การเพิ่มความเสถียรของเม็ดเจลอัลจิเนตด้วยยางธรรมชาติและไฮดรอกซีอะพาไทท์ชนิดนาโนเพื่อการตรึงเอนไซม์

นายอภิวัฒน์ สัตย์ซ้ำ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ปีการศึกษา 2555

STABILITY IMPROVEMENT OF ALGINATE BEADS USING NATURAL RUBBER/HYDROXYAPATITE NANOCOMPOSITE FOR ENZYME IMMOBILIZATION

Apiwat Satsum

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology

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STABILITY IMPROVEMENT OF ALGINATE BEADS USING NATURAL RUBBER/HYDROXYAPATITE NANOCOMPOSITE FOR ENZYME IMMOBILIZATION

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirement for a Master's Degree.

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อภิวัฒน์ สัตย์ซ้ำ : การเพิ่มความเสถียรของเม็ดเจลอัลจิเนตด้วยยางธรรมชาติและไฮดรอกซีอะพาไทท์ชนิดนาโนเพื่อการตรึงเอนไซม์ (STABILITY IMPROVEMENT OF ALGINATE BEADS USING NATURAL RUBBER/HYDROXYAPATITE NANOCOMPOSITE FOR ENZYME IMMOBILIZATION) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.โชคชัย วนภู, 93 หน้า.

แคลเซียมอัลจิเนต, ไฮดรอกซิอะพาไทท์และยางพาราเป็นวัตถุดิบที่ถูกนำไปใช้อย่างแพร่หลาย ทั้งในการประยุกต์เพื่อการตรึงเอนไซม์และชะลอการปลดปล่อยหรือเพื่อควบคุมการปลดปล่อยของตัวยา โดยในการทดลองครั้งนี้ได้ทำการผสมแคลเซียมอัลจิเนตเข้ากับไฮดรอกซีอะพาไทท์ซึ่งเตรียมได้จากส่วนผสมทางเคมีแบบเปียกและน้ำยางพารา ทำการแบ่งกลุ่มการทดลองออกเป็น 2 กลุ่มคือ แคลเซียมอัลจิเนต/ยางพารา และแคลเซียมอัลจิเนต/ไฮดรอกซีอะพาไทท์/ยางพารา โดยทำการปรับเปลี่ยนปริมาณของน้ำยางพารา ซึ่งปริมาณของน้ำยางพาราในการทดลองครั้งนี้คือ 2.5%, 5%, 10%, 15% และ 20% จากการทดลองการเตรียมเม็ด เจลบีดพบว่า ปริมาณน้ำยางพาราที่ไม่สามารถใช้ได้คือ 15% และ 20% เพราะจะทำให้เม็ดเจลบีดที่ได้ลอยน้ำ ดังนั้นจึงใช้ปริมาณน้ำยางพาราตั้งแต่ 2.5-10% ในทุกๆการทดลองต่อไป

จากการทดสอบหาค่าความหนืดของสารละลายผสมทั้งหมดก่อนที่จะทำการขึ้นรูปเม็ดเจลบีดแสดงให้เห็นว่า เมื่ออุณหภูมิของสารละลายผสมเพิ่มขึ้น ความหนืดของสารละลายจะลดลง จากนั้นได้ทำการทดสอบค่าความแข็งแรง และค่าความคงตัวของเม็ดเจลบีด ผลปรากฏว่าที่การผสมยางพาราลงไป 10% มีผลทำให้เม็ดเจลบีดของทั้งกลุ่มการทดลอง แคลเซียมอัลจิเนต/ยางพารา และแคลเซียมอัลจิเนต/ไฮดรอกซิอะพาไทท์/ยางพารา มีค่าสูงที่สุด โดยความแตกต่างกันคือ ลักษณะการแตกของเม็ดเจลบีดที่ต่างกัน กล่าวคือลักษณะการแตกแบบครั้งเดียวพบในกลุ่มตัวอย่างแคลเซียมอัลจิเนต/ยางพารา และลักษณะการแตกสองครั้งพบในกลุ่มตัวอย่าง แคลเซียมอัลจิเนต/ไฮดรอกซิอะพาไทท์/ยางพารา หลังจากนั้นทำการทดลองค่าการดูดซับน้ำของตัวอย่างทั้งสองกลุ่มการทดลอง พบว่าเมื่อมีการผสมยางพาราลงไป ค่าการดูดซับน้ำของตัวอย่างลดลงตามสัดส่วนของยางพาราที่เพิ่มขึ้น และค่าการดูดซับน้ำลดลงมาที่สุดที่ปริมาณของยางพาราเท่ากับ 10% ทั้งนี้เนื่องมาจากยางพารานั้นดูดซึมน้ำได้น้อยมาก ซึ่งไปขัดขวางการดูดซับน้ำของเม็ดเจลบีด ยิ่งไปกว่านั้นอุณหภูมิ และ pH ก็ยังส่งผลต่อค่าการดูดซับน้ำของเม็ดเจลบีดอีกด้วยโดยค่าของอุณหภูมิ และ pH ที่ส่งผลดีที่สุดต่อการดูดซับน้ำของเม็ดเจลบีด คือ pH 4-7 และอุณหภูมิที่ 25 องศาเซลเซียส จากการใช้กล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราด และกล้องจุลทรรศน์อิเล็กตรอนแบบส่องส่องผ่านพบว่า เนื้อของยางพาราและอัลจิเนตเข้ากันได้ดี แต่ไฮดรอกซีอะพาไทท์นั้นมีลักษณะเป็นแท่งคริสตัลอยู่ทั่วบริเวณ ซึ่งการผสมกันของสารดังกล่าวได้ถูกนำไป

ทดสอบด้วยเทคนิค FT-IR เพื่อยืนยันการผสมกันจากการเปลี่ยนแปลงของพันธะเคมี ซึ่งพบว่าพันธะเคมีของยางพารามีการเปลี่ยนแปลงโดยมีลักษณะการย้ายตำแหน่ง

การทดสอบการตรึงเอนไซม์ จากการทดสอบดังกล่าวพบว่าในการเปลี่ยนแปลงปริมาณยางพาราและอุณหภูมิในระหว่างการขึ้นรูปเม็ดเจลบีดนั้น ที่ปริมาณยางพาราและอุณหภูมิสูง (40 และ 50 องศาเซลเซียส) พบว่าแทบจะไม่มีกิจกรรมของเอนไซม์เกิดขึ้น นั้นเป็นเพราะเมื่อยางพาราได้รับอุณหภูมิสูง (40 และ 50 องศาเซลเซียส)ในขณะขึ้นรูป จะมีผลทำให้ยางพาราเกิดการขยายหรือพองตัวจนไปปิดรูพรุนภายในโครงสร้างของเม็ดเจลบีดจนหมด และทำให้ไม่พบกิจกรรมของเอนไซม์เกิดขึ้น ซึ่งเหตุการณ์ดังกล่าวกลับไม่พบเมื่อมีการเติมไฮดรอกซิอะพาไทท์ลงไป โดยไฮดรอกซิอะพาไทท์นั้นไปทำหน้าที่ในการขัดขวางการปิดกั้นรูพรุนของยางพารา และสอดคล้องกับการทดสอบความเร็วเริ่มต้นของปฏิกิริยาซึ่งพบว่ากลุ่มตัวอย่างที่มีไฮดรอกซีอะพาไทท์มีความเร็วมากกว่ากลุ่มตัวอย่างที่ไม่มีการเติมไฮดรอกซิอะพาไทท์ และผลการทดสอบการนำกลับมาใช้ของเม็ดเจลบีดที่ตรึงเอนไซม์พบว่าไม่มีความแตกต่างกันมานักสำหรับเม็ดเจลบีดชนิด แคลเซียมอัลจิเนต, แคลเซียมอัลจิเนต/ไฮดรอกซิอะพาไทท์/ยางพารา และ แคลเซียมอัลจิเนต/ไฮดรอกซิอะพาไทท์/ยางพารา (10%) เมื่อใช้ไป 8 ครั้ง แต่สำหรับเม็ดเจลบีดชนิด แคลเซียมอัลจิเนต/ยางพารา (10%)สามารถนำกลับมาใช้ได้เพียงสองครั้ง

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2555 ลายมือชื่ออาจารย์ที่ปรึกษา_____

APIWAT SATSUM : STABILITY IMPROVEMENT OF ALGINATE BEADS USING NATURAL RUBBER/HYDROXYAPATITE NANOCOMPOSITE FOR ENZYME IMMOBILIZATION. THESIS ADVISOR : ASST. PROF. CHOKCHAI WANAPU, Ph.D., 93 PP.

CALCIUM ALGINATE/HYDROXYAPATITE/LATEX/NANOCOMPOSITE

Calcium alginate (CA) is applied to enzyme immobilization for prolonging enzymatic activity. In this study CA composite with hydroxyapatite (HA) was prepared by using a wet chemical method and then mixed with various Latex (LX) concentrations into groups of CA/LX and CA/HA/LX in various LX concentrations. LX solutions at 2.5%, 5%, 10%, 15% and 20% were used and it was found that between 15% and 20% of their beads were floating. Thus 2.5-10% LX with CA and CA/HA were used for the entire experiment. At the gel forming stage, all solutions showed decreased viscosity when the temperature was increased.

