted into a product of glucose. The glucose produced were measured over time for both with/without cellulose nanoparticle.

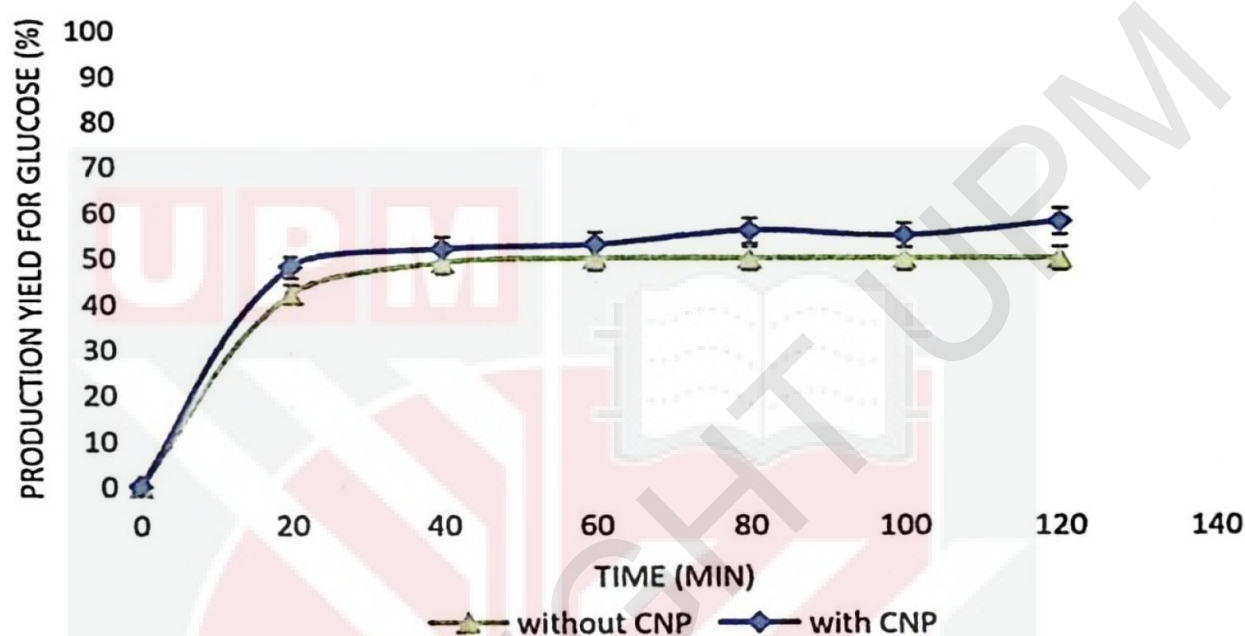


Figure 8: Comparison of Glucose Production Yield for With/without Cellulose Nanoparticle

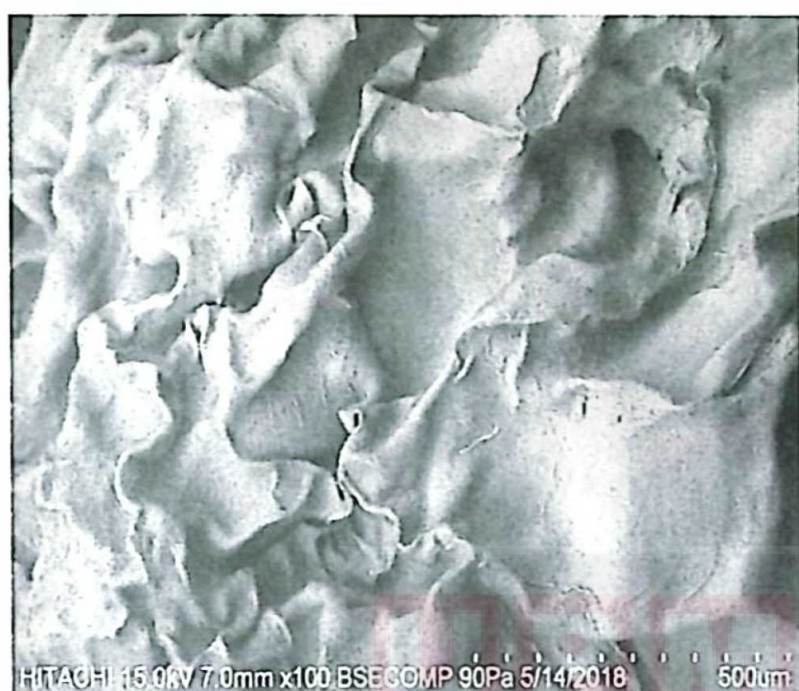
From figure 8, it can be shown that there was a difference between the immobilized enzyme for with/without cellulose nanoparticle. As it can be seen from the figure above, immobilized bead with cellulose nanoparticle show higher glucose production yield up to 60% while without cellulose nanoparticle yield only 50% glucose respectively. The constant amount of glucose produced in the graph were due to substrate limitation.

4.2 (SEM) Analysis for Surface Morphology Observation

Scanning Electron Microscope (SEM) also known as SEM analysis is used very effectively in microanalysis. SEM is performed at high magnification, generates high-resolution images and precisely measures very small features and objects.