The results of a texture profile showed that both the strength and stability of the bead structure at 10% LX addition from each group of CA/LX and CA/HA/LX were higher than the control group. There were differences in the texture profile for CA/LX and CA/HA/LX groups which showed different cracking pattern. A single crack was found in the CA/LX group and a double crack was found in the CA/HA/LX group. Bead swelling of 10% LX decreased because the LX's hydrophobic character obstructed the swelling of defective CA/LX and CA/HA/LX beads. Moreover, pH and temperature had an affect on the swelling of the beads. The best conditions were found between pH 4-7 at 25°C. The morphology of all the groups was studied by SEM and TEM and it was found that the LX material was homogeneous in CA and

CA/HA, and that the HA in CA/LX resulted in crystallization. The material was proved by the result of FT-IR analysis when it was found that the CA/LX and CA/HA/LX had changed the wave number at 1325-1380 cm^{-1} which was the character's bands of CH_2 from LX. The bands shifted towards a higher number of waves in the composite beads.

Finally, the enzyme was immobilized in all the composite beads. The most important result of the enzyme immobilization experiment was shown in the high concentration of LX at 40°C and 50°C at the gel forming stage. The LX in CA/LX was inflated and blocked the flow of the substrate. However, this did not occur in CA/HA/LX. It is possible that the HA in CA/HA/LX bead expanded the pores inside the beads, whereas the LX expansion was inadequate to seal the expanded pores. In addition, LX determined the rate of relative enzyme activity for CA/LX and CA/HA/LX. HA also caused the enzymatic initial velocity of CA/HA/LX bead to be higher than that for the CA/LX bead. The results of reused immobilized beads showed no differences in the relative activity of CA, CA/HA and CA/HA/(10%) LX for 8 cycles, but for CA/(10%) the LX beads were only reused for 2 cycles.

School of Biotechnology Student's Signature_____

Academic Year 2012 Advisor's Signature_____

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Apiwat Satsum

CONTENTS

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF TABLE

Table **Page**

LIST OF FIGURE

Figure **Page**

LIST OF FIGURE (Continued)

Figure **Page**

LIST OF FIGURE (Continued)

Figure **Page**

LIST OF ABBREVIATIONS

°C	=	degree Celsius
ng	=	nanogram
µg	=	microgram
mg	=	milligram
g	=	gram
kg	=	kilogram
m^2	=	square meter
s	=	second
min	=	minute
h	=	hour
d	=	day
µl	=	microliter
ml	=	milliliter
mM	=	millimolar
M	=	molar

CHAPTER I

INTRODUCTION

1.1 Significance of the study

Alginate is of considerable interest in the fields of biotechnology and biomedical engineering due to biodegradability, biocompatibility, bioreactor, natural abundance, unique chemical structures and physical of chemical/biological properties (Yang et al., 2011). Alginate was firstly discovered in the late 19^{th} century by an English chemist, E.C.C. Standford that is quiet abundant in nature occur as a structure component in marine brown algae (*Laminaria, Phaeophyceae* and *Macrocystis*) (Gasesa, 1998). Alginate, a linear polysaccharide consisting of mannuronic acid (M) and guluronic acid (G) residues, is considered as a stimuli-responsive polymer which gel forming processed in the presence of di-valent or multi-valent cations such as Ca^{2+} and changes polymer hydrophilicity in response to external pH and temperature environment (Yang et al., 2009). Physically crosslinked alginate gel is widely used for immobilizing purposes, and has been studied for many years in an effort to relate composition of the polymer to its gel properties such as pore size, swelling and mechanical stability (Sand et al., 2010). However, since natural materials have soft structures, the ability to modify polymer properties are applied (Boisseson et al., 2004). Therefore, there is considerable interested in this natural material and in the ability to physical property have been applied to improve the properties of the biopolymer vehicles.

Hydroxyapatite is a famous nano-material to apply in biotechnology its material is considerable interest to improve other physical properties such as porous ability cause of the hydroxyapatite crystal can induce a porous structure and crystal network in side gel beads (Huang et al., 2011; Pallela et al., 2011). And an important material to improve strength and gel beads stability is natural rubber or latex (*cis*-1, 4-polyisoprene) occurs in several plant species (Mark et al., 2005), the most important source is the '*Hevea brasiliensis*' tree which accounts for over 99% of the world's natural rubber production. Natural rubber is extracted as a latex aqueous emulsion or dispersion of the natural polymer (~96 % of solids) and other substances, such as proteins (~1%) and lipids (~3%) (Kohjiya et al., 2006; Oberdisse et al., 2005).

This research was to develop a new co-biopolymer from an ordinary alginate polymer, expected with new properties, and to consider the possibility of applications as enzyme retentive, controlled and prolong device. Finally, tested invertase enzyme was immobilized with these nano-composite beads for controlling its activity in some conditions.

1.2 Research objectives

1.2.1 To optimize condition for nano-composite beads production such as pH, temperature and ratio of each polymer.

1.2.2 To increase some structural properties of nanocomposite beads production such as porous ability and strength.

1.2.3 To apply nanocomposite beads enzyme immobilization using invertase as model enzyme and test efficiency of immobilization.

1.3 Research hypothesis

The structure of alginate beads is improved structural quality by hydroxyapatite and latex materials and is used in invertase enzyme immobilization.

1.4 Scope and limitation of the study

To produce and optimize nanocomposite beads with latex and hydroxyapatite. The physical properties of nanocomposite beads were characterized and then the efficiency of immobilized invertase enzyme was analyzed.

1.5 Expected results

The high strength and porus ability could be obtained from nanocomposite beads and showed prolong enzymatic invertase activity in immobilization stage.

1.6 References

Boisseson, M. R. D., Leonard, M., Hubert, P., Marchal, P., Stequert, A., Castel, C., et al. (2004). Physical alginate hydrogels based on hydrophobic or dual hydrophobic/ionic interactions: Bead formation, structure, and stability. **Journal of Colloid and Interface Science.** 273, 131-139.

Gacesa, P. (1998). Bacterial alginate biosynthesis recent progress and future prospects. **Microbiology.** 144: 1133-1143.

Huang, Y.T., Imura M., Yoshihiro N., Cheng C.H. and Yusuke Y. (2011). Block copolymer-assisted synthesis of hydroxyapatite nanoparticles with high surface area and uniform size. **Science Technology Advance Material**. 12**:**450-505.

Kohjiya S., Katoh A., Suda T., Shimanuki J., Ikeda Y. (2006). Visualisation of carbon black networks in rubbery matrix by skeletonisation of 3D-TEM image. **Polymer.** 47(10): 3298-301.

Mark, J.E., Abou-Hussein R., Sen, T.Z., Kloczkowski A. (2005). Some simulations on filler reinforcement in elastomers. **Polymer**. 46(21): 8894-904.

Oberdisse J., El Harrak A., Carrot G., Jestin J., Boue F. (2005). Structure and rheological properties of soft-hard nanocomposites: influence of aggregation and interfacial modification. **Polymer.** 46(17): 6695-705.

Pallela, .R, Venkatesan, J. Kim, S.K. (2011). Polymer assisted isolation of hydroxyapatite from *Thunnus obesus* bone. **Ceramics International.** 37: 3489-3497.

Sand, A., Yadav, M., & Behari, K. (2010). Synthesis and characterization of alginateg-vinyl sulfonic acid with a potassium peroxydiphosphate/thiourea system. **Journal of Applied Polymer Science**. 118: 3685-3694.

Yang, J. S., Chen, S. B., & Fang, Y. (2009). Viscosity study of interactions between sodium alginate and CTAB in dilute solutions at different pH values. **Carbohydrate**. 75: 333-337.

Yang, J.S., Xie, Y.J., & He, W. (2011). Research progress on chemical modification of alginate: A review. **Carbohydrate Polymers**. 84: 33-39.

CHAPTER II

LITERATURE REVIEW

2.1 Alginate

2.1.1 Chemical properties

Alginate is a linear copolymer of β-D-mannuronic acid (M) and C-5 epimer, α-L-guluronic acid (G) residues arranged in irregular blocks along the linear chain (Figure 1). These monomers can be organized in blocks of consecutive G-residues (G-blocks), consecutive M-residues (M-blocks), alternating M-residues and G-residues (MG-blocks) or randomly organized blocks (Figure 2) (Valla et al., 1996). The relative amount of each block type varies both with the origin of the alginate. Due to these properties, alginate has various industrial uses as viscosifiers, stabilizers and gel-forming, filming-forming or water-binding agents. These applications range from textile printing and manufacturing of ceramics to produce of welding rods and water-treatment. The polymer is soluble in cold water and forms thermo stable gels. These properties are utilized in the food industry in products like custard creams and restructured food (Yang et al., 2011). The polymer is also used as a stabilizer and thickener in a variety of beverages, cream, emulsions and sauces. The pharmaceutical industry uses alginate as wound dressings and dental impression materials. The polysaccharide is also used as a tablet binder or disintegrant, and by carefully choosing the optimal alginate quality one can control release of the drug (Onsoien, 1996). The biomedical used alginate to produce such as prosthetics dentistry and

artificial organs (Change, 2003). In recent year alginate is by far the most widely used polymer for immobilization and microcapsulation technologies (Funduenanu, et al., 1999). Within the food industry, alginate encapsulation and immobilization technologies are also used for many purposes, including food processing, food functionality and product acceptability. These immobilization technologies can enhance productivity as a result of continuous operation and reuse of the entrapped cells or enzymes (Yang et al., 2011). Immobilization or encapsulation technology is used to produce a wide range of bacterial metabolites, including enzymes, cells, amino acids, organic acids and alcohols (Nazzaro et al., 2012).