SEM uses a focused beam of high-energy electrons to generate a variety of signals at the surface of solid specimens. In most SEM microscopy applications, data is collected over a selected area of the surface of the sample and a two-dimensional image is generated that displays spatial variations in properties including chemical characterization, texture and orientation of materials. All the figures below show the morphology of samples that are being analyzed by SEM.

4.2.1 Comparison of Immobilized bead at 100X



(a) Immobilized bead without CNP



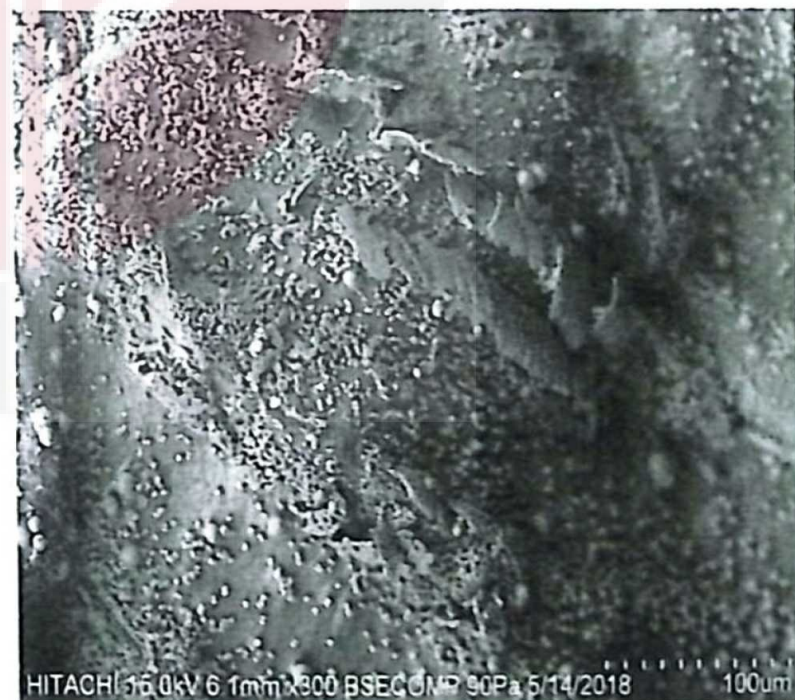
(b) Immobilized bead with CNP

Figure 9: Comparison Immobilized Bead With/Without Presence of Cellulose Nanoparticle at 100X

4.2.2 Comparison of Immobilized bead at 300X



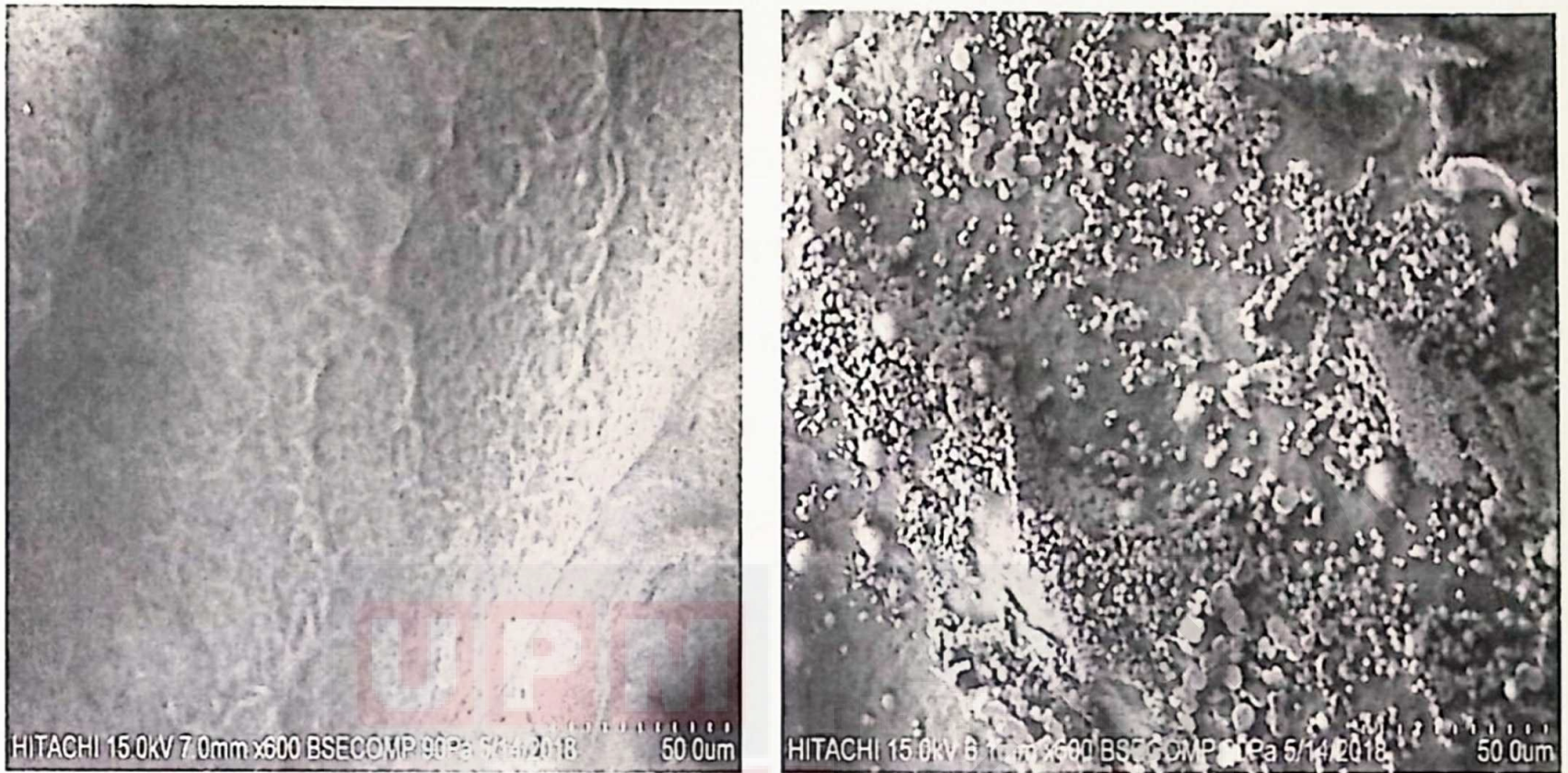
(a) Immobilized bead without CNP



(b) Immobilized bead with CNP

Figure 10: Comparison Immobilized Bead With/Without Presence of Cellulose Nanoparticle at 300X

4.2.3 Comparison of Immobilized bead at 600X

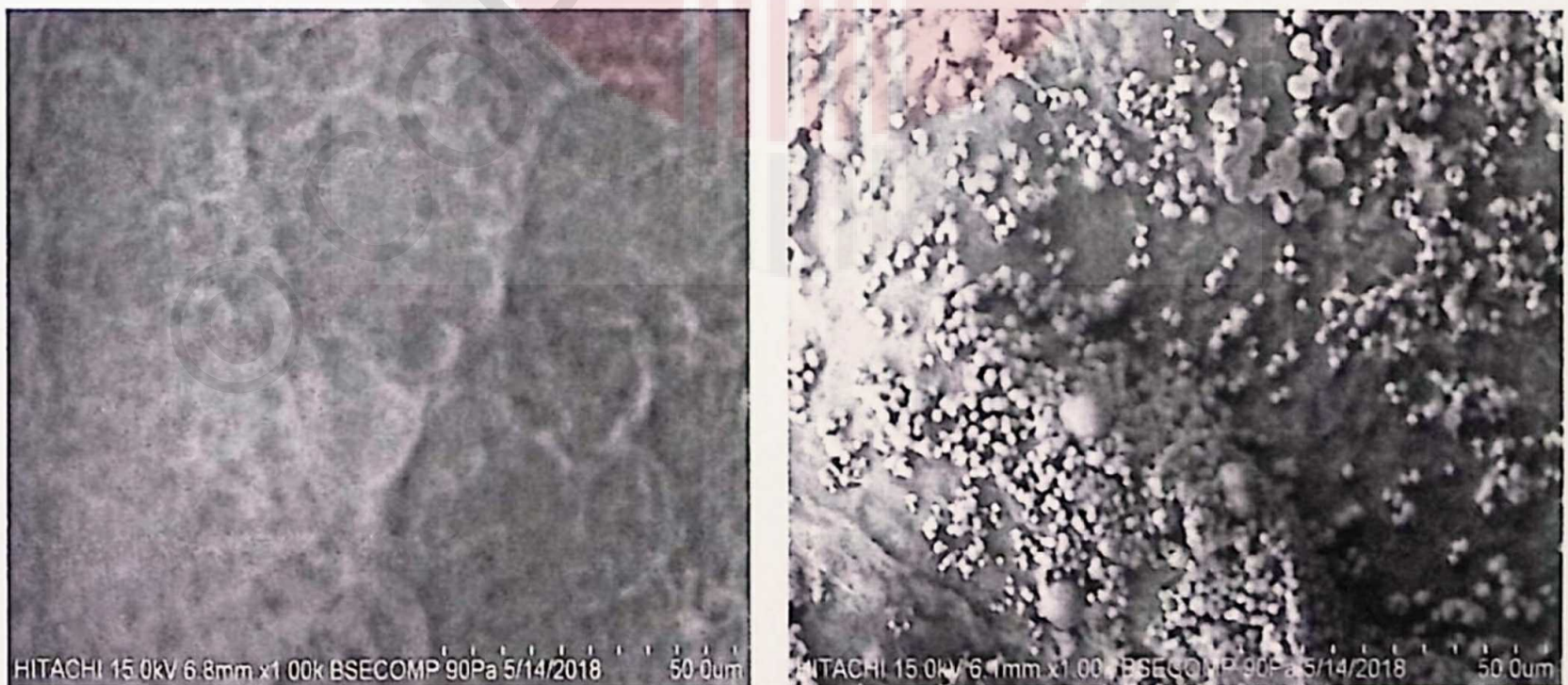


(a) Immobilized bead without CNP

(b) Immobilized bead with CNP

Figure 11: Comparison Immobilized Bead With/Without Presence of Cellulose Nanoparticle at 600X

4.2.4 Comparison of Immobilized bead at 1000X



(a) Immobilized bead without CNP

(b) Immobilized bead with CNP

Figure 12: Comparison Immobilized Bead With/Without Presence of Cellulose Nanoparticle at 1000X