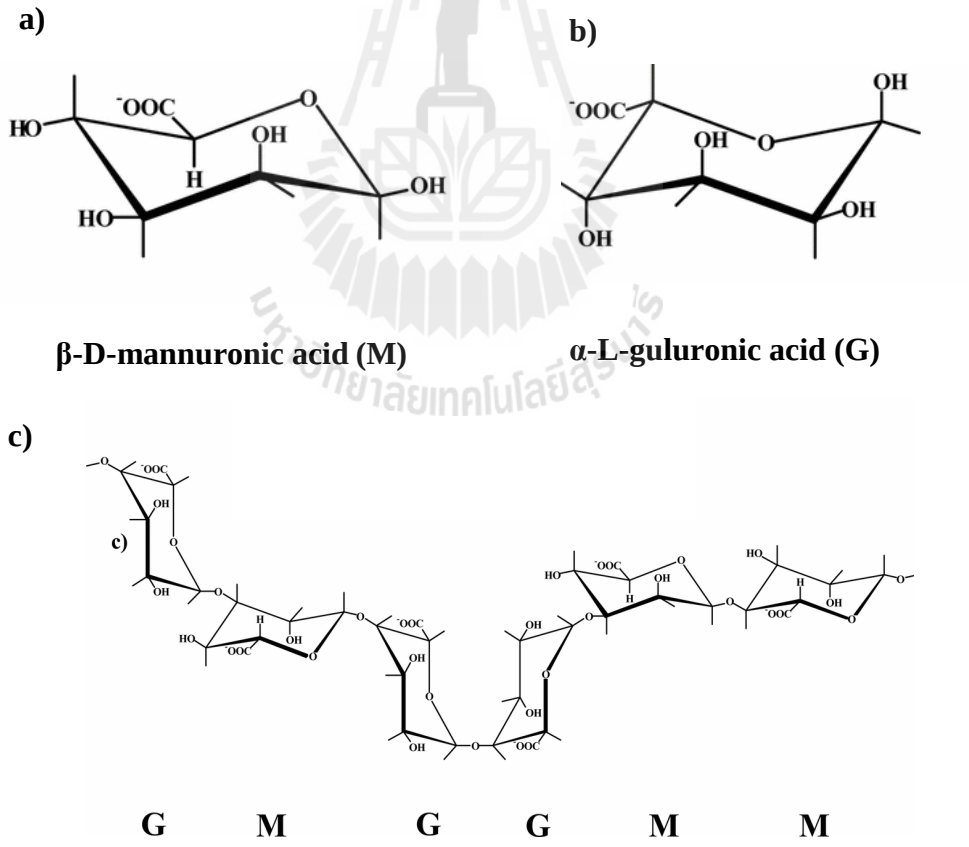


Figure 1. Structure of a) β-D-mannuronic acid or M-block, b) α-L-guluronic acid or G-block and c) alginate (Avella, et al., 2007).

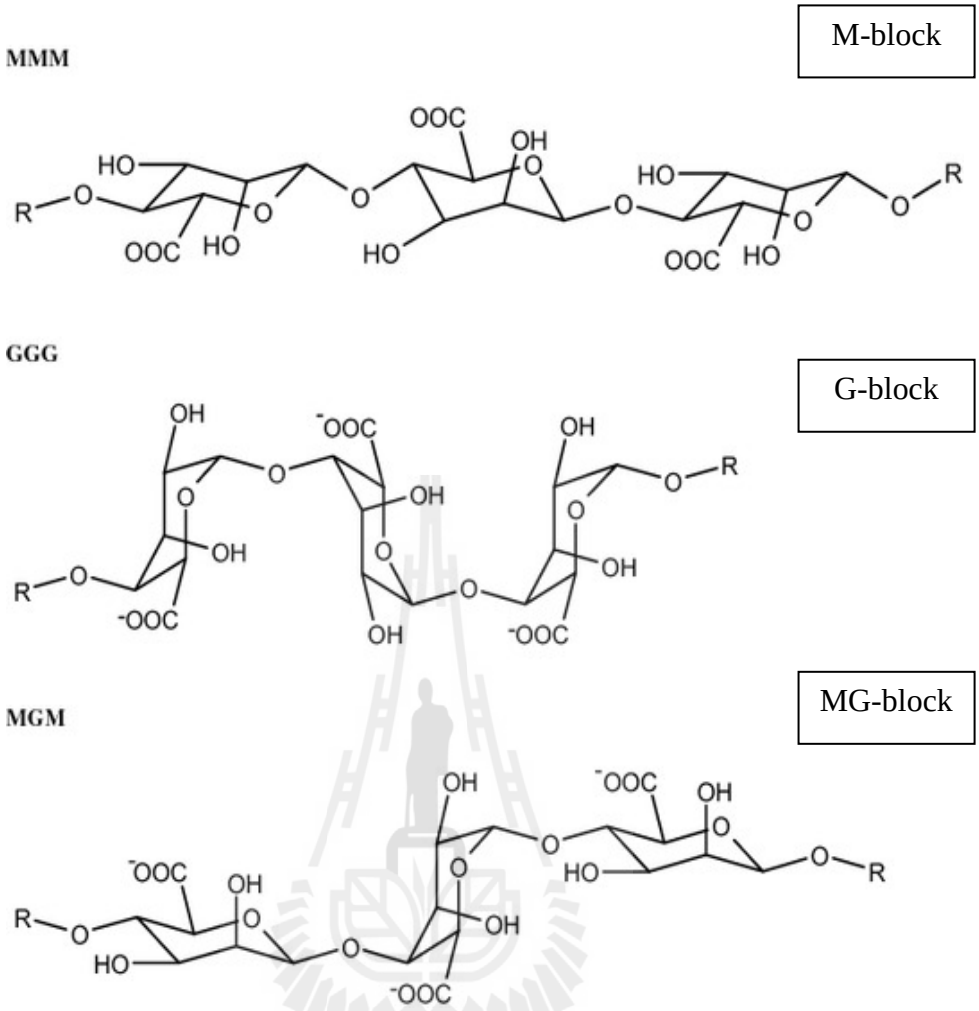


Figure 2. Block polymers structure of alginate (Avella, et al., 2007).

2.1.2 Physical properties

2.1.2.1 Rheological and solubility properties

Alginates are soluble in aqueous solution at pH ranging from 5-10. Alginate solution concentrations of less than 0.5% w/v show the Newtonian property, whereas solutions of 0.5-2.5% w/v behave as pseudo-plastic fluids over a wide range of shear rates (10-10,000 sec^{-1}). Alginate concentration increases when the solution passes through stages of a viscous liquid to a thick paste, any further addition of alginate to the suspension medium may not be properly dispersed and hydrated

(Kourkoutas et al., 2002). Alginate solution viscosity depends on polymer molecular weight, composition and sequence of M and G residues, and decreases with increasing temperature. There are three essential parameters determining and limiting the solubility of alginates in water. (I) The pH of the solvent is important because of its electrostatic charges on the mannuronic acid and guluronic acid residues. (II) Total ionic strength of the solute also plays an important role (salting-out effects of non-gelling cations), and obviously, the content of gelling ions in the solvent limits the solubility. And (III) in the latter case, the structure is depend on the water and content of Ca^{2+} ions are to be the main structure forming (Steinbuchel et al., 2001).

2.1.2.2 Alginate on pH properties

Alginate contains a carboxylic acid moiety per uronic acid monomer. The acid dissociation constants (pKa) of alginate polymers differ only slightly from those of the monomeric residues which were determined to be 3.38 and 3.65 for mannuronic and guluronic acids, respectively, determined from potentiometric titration. The pKa of alginate is ionic strength dependent and affected by the monomeric block compositions. Alginates are susceptible to both acid and alkaline degradation, via cleavage of the glycosidic linkages. Compared to other sugars, glycosidic linkages involving uronic acids such as M and G are more strongly resistant toward hydrolysis in very strong acid, conditions normally used to fully hydrolyse polysaccharides to monosaccharides (Lee et al., 2000). At very low pH, the homopolymeric blocks of alginates have tendency to crystallize due to inductive effects, protecting the polymer from acid hydrolysis. However, the rate of acid degradation near the pKa (pH range 1-4) is much higher than for neutral polysaccharides. In this range, the carboxylic acid of M and G contributes to the hydrolysis by intramolecular catalysis in addition to the free H^+ ions, making alginate

less stable than neutral polysaccharides such as methylcellulose. The rate of degradation is approximately 3 times higher for MM blocks compared to GG blocks, but is almost independent of pH within. Optimum stability is at pH 7-12 (Rao et al., 1989; Velten et al., 1999).