From figure 9, 10, 11 and 12, all of the SEM images shown are immobilized enzyme with or without the presence of cellulose nanoparticle support. As a comparison, the immobilized enzyme without cellulose nanoparticle support only has sodium alginate as their main support while for immobilized enzyme with cellulose nanoparticle support has both sodium alginate and cellulose nanoparticle. From all the comparison of SEM images, it shows that the surface of the immobilized enzyme without cellulose nanoparticle is smooth while surface of immobilized enzyme with cellulose nanoparticle is rough. The rough surface of immobilized enzyme with presence of cellulose nanoparticle will help to give extra strength to alginate structure thus holding the glucoamylase enzyme stronger. The size of cellulose nanobead is 8 μm .

4.3 Strength Analysis of Immobilized Bead

The strength of the immobilized bead with/without cellulose nanoparticle were tested by using texture analyzer equipment. The immobilized bead were put under a load of 10 kg and their break point were measured. The compression test were carried out for 2 seconds. The results of compression test were tabulated as below:

Table 3: Comparison of Compression Test Results for Different Support

Type of support	Break force (N)
Without cellulose nanoparticle	1.548
With cellulose nanoparticle	1.763

From table 3, it can be shown that immobilized enzyme with cellulose nanoparticle support need higher force to break the surface of the immobilized bead. This due to the more rigid and stronger bead that were caused by the cellulose nanoparticle. The extra strength that were provided by the cellulose nanoparticle towards the immobilized bead has been proved by using the compression test.

4.4 Reusability of Immobilized Glucoamylase

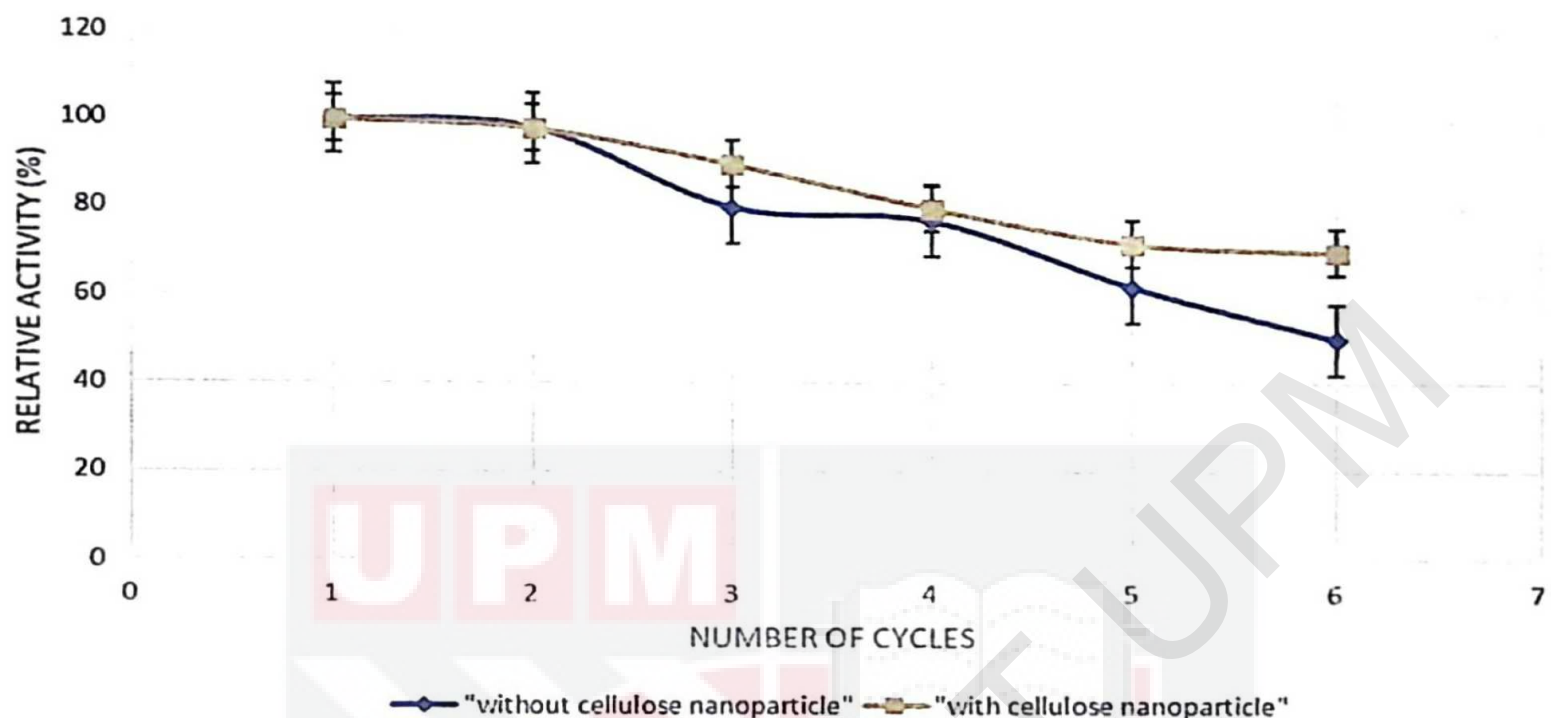


Figure 13: Graph of Reusability of Immobilized Glucoamylase

Figure 13 shows the reusability of immobilized glucoamylase which is 6 cycles of repeated batch process. This repeated batch process was done at 55°C which is the optimum temperature of glucoamylase. The result shows that the residual activity for immobilized glucoamylase without cellulose nanoparticle has gradually decreased and can retained its activity for about 50% after 6 cycles of reaction. In contrast, the residual activity for immobilized glucoamylase with cellulose nanoparticle has slightly decreased and can retained its activity for about 70% after 6 cycles of reaction.

It was observed that after the first batch, the catalytic activity of immobilized glucoamylase was started to decrease and was followed by the subsequent batch. This condition was similar for both immobilized enzyme with cellulose nanoparticle and without cellulose nanoparticle. The relative activity of immobilized glucoamylase that decreased

every cycle as shown in figure 13 is because of leaching of enzyme from support matrix by excessive washing of glucoamylase immobilized pieces after each batch or may due to conformational changes of repeatedly reusing the enzymes (Alkorta I et al., 1998).

Apart from that, this reusability study showed that the benefits of immobilized enzyme which have operational stability and can be re-used since it is easily recovered or separated from reaction medium compared to free glucoamylase that can only react for one-time production.



CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

After studying the observations, it was found that immobilization of glucoamylase onto sodium alginate and cellulose nanoparticle by entrapment method resulted about 62.10% of immobilization retained enzyme activity with 45.62% protein loading. The results show high percentage of protein loading and enzyme activity retained and this indicates the successful interaction between glucoamylase and sodium alginate and cellulose nanoparticle. This result was also contributing a better performance of enzymatic reaction. Furthermore, this was proven in reusability test which it was found that in the reaction catalyzed by immobilized glucoamylase can retained its activity after 6 cycles more than 50%. This clearly indicates that immobilized enzyme onto sodium alginate and cellulose nanoparticle contributes retention of enzyme activity for longer periods. Since immobilized enzymes can be recovered from reaction mixture and can be made available for reuse again, they are more preferred compared to free enzyme which can only be use once. Moreover, the strength of

immobilized glucoamylase with cellulose nanoparticle are stronger as compared to immobilized glucoamylase without cellulose nanoparticle. The structure of immobilized glucoamylase with the presence of cellulose nanoparticle is more rigid and stronger.

Lastly, it can be concluded that glucoamylase enzyme is able to be immobilize on calcium alginate with the presence of cellulose nanoparticle which improve the structure of bead.

5.2 Recommendations

Finding in this study are directive towards several research areas which can be developed further. Some of them are listed as below:

- i. The effect of temperature and pH on enzyme activity of free and immobilized glucoamylase onto support by optimization
- ii. Different method of immobilization to compare the resulting effect based on enzyme activity and enzyme loading rate

REFERENCES

- Abraham, T. E. (2004). Crosslinked enzyme crystals of glucoamylase as a potent catalyst for biotransformations. *Carbohydrate Research*, 1099-1104.
- Alkorta, I., Garbisu, C., Llama, M.J., & Serra, J.L. (1998). Industrial applications of pectic enzymes: A review. *Process Biochemistry*, 33(1), 21-28.
- [https://doi.org/10.1016/S0032-9592\(97\)00046-0](https://doi.org/10.1016/S0032-9592(97)00046-0)
- Dr. Sikander Ali, W. Z. (2017). Enzymes Immobilization: An Overview Of Techniques, Support Materials And Its. *INTERNATIONAL JOURNAL OF SCIENTIFIC & TECHNOLOGY RESEARCH*.
- Hesam, F. (2015). Evaluation of α -amylase Activity of Sweet Potato (*Ipomoea batatas*) Cultivated in Iran. *Evaluation of α -amylase Activity of Sweet Potato (Ipomoea batatas) Cultivated in Iran*, 41-48.
- Johnson, R. (2009). Comparative production of glucose and high fructose syrup from cassava and sweet potato roots by direct conversion techniques. *Innovative Food Science and Emerging Technologies*, 616-620.
- Lee, K. Y. (2012). Alginate: Properties and biomedical applications. *Progress in Polymer Science (Oxford)*, 106-126.
- Lee, K. Y. (2012). Alginate: Properties and biomedical applications. *Progress in Polymer Science (Oxford)*, 106-126.
- Nielsen, T. H. (1997). A β -Amylase in Potato Tubers Is Induced by Storage at Low Temperature. *Plant physiology*, 503-510.
- Noda, T. (2001). Sweet potato β -amylase immobilized on chitosan beads and its application in the semi-continuous production of maltose. *Carbohydrate Polymers*, 189-195.
- Pawar, S. N. (2012). Alginate derivatization: A review of chemistry, properties and applications. *Biomaterials*, 3279-3305.
- Presecki, A. V. (2013). Complete starch hydrolysis by the synergistic action of amylase and glucoamylase: Impact of calcium ions. *Bioprocess and Biosystems Engineering*.
- Sikander, A. (2017). Enzymes Immobilization: An Overview Of Techniques, Support Materials And Its Applications. *International Journal of Scientific & Technology Research*, 64-72.