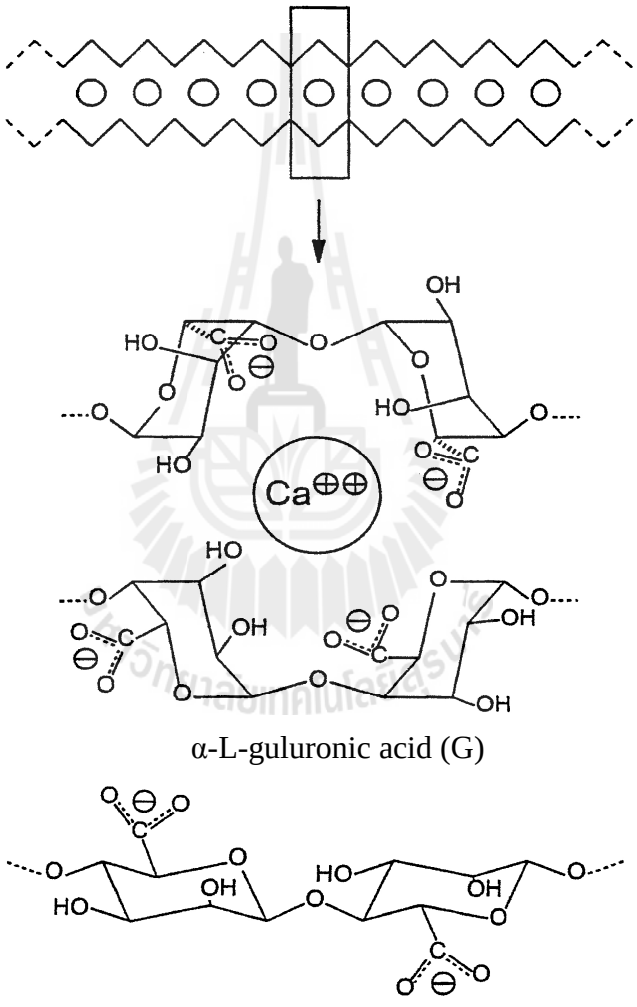
2.1.2.3 Properties of ion binding

Alginates have limited solubility in solution containing gelling ions such as Ca^{2+}, Ba^{2+}, and Zn^{2+}. However, pre-dissolved alginates in aqueous solution at physiological pH are polyanionic and can undergo solution gel transition in the presence of divalent or multi-valent cations, most commonly with Ca^{2+} or form interpenetrating polymer complex with polycations such as chitosan. The basis for the gelling properties of alginates is their specific ion-binding characteristics (Haug, 1964; Smidsrod, 1973). Experiments involving equilibrium dialysis of alginate have shown that the selective binding of certain alkaline earth metals ions (e.g. strong and cooperative binding of Ca^{2+} relative to Mg^{2+}) increased apparent with increasing content of α-L-guluronate residues in the chains. Poly-mannuronate blocks and alternating blocks were almost without selectivity.

The high selectivity between similar ions such as those from the alkaline earth metals indicates that some chelating caused by structural features in the G-blocks takes place. Attempts were made to explain this phenomenon by the so-called “egg-box” model (Grant et al., 1973), based upon the linkage conformation of the guluronate residues (Figure 3).

The selectivity of alginates for multivalent cations is also dependent on the ionic composition of the alginate gel, as the affinity toward a specific ion increases with increasing content of the ion in the gel (Skjak-Braek et al., 1986). Thus, a Ca-alginate (CA) gel has a markedly higher affinity toward Ca^{2+} ions than its

Na-alginate (NA) solution. This has been explained theoretically (Smidsrod, 1973; Skjak-Braek et al., 1986) by a near-neighbor auto-cooperative process (Ising model) and can be explained physically by the entropically unfavorable binding of the first divalent ion between two G-blocks and the more favorable binding of the next ions in the same dimension.



α-L-guluronic acid (G)

β-D-mannuronic acid (M)

Figure 3. The calcium ion-dependent epimerization process and the formation of the so called “egg-box model” (Steinbuchel et al., 2001).

2.1.2.4 Alginate swelling behavior

For the swelling behavior of dry alginate powder in aqueous media with different concentrations of Ca^{2+}, there seems to be a limit at approximately 3 mM free calcium ions. Alginate can be solubilized at above 3 mM [Ca^{2+}] by the addition of complexing agents, such as polyphosphates or citrate, before addition of the alginate powder (Skjak-Braek et al., 1986).

2.1.2.5 Gel formation

A direct mixing of alginate and multivalent cations rarely produces homogeneous gels due to the very rapid and irreversible binding of such ions. A controlled introduction of cross-linking ions is possible by the two fundamental methods of preparation sach as the diffusion method and the internal setting method (Smidsrod and Draget, 1996). The diffusion method is characterized by allowed a cross-linking ion (e.g., Ca^{2+}) to diffuse from a large outer reservoir into an alginate solution. Diffusion setting is characterized by rapid gelling kinetics and is utilized for immobilization purposes where each droplet of alginate solution makes one single gel bead with entrapped active agent (Smidsrod and Skjak-Braek, 1990; Funduennanu, 1999). This setting is High-speed also beneficial, e.g., in restructuring of the food when a given size and shape of the final product is desirable (Smidsrod, 1973).

The internal setting method differs from the diffusion method in that the Ca^{2+} ions are released in a controlled fashion from an inert calcium source within the alginate solution. Controlled release usually is obtained by a change in pH, by a limited solubility of the calcium salt source, and/or the by presence of chelating agents. The main difference between internal and diffusion setting is the gelling kinetics that is not diffusion-controlled in the former case (Draget et al., 1991).

2.1.3 Applications of alginate

Given the large number of different applications, alginates must be regarded as one of the most versatile polysaccharides. These applications span from traditional technical utilization to foods and biomedicine (Nazzaro et al., 2012).

2.1.3.1 Biotechnology technology

In recent years, entrapment within spheres of calcium alginate gel has become the most widely used technique for immobilizing living cells such as bacteria, cyanobacteria, algae, fungi, plant protoplast, plant and animal cells. Alginate immobilized cell system is used as biocatalysts in several industrial processes ranging from ethanol production by yeast (Crescenzi, 1995), stimulating immune cells to secrete cytokines, such as Tumor Necrosis Factor-α (TNF-α), Interleukin-1 (IL-1) and Interleukin-6 (IL-6) (Otterlei et al., 1991). Alginate gel also has a potential as implantation material for hormone-production cells and encapsulated langerhans islets are currently being evaluated as a bio-artificial endocrine pancreas (Skjak-Braek and Moe, 1992; Crescenzi, 1995; Clementi, 1995).

2.1.3.2 Textile industry

The quantitatively most important technical application of alginates is as a shear-thinning viscosity in textile printing, in which alginates has gained a high popularity because of the resulting color yield, brightness, and print levelness (Steinbuchel et al., 2001). In the textile printing, alginates are used as thickeners for the paste containing the dye. These pastes may be applied to the fabric by either screen or roller equipment. Alginates became important thickeners with the advent of reactive dyes (Sutherland and Ellwood, 1979). These combine chemically with cellulose in the fabric. Many of the usual thickeners such as starch react with the

reactive dyes and this leads to lower color yields and sometimes by-products that are not easily washed out. Alginates do not react with the dyes; they easily wash out the finished textile and are the best thickeners for reactive dyes. Alginates are more expensive than starch and recently starch manufacturers have made efforts to produce modified starches that do not react with the reactive dyes, so it is becoming a more competitive market. The types of alginate required vary from medium to high apparent viscosity with older screen printing equipment, to low apparent viscosity if modern, high speed, roller printing is used (Steinbuchel et al., 2001).

2.1.3.3 Paper industry

The main use for alginate in the paper industry is in surface sizing Alginate added to the normal starch sizing gives a smooth continuous film and a surface with less fluffing. The oil resistance of alginate films gives a size with better oil resistance and enhances greaseproof properties. An improved gloss is obtained with high gloss inks. If papers or boards are to be waxed, alginate in the sized will keep the wax mainly at the surface. So give better coating run ability than other thickeners especially in hot, on machine coating applications. Cottrell and Kovacs (1980) give examples of formulations for a kraft lineboard sizing and for corrugating adhesives. An improved sizing with alginate has been obtained by using a paper containing 5-25% of calcium carbonate filler; the calcium alginate film which forms gives better solvent resistance. The alginate is blended with 6-20 parts of starch or it may be combined with polyvinyl alcohol.

Paper coating methods have developed significantly since the late 1950s as the demand for a moderately priced coated paper for high quality printing. Trailing blade coating equipment runs at 1,000 meters per minute or more so the

coating material, usually clay plus a synthetic latex binder, must have consistent rheological properties under the conditions of coating. Up to 1% alginate will prevent change in viscosity of the coating suspension under the high shear conditions where it contacts the roller. The alginate also helps to control water loss from the coating suspension into the paper, between the point where the coating is applied and the point where the excess is removed by the trailing blade. The viscosity of the coating suspension must not be allowed to increase by loss of water into the paper because this leads to uneven removal by the trailing blade and streaking of the coating. Medium to high viscosity alginates are used, at a rate of 0.4-0.8% of the clay solids. A new modified form of sodium alginate has been reported to be more effective than existing alginate and results in lower processing costs (Sutherland and Ellwood, 1979).