- sulaiman, S. (2015). tudy on the Preparation of Cellulose Nanofibre (CNF) from Kenaf Bast Fibre for Enzyme Immobilization Application. 1541-1550.
- Sulaiman, S. (2016). Development of cellulose nanofibre (CNF) derived from kenaf bast fibre and it ' s potential in enzyme immobilization support KENAF BAST FIBRE AND IT ' S POTENTIAL IN ENZYME.
- Tavano, O. L. (2013). Optimization of the immobilization of sweet potato amylase using glutaraldehyde-agarose support. Characterization of the immobilized enzyme. *Process Biochemistry*, 1054-1058.
- Teotia, S. (2001). An efficient purification process for sweet potato beta-amylase by affinity precipitation with alginate. *Enzyme and Microbial Technology*, 792-795.
- THIVEND, P. (1972). Determination of Starch with Glucoamylase. *General Carbohydrate Method*, 100-105.
- Xiao, Z. (2007). Corrigendum to "A quantitative starch-iodine method for measuring alpha-amylase and glucoamylase activities" [Anal. Biochem. 351 (2006) 146-148] (DOI:10.1016/j.ab.2006.01.036). *Analytical Biochemistry*, 154.

APPENDICES

Appendix A

Absorbance value for standard BSA using UV/VIS Spectrophotometer at wavelength 595 nm.

Table 4: Absorbance Value for Standard BSA

Protein Concentration ($\mu\text{g/ml}$)	Absorbance value at wavelength of 595 nm			
	1	2	3	Average
0	0.000	0.000	0.000	0.000
10	0.062	0.069	0.069	0.067
20	0.152	0.135	0.159	0.149
40	0.309	0.309	0.319	0.312
60	0.450	0.476	0.467	0.464
80	0.617	0.625	0.639	0.639
100	0.831	0.842	0.854	0.842

(Adapted from Ciehl, 2012)

Appendix B

Table 5: Absorbance Value for Calibration Curve Of HPLC-Ri

Glucose Concentration ($\mu\text{g/ml}$)	Absorbance at 500 nm
	Average
0	0.000
230	0.0856
260	0.0976
500	0.194
750	0.294
1000	0.394
2000	0.794

Appendix C

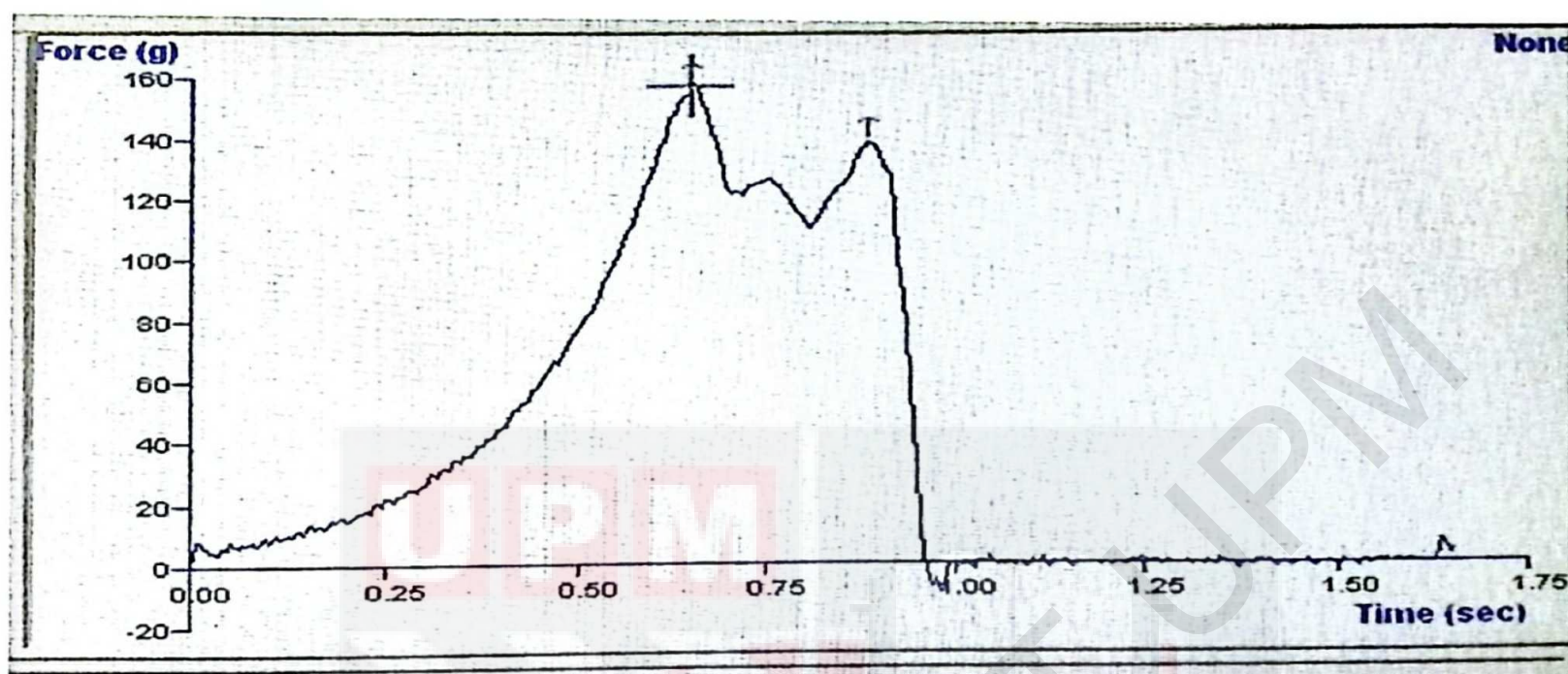


Figure 14: Compression Test Graph Result for Immobilized Bead Without Cellulose Nanoparticle