2.1.3.4 Welding rods

Coatings are applied to welding rods or electrodes to act as a flux and to control the conditions in the intermediate vicinity of the weld such as temperature or oxygen and hydrogen availability. The dry ingredients of the coating are mixed with sodium silicate (water-glass) which gives some of the plasticity necessary for extrusion of the coating onto the rod; it also acts as the binder for the dried coating on the rod. However, the wet silicate has no binding action and does not provide sufficient lubrication to allow effective and smooth extrusion. An additional lubricant is shape of the coating on the rod during drying and baking. Alginates are used to meet these requirements. The quantities of alginates used are very dependent on the type of welding rod being coated and the extrusion equipment being used. Alginate manufacturers are the best source of information for using alginates in welding rod applications (Steinbuchel et al., 2001).

2.1.3.5 Binders for animal feed

The worldwide growth in aquaculture has led to the use of crude alginate as a binder in salmon and other animal feeds, especially moist feed made from fresh waste fish with various dry components. Alginate binding can lower consumption by up to 40 percent and pollution of culture ponds is sharply reduced (Steinbuchel et al., 2001).

2.1.3.6 Release agents

The poor adhesion of films of alginate to many surfaces, together with their insolubility in non-aqueous solvents, have led to their use as mould release agents, originally for plaster moulds and later in the forming of fiberglass plastics. Sodium alginate (SA) also makes a good coating for anti-tack paper which is used as a release agent in the manufacture of synthetic resin decorative boards (Sumitomo, 1981). Films of calcium alginate (CA), formed in situ on a paper, have been used to separate decorative laminates after they have been formed in a hot-pressing system.

2.1.3.7 Medicine and Pharmacy

Alginates have been used for decade as helping agents in various human-health applications. Some examples include use in traditional would dressing, in dental impression material, and in some formulations preventing gastric reflux. Alginate was used as an immobilization matrix in various biotechnological processes. Entrapment of cells within Ca-alginate spheres has become the most widely used technique for the immobilization of living cells (Smidsrod and Skjak-Braek, 1990). This immobilization procedure can be carried out a single-step process under very mild conditions and is therefore compatible with most cells. The cell suspension is mixed with a SA solution and the mixture is dripped into a solution containing multivalent (usually Ca^{2+}). The droplets then instantaneously from gel-spheres

entrapping the cells in a three-dimensional lattice of ionic ally cross-linked alginate. The possible uses for such systems in industry, medicine, and agriculture are numerous, ranging from production of ethanol by yeast, to production of monoclonal antibodies by hybridoma cells, to mass production of artificial seed by entrapment of plant embryos (Smidsrod and Skjak-Braek, 1990).

The most exciting prospect for alginate gel immobilized cells is their potential use in cell transplantation. The main purpose of the gel is to act as a barrier between the transplant and the immune system of the host. Different cells have been suggested for gel immobilization, including parathyroid cells for treatment of hypocalcemia and dopamine-production adrenal chromaffin cells for treatment of Parkinson's disease (Aebisher et al., 1993; Change, 2003). However, major interest has been focused on insulin-producting cells for the treatment of type I diabetes. Alginate/poly-L-lysine capsules containing pancreatic Langerhans islets have been shown to reverse diabetes in large animals and currently are being clinically tested in humans (Soon-Shiong et al., 1993).

2.1.3.8 Foods

In food industry, alginate about 50% of world production it is used as food additives to improve, modify and stabilize the texture of foods. This is valid for such properties as apparent enhancement, gel-forming ability and stabilization of aqueous mixtures, dispersions and emulsions. In many industries it used full of alginate directly and apply for each industry (Cottrell and Kovacs 1980; Sime, 1990 and Littlecott, 1982).

2.1.4 Hydroxyapatite

Hydroxyapatite (HA) is the most biocompatible material due to chemical and crystallographic similarities to the apatite component of bone (Inkyo et al., 2008). However synthesis of HA has been obtained by

$$10Ca(NO_3)_2 + 6(NH_4)_2HPO_4 + 8NH_4OH \longrightarrow Ca_{10}(PO_4)_6(OH)_2 + 6H_2O + 20NH_4NO_3$$

For production of HA to produce materials for the hard tissue implantation due to its excellent biocompatibility and osteoconductivity as well as close similarity to the human bone structure (Monmaturapoj, 2008). Many processing routes have been used for the fabrication of porous HA granules, such as hydrothermal conversion of natural corals (Roy, 1974) and crushing of sintered blocks, granulation by vibration and rolling (Zhang, 1989), dripping procedure (Fabbri et al., 1994), casting in plaster mould (Liu, 1996) emulsion methods (Takahashai et al., 2008) and the spray drying process (Ikoma et al., 2008). Most of the HA biopolymer have been prepared according to the following two-step procedure. The HA particles with various nanocomposite were first prepared and then introduced into the biopolymer matrices under proper conditions. Although the pregenerated HA particles may be at the nano scale. HA is an ideal material for the preparation of drug scaffolds because of its excellent properties, such as the ability to absorb a variety of chemical species and biocompatibility. However, the release of drugs from HA has been proved to be initially very fast (Mizushima et al., 2006), similar to the weak of enzyme and cell immobilized.

Entrapment has been extensively used for the immobilization of enzymes or cell major limitation of this technique for the immobilization of enzymes is the

possible prolong during continuous use because of the small molecular size and stability of alginate gel. One of the major limitations of entrapment technique is the diffusion limitation as well as the hindrance, especially when the macromolecular substrates like starch and proteins are used. Diffusion problems can be minimized by using an open pore matrix and the all the above problem can be used hydroxyapatite for improve capability. Recently, the preparation and application of novel biopolymer/ inorganic material composites as immobilize enzyme or cell have attracted much attention owing to their unique structure and properties. Hydroxyapatite has been used for the preparation of this class of composites. The synergistic effect of biopolymer and inorganic material as well as the strong interfacial interactions between them via electrostatic interaction and hydrogen bonding could improve the mechanical properties, swelling behavior, porousability and slow release.

2.1.5 Natural rubber

Natural rubber or latex (LX) is obtained from the milky secretion of various plants, but the only important commercial source of natural rubber is the tree *Hevea brasiliensis.* The only other plant under cultivation as a commercial rubber source is guayule (*Parthenium argentatum*), a shrub native to the arid regions of South-east Asia such Malaysia and Thailand. All natural rubber materials are polymer, which are high molecular weight compounds consisting of long chains of one or more types of molecules, such as monomers. Vulcanization (or curing) produces chemical links between the loosely coiled polymeric chains. Elasticity occurs because the chains can be stretched and the crosslink cause them to spring back when the stress is released. Natural rubber is a polyterpene, i.e., it consists of isoprene molecules linked into loosely twisted chains (Crowther et al., 1988). The monomer units along the backbone

of the carbon chains are in a *cis* arrangement (Figure 4) and it is this spatial configuration that gives rubber its highly elastic character. From special property such as high strength, wider temperature range and highly elastic character of natural rubber may be suitable for use the various properties to improve the stability of alginate gel beads.

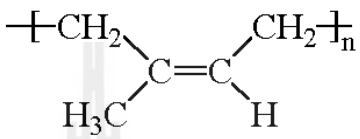


Figure 4. structure of natural rubber.

Polymer is something made of many units. The monomers are small molecules that usually contain 10 or less atoms in a row. Carbon and hydrogen are the most common atoms in monomers, but oxygen, nitrogen, chlorine, fluorine, silicon and sulfur may also be present. Chain in the monomers are polymerized together to make a chain with at least 1000 atoms in a row. It is this feature of large size that gives polymers their special properties macromolecules or polymers are found in the human body, animals, plants, minerals and manufactured products. For elastic, strength and stability properties of natural rubber are interest to enhance alginate beads in secondary biopolymer. When use the alginate beads in fermenter strength and thermal stability is important to properties requirement.

2.2 Immobilization

Alginate is commonly used as an immobilization medium for whole isolated enzyme preparations in industrial processes requiring enzymatic activity. Immobilization in this manner is a simple means of reducing enzyme loss, and can improve the heat stability of entrapped enzymes. As alginate (particularly their calcium salts) form ionotropic gels spontaneously under low-temperature conditions, it is ideal for entrapment of whole enzymes, which would otherwise be damaged under more stringent conditions (Navratil et al. 2002). However, as alginate or other viscous entrapment media act as a diffusion barrier, they may also reduce enzyme access to the substrates or decrease the rates of release of end products. Therefore, this may results in a reduction in the efficiency of this type of process (Mishra and Kar 2003). This must be taken into account when choosing an immobilization medium for large-scale industrial processes. While enzyme immobilization is suitable for several applications, the use of whole cell immobilization is necessary to carry out multiple steps, enzyme reactions, especially those dependent on the presence of enzyme cofactors. Alginate is the most frequently described immobilization agent in previous literature (Boyaval and Goulet 1988; Brandenberger and Widmer 1998).