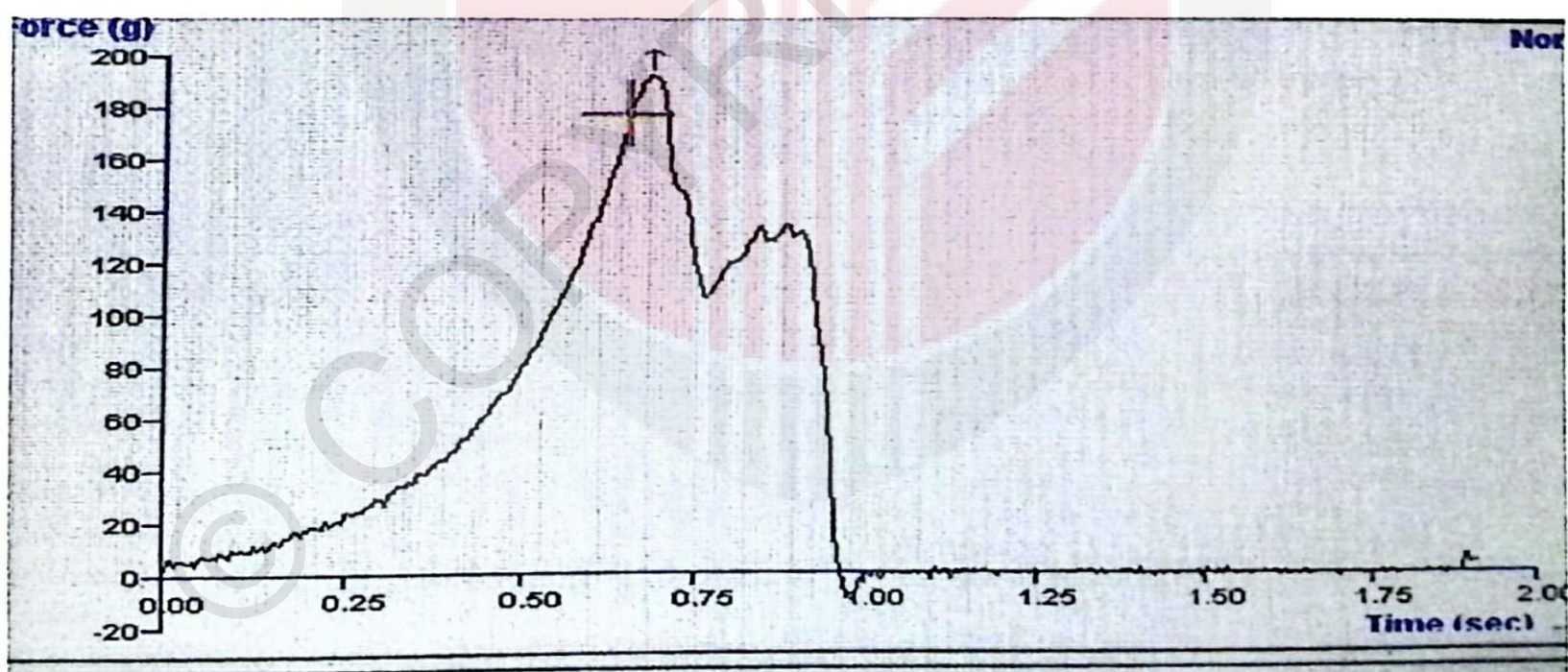
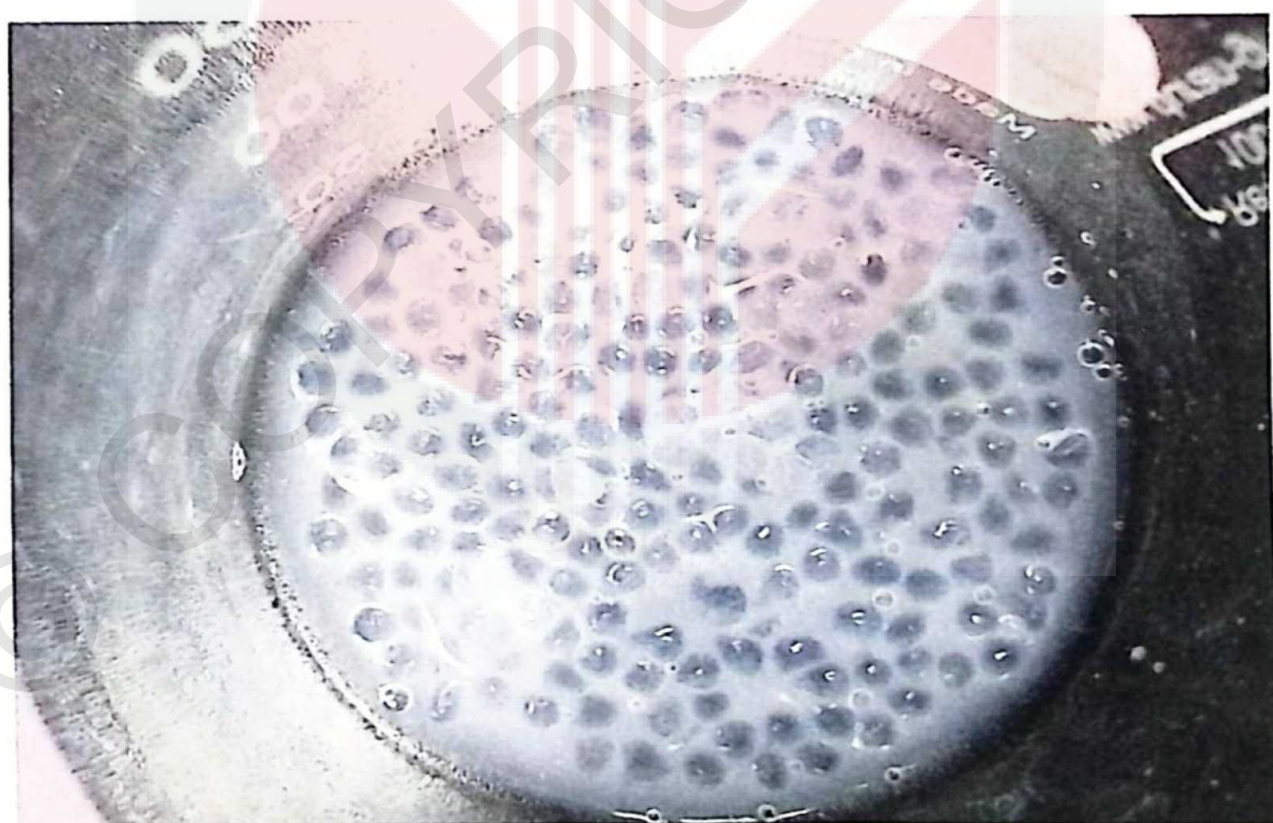