Enzymes are macromolecules that act as a catalyst to induce chemical changes in other substances, while their self remaining apparently unchanged by the process. They are three-dimensional biological catalysts, only produced in living organisms. The variety of chemical transformations catalyzed by enzymes has made these molecules a prime target of exploitation by the emerging biotechnology industries such as fine chemistry, food chemistry, therapeutics applications, decontamination processes and protein engineering. The use of enzymes in these applications has been limited by some factors, mainly their stability, high cost of production and availability

in small amounts. It is difficult and expensive to recover them from reactor effluents at the end of the catalytic process because of their solubility. From this point of view, the transformation of chemical processes that from the laboratory to industry catalyzed by enzymes one of the most complex and exciting goals in biotechnology (Bugg 2001). Over last few decades, intense research in the area of enzyme technology has provided many techniques that make easy their practical applications. Some of these techniques are protein engineering of existing enzymes, chemical modification of enzymes, addition of additives and immobilization (Iyer and Ananthanarayan, 2008). Among them, the newer technological development in the field of immobilized biocatalysts can offer the possibility of a wider and more economical exploitation of biocatalysts. Immobilized biocatalysts are not only enzymes, but also cells or organelles (or combinations of these). For many industrial applications, enzymes and cells have to be immobilized, via very simple and cost-effective protocols, in order to be re-used over very long periods of time (Meena and Raja, 2006).

Advantages of Immobilizaed enzymes, in addition to their excellent functional properties (activity, selectivity, specificity, able to catalyze the most complex chemical processes under the most benign experimental and environmental conditions), also have some characteristics that are not very suitable for industrial applications, many industries it used full of alginate directly and apply for each industry. Alginate immobilized cell and enzyme system is famous material in several industrial processes ranging from ethanol production by yeast cells to the production of monoclonal antibodies from hybridoma cells (Crescenzi, 1995), for alginate in the paper industry is in surface sizing (Sutherland and Ellwood, 1979). Alginate added to the normal starch sizing gives a smooth continuous film and a surface with less

fluffing. The quantity of alginate used is usually 5-10 percent of the weight of starch in the size (Steinbuchel et al., 2001). Alginate is a mean material to matrix immobilization in various biotechnological processes. Entrapment of cells within Ca-alginate spheres matrix has become the most widely used technique for living cells (Smidsrod and Skjak-Braek, 1990).

The main components of an immobilized enzyme system are the enzyme, the matrix, and the mode of attachment or entrapment. Ideal matrix properties include physical resistance to compression, hydrophilicity, stability under the conditions to be used, biocompatibility, resistance to microbial attack, and availability at low cost. Supports can be classified as inorganic and organic according to their chemical composition. The organic supports can be subdivided into natural and synthetic polymers.

Organic

Natural polymers

Polysaccharides: Cellulose, agar, agarose, chitin, alginate dextrans.

Proteins: Collagen, albumin.

Other polymers: Polyacrylate polymethacrylates, polyacrylamide, polyamides, vinyl, Polystyrene and all-polymers.

Inorganic

Natural minerals: Bentonite, silica, sand.

Processed materials: Glass (nonporous and controlled pore), metals, controlled pore Metal oxides (e.g. ZrO_2, TiO_2, Al_2O_3) (Guisan 2006).

Chemical and physical qualities of the support material including porosity, particle size and functional group on the surface have to be considered when making a choice for the immobilization technique (Guisan 2006). The selection of the immobilization technique (the mode of attachment or entrapment) is based on the process specifications for the enzyme used because of affects of some kinetic parameters of the immobilized enzyme. The most commonly used immobilization methods are show in Figure 5.

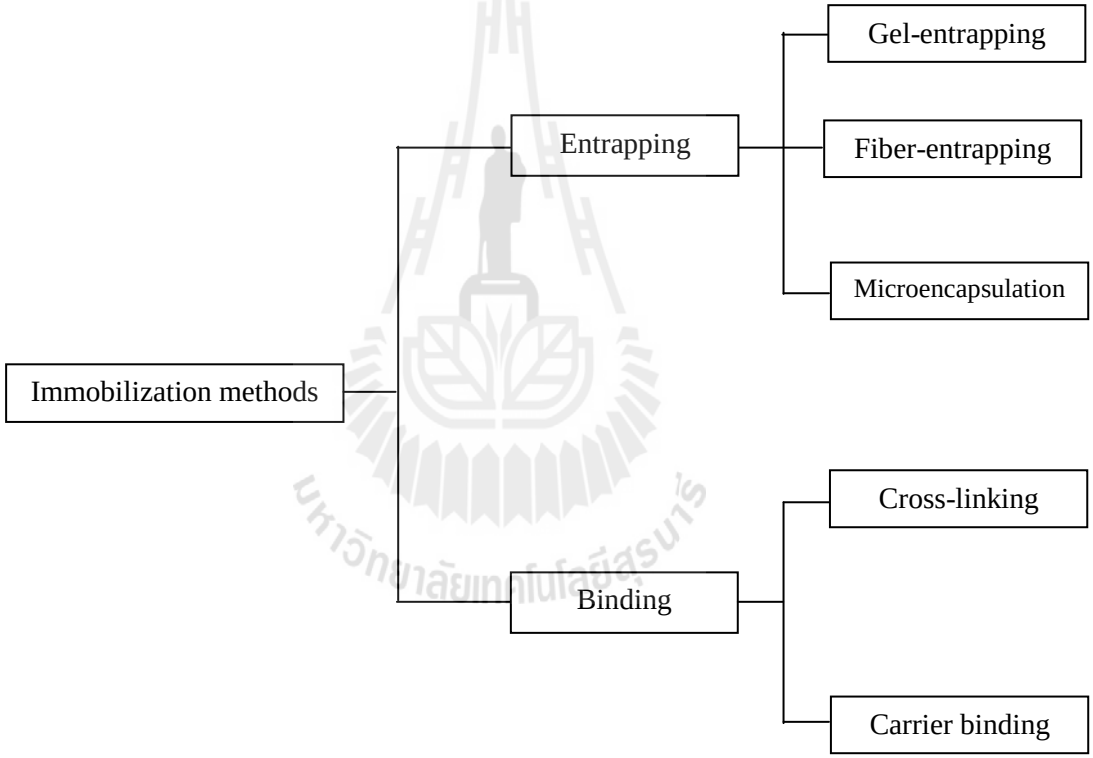


Figure 5. Immobilization methods for insoluble enzymes.

2.2.1 Immobilization Methods

These immobilization techniques are sorted into as chemical and physical methods. Chemical methods involve the formation of covalent bonds between the functional group on the enzyme and functional groups on the support material while

physical methods do not involve covalent bonding with the enzyme. Methods for enzyme immobilization can be classified into two main categories (Guisan 2006):

1. Binding (modified biocatalyst)

2. Entrapment (free biocatalyst)

2.2.1.1 Binding

This method of immobilization involves the formation of a covalent bond between the enzyme and a support material. It method can be sub-classified into cross linking and binding (Woodward, 1985; Porath and Axen, 1976; Cabral and Kennedy, 1991).

2.2.1.2 Cross linking

This type of immobilization is support-free and involves joining the enzymes to each other to form a large, three-dimensional complex structure, and can be achieved by chemical or physical methods (Broun, 1976). Cross-linking is based on the formation of chemical bonds, as in the covalent binding method, but water-insoluble carriers are not used. It can be explained as the intermolecular cross-linking of enzymes by biofunctional or multifunctional reagents. Glutaraldehyde is the most common reagent that used for cross-linking. Crosslink is a simple and rapid procedure to immobilized enzymes. It can be usefully employed in conjunction with other methods, namely to minimize leakage of enzymes already immobilized by adsorption. However the cross linking reaction is not easily controlled and so it is very difficult to obtain large enzyme aggregates with high activity retention. Cross-linking reactions are carried out under relatively severe conditions (Figure 6). These rough conditions can change the conformation of active center of the enzyme; and so may lead to significant loss of activity (Sheldon et al., 2006).

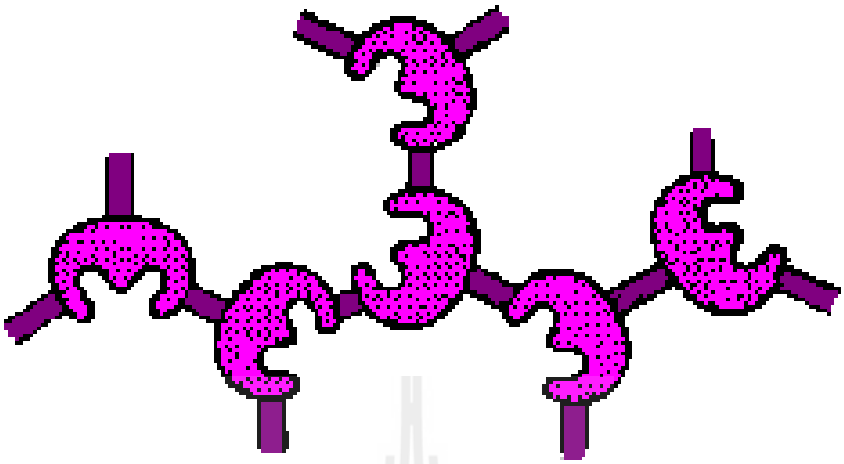


Figure 6. Schematic illustration of cross-linking method. (Source: www.rpi.edu).

2.2.1.3 Carrier Binding

Carrier binding method is described as the binding of enzymes to waterinsoluble carriers. It is the oldest immobilization method for enzymes. In this method shown in Figure 7, the amount of enzyme bound to the carrier and the activity depends on the nature of the carrier. The most commonly used carriers for enzyme immobilization are polysaccharide derivatives such as cellulose, agarose, dextran and polyacrylamide gel. The important factors of choosing carrier are (Cao, 2006).

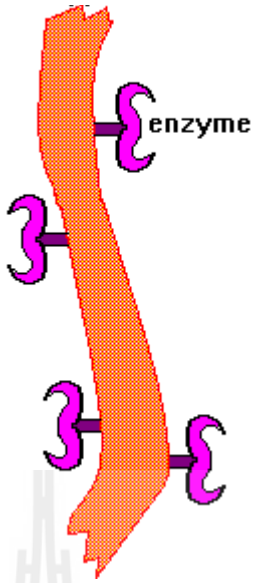


Figure 7. Schematic illustration of carrier binding method. (Source: www.rpi.edu).

2.2.2 Entrapment

2.2.2.1 Entrapment method

The entrapment method is based on the sequestering of an enzyme (or a cell or a multi enzyme) within a polymeric network that allows the substrate and products to pass through but retains the enzyme. This method differs from the binding, in that the enzyme is not bound to the matrix or membrane. Enzyme molecules are free in solution, but entrapped by lattice or membrane. This membrane can be alginate, carrageenan, agarose, polyacrylamide, pectin, gelatin, chitin or chitosan (Wadiack and Carbonell, 1975). There are some advantages of entrapment (Guisan, 2006):

• Enzymes are immobilized without a chemical or structural modification that prevents hindrance of active side of the enzyme so there is no loss of activity because of the intermolecular linking. Denaturation is usually avoided.

• Entrapment is a general technique which has a wide applicability.

• Possibility of simultaneous immobilization of different enzymes in a single step and combination of other methods such as cross linking.

• Large specific area for the effective contact of the substrate and the enzyme.

• Biocompatibility. For example calcium alginate gels are reported to be nontoxic for cells and hence suitable for cell immobilization.

• Easy, simply and low cost.

Despite these advantages, this method also has limitation such as possible leakage of enzyme during repeated use because of semi-permeable surface of matrix. The practical use of entrapment methods is limited by mass transfer limitations through membranes or gels. The diffusion rate is sometimes the limiting parameter according as molecular weight of the substrate and the product. Generally, high substrate concentrations are necessary in order to limit its influence. Entrapped enzymes are better used with small substrates since larger ones may not be able to pass the membrane and reach the active site of the biocatalyst (Won et al., 2005).

2.2.2.2 Entrapped in a matrix

Some synthetic polymers such as polyacrylamide, polyvinyl alcohol and natural polymer have been used to immobilize enzymes using this technique that called lattice-type entrapment. Lattice-Type entrapment involves entrapping enzymes within the interstitial spaces of a cross-linked water-insoluble polymer. This method offers several advantages as resistant to weak acids and alkali,

high ionic strength, some organic solvents. However their use is limited to low-molecular weight substrates due to steric hindrance and inactivation of the enzyme may occur as a result of the use of precipitating agents, polymer solvents and water immiscible liquids (Guisan, 2006).

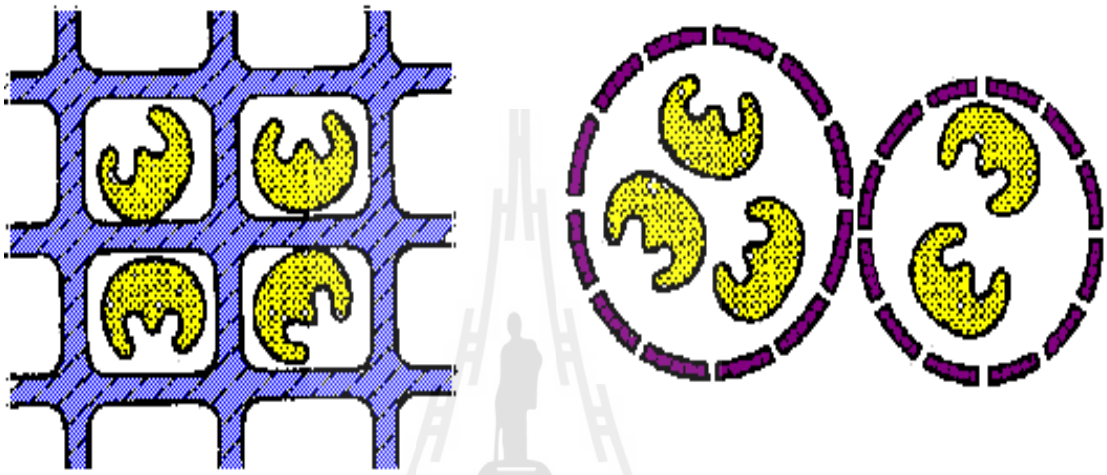


Figure 8. Enzyme entrapped in a matrix (Source: www.rpi.edu).

2.2.2.3 Micro-capsulation

Microcapsule-entrapping involves enclosing the enzymes within semi permeable polymer membranes. The preparation of enzyme micro-capsules requires extremely well-controlled conditions and the procedures for micro-capsulation of enzymes can be classified as:

- Interfacial polymerization method: In this procedure, enzymes are enclosed in semi-permeable membranes of polymers. An aqueous mixture of the enzyme and hydrophilic monomer are emulsified in a water-immiscible organic solvent. Then the same hydrophilic monomer is added to the organic solvent by stirring. Polymerization of the monomers then occurs at the interface between the

aqueous and organic solvent phases in the emulsion. The result is that the enzyme in the aqueous phase is enclosed in a membrane of polymer.

- Liquid drying: In this process, a polymer is dissolved in a water-immiscible organic solvent which has a boiling point lower than that of water. An aqueous solution of enzyme is dispersed in the organic phase to form a first emulsion of water-in-oil type. The first emulsion containing aqueous micro droplets is then dispersed in an aqueous phase containing protective colloidal substances such as gelatin, and surfactants, and a secondary emulsion is prepared. The organic solvent in then removed by warming in vacuum. A polymer membrane is thus produced to give enzyme micro capsules.

The most commonly used microencapsulation coating materials are gums (gum arabic, sodium alginate, and carrageenan), carbohydrates (starch, dextran, and sucrose), Celluloses (carboxymethylcellulose, methycellulose), lipids (bees wax, stearic acid, phospholipids). Selection of the most convenient coating material for core is the primary important factor for application of this method. It is common to define microencapsulation as the embedding of at least one ingredient (active agent) into at least one other (shell material). The active agent is usually not suitable to use it directly due to several reasons: low solubility, reactivity (to low or high), stability. On the other hand, it may be of advantage to optimize the properties of the active agents, e.g. by using controlled release. For all practical aspects a common classification into two basic methods can be made:

- Matrix-encapsulation
- Core-shell-encapsulation

2.2.2.4 Matrix-encapsulation

Matrix-encapsulation is used for distributed homogeneous active agent in the shell material as shown in Figure 9. A microsphere is a spherical particle of a homogeneous mixture. These microspheres are able to release the encapsulated active agents over a defined time. Controlled release microspheres are optimized by a suitable choice of the shell material and the chosen particle size. The choice of shell materials suitable for the application is waxes, hydrocolloids or other organic or inorganic materials. Wax embedded active agents with defined controlled release curves and an enhanced stability of the embedded chemicals are produced for cosmetic or pharmaceutical applications. In other fields of application like food industries the retention time of the agent (e.g. a flavor) needs to be enhanced.

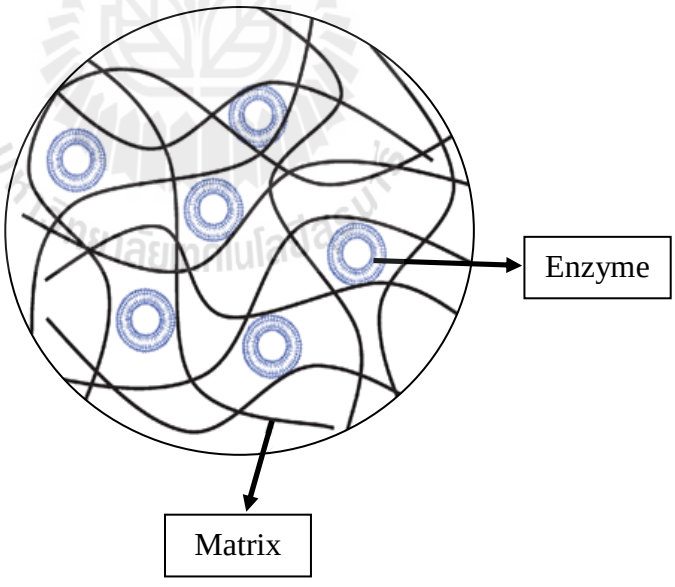


Figure 9. Enzyme Matrix-encapsulation.

2.2.2.5 Core-shell-encapsulation

Spherical particle with a solid shell is commonly denominated as microcapsule (Figure 10). They are used in applications like chewing gum with an extraordinary taste or anti-allergic bedware which keeps its features over years and even after a hot wash. The suitable range of shell materials is very broad, since most gelling systems can be used. In general the most commonly used shell materials are hydrocolloids like alginate, agar-agar, gelatin but also gum arabicum, latices, polymethacrylates, polyethersulfones, wax and many more. The core material needs to be liquid which makes hydrophilic (e.g. aqueous solutions) and also hydrophobic agents (e.g. oils) suitable for the process. For even finer release tuning, the microcapsules can be processed after the production by coating, polymerization or cross-linking to adjust the properties to the desired needs.