Figure 15: Compression Test Graph Result for Immobilized Bead With Cellulose Nanoparticle



(a) Immobilized enzyme without cellulose nanobead



(b) Immobilized enzyme with cellulose nanobead

Figure 16: Comparison of Immobilized Bead with Different Support

Appendix E

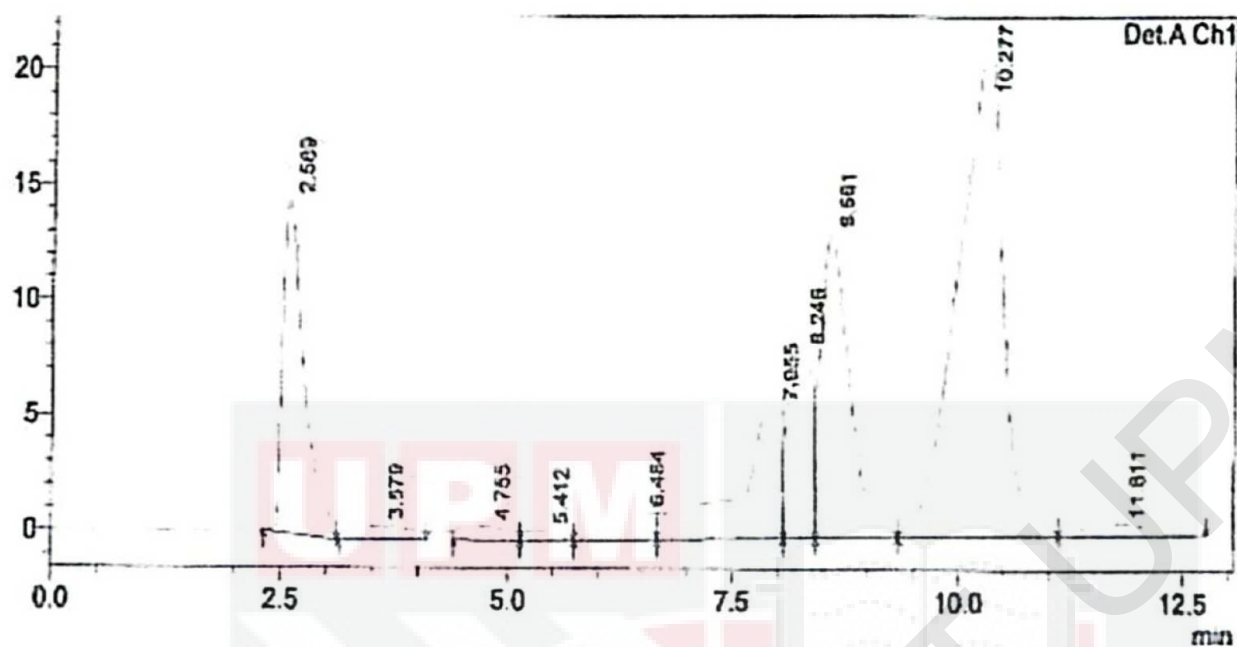


Figure 17: HPLC Chromatogram of Glucose Produced with Soluble Starch