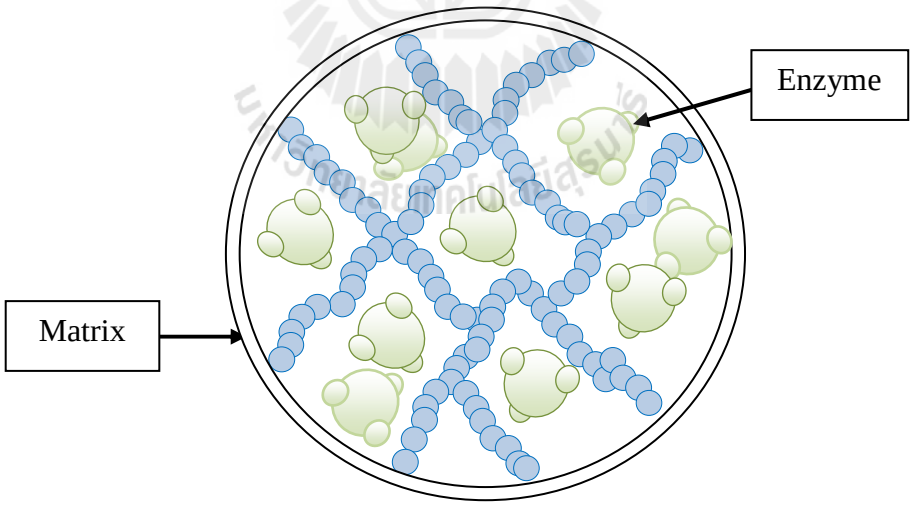


Figure 10. Core-shell-encapsulation (Source: Nazzaro et al., 2012).

The variety of chemical transformations catalyzed by enzymes has made these molecules a prime target of exploitation by the emerging

biotechnology industries such as fine chemistry, food chemistry, therapeutics applications, decontamination processes and protein engineering. The use of enzymes in these applications has been limited by some factors, mainly their stability, high cost of production, availability in small amounts. It is difficult and expensive to recover them from reactor effluents at the end of the catalytic process because of their solubility. From this point of view, the transformation of chemical processes that from the laboratory to industry catalyzed by enzymes one of the most complex and exciting goals in biotechnology (Bugg, 2001).

2.3 Invertase enzyme

Invertase is one type of thermophilic enzymes mainly used in the food industry where fructose is preferred over sucrose because it is sweeter and does not crystallize as easily. Invertase is a yeast-derived enzyme can splits sucrose into glucose and fructose (invert syrup) and can be applied for any inversion of sucrose especially liquefied cherry centers, creams, mints, truffles, marshmallow, invert syrup and other fondants. Invertase is used to improve shelf life of confections. The invertase catalyses the hydrolysis of sucrose, commonly known as table sugar, is a disaccharide composed of an alpha-D-glucose molecule and a beta-D-fructose molecule linked by an alpha-1,4-glycosidic bond (Barlikova et al., 1991). When this bond is cleaved in a hydrolysis reaction, an equimolar mixture of glucose and fructose is generated. This mixture of monosaccharides is called invert sugar, which is derived from the fact that sucrose rotates plane polarized light to the right i.e., dextrorotatory, +66.5º, whereas the hydrolysis products rotates plane polarized light to the left i.e., levorotatory, -20º for the mixture (+52.5º for D(+)-glucose and -92º for D(-)-fructose).

$$\text{Sucrose} + H_2O \longrightarrow \text{glucose} + \text{fructose}$$

The official name of invertase is beta-D-fructofuranosidase (EC3.2.1.26), which widely used in the food and drink industry. Enzymatic hydrolysis of sucrose by invertase results in the cleavage of alpha 1, 4-glycosidic bonds of sucrose (Marquez et al., 2008). Note that sucrose can be hydrolyzed relatively easily; the reaction proceeds in acid hydrolysis of sucrose, is not preferable over invert syrup produced by invertase (Sanchez et al., 2001). Invertase is mainly used in the food industry where fructose is preferred over sucrose because it is sweeter and does not crystallize as easily. A wide range of microorganisms produces invertase and utilizes sucrose as a nutrient. Commercially, invertase is biosynthesized chiefly by yeast strains of *Saccharomyces cerevisiae* (Bokosa et al., 1992) is an important source of enzymes that have found many applications in the food and drink industry (Aleksandra et al., 2005). Even within the same yeast culture, invertase exists in more than one form. For example, the intracellular invertase has a molecular weight of 135,000 Daltons, whereas the extracellular variety has a molecular weight of 270,000 Daltons (Aziz and Senay, 2001). In contrary to most other enzymes, invertase exhibits relatively high activity over a broad range of pH (3.5-5.5), with the optimum pH 4.5 and enzyme activity reaches a maximum at about 55ºC.

The enzymatic sucrose hydrolysis processes utilizes invertase in their free or immobilized forms. There are many benefits in using the immobilized enzyme in comparison with their soluble. The use of free enzyme in industrial processes is restricted due to its high cost, to its recovery difficulty at the end of the process and also its instability. The application of the enzyme in its immobilized form may have some advantages in relation to its free form, such as cost reduction, possibility of

better process control, continuous operation and stability increases (Marquez et al., 2008). In order to examine the suitability of this immobilized invertase enzyme in nano-composite beads. The nano-composite bead was improved structure crosslinker, forming network hydrogel in gel forming state by both HA and LX materials to prolong or control enzyme activity.

2.4 References

Aziz, T., Senay D. (2001). Immobilization of invertase within calcium alginate gel capsules. **Process Biochemistry**. 36: 1081-1083.

Aleksandra Milovanovic, Natasa Bozic, Zoran Vujcic. (2007). Cell wall invertase immobilization within calcium alginate beads. **Food Chemistry.** 104: 81-86.

Aebischer, P, Goddard, M. and Tresco, P.A. (1993). Cell encapsulation for the nervous system, in: Fundamentals of Animal Cell Encapsulation and Immobilization. **Boca Raton, Florida.** 197-224.

Avella, M., Pace, E.D., Immirzi, B. and Impallomeni, G. (2007). Addition of glycerol plasticizer to seaweeds derived alginates: Influence of microstructure on chemical-physical properties. **Carbohydrate Polymer**. 69: 503-511.

Barlikova, A., J. Svorc and S. Miertus. (1991). Invertase for inverted syrup production and sugar determination. **Analytical Chimical Acta**. 247: 83-87.

Broun, G.B. (1976). **Chemically aggregated enzymes,** in Methods in Enzymology, Vol. XLIV (Mosbach, K., ed.), Academic, New York, p.263-280.

Bokosa, I., A. Krastanov and Z.I. Roshkova. (1992) Invertase biosynthesis by *Saccharomyces cerevisiae***. Nauchni Trudove Na Institut Journal**. 39: 269-279.

Boyaval P, Goulet J. (1988). Optimal conditions for production of lactic acid from cheese whey permeate by Ca-alginate-entrapped *Lactobacillus helveticas*. **Enzyme Microbial Technology.** 10: 725-728.

Brandenberger H, Widmer F. (1998). A new multinozzle encapsulation/immobilisation system to produce uniform beads of alginate . **Journal of Biotechnol**. 63: 73-80.

Bugg, D. H. T. 2001. The development of mechanistic enzymology in the 20th century. **Natural Product Report**. 18: 465-493.

Crescenzi, V. (1995). Microbial polysaccharide of applied interest. Ongoing research activities in Europe. **Biotechnology and Program.** 11: 251-259.

Clementi, F., Fantozzi, P., Mancini, F. and Moresi, M. (1995). Optimum conditions for alginate production by *Azotobacter vinelandii*. **Enzyme and Microbial Technology.** 17: 983-988.

Cottrell, I.W. and Kovacs, P. (1980). Handbook of Water-Soluble Gums and Resins**,** **Auckland**, New Zealand: McGraw-Hill, 21-43.

Change, T.M.S. (2003). Artificial cells for cell and organ replacements. **Artificial Organisum**. 28: 265-270.

Change, T.M.S. (2003). Artificial cells for cell and organ replacements. **Artificial**.

Cabral, J.M.S. and Kennedy, J. F. (1991). Covalent and coordination immobilization of proteins, in Protein Immobilization. **Marcel Dekker**, New york, p.73-138.

Cao, L. 2006. Carrier-bound Immobilized Enzymes. **Wiley-vch.** p.125-209.

Crowther BG, Lewis PM, Metherell C. Compounding In: Roberts AD. (1988). Natural rubber science and technology. **Oxford University**. New York p. 177.

Draget, K.I., Smidsrod, O. and Ostgaard, K. (1991). Homogeneous alginate gels; a technical approach. **Carbohydrate and Polymer**. 14: 159-178.

Funduenanu, G., Nastruzzi, C., Carpov, A., Desbrieres, J. and Rinaudo, M. (1999) Physico-chemical characterization of Ca-algiante microparticles produced by different methods. **Biomaterials.** 20: 1427-1435.

Fabbri, M., Celotti, G. C. and Ravaglioli, A., (1994). Granulated based on calcium phosphate with controlled morphology and porosity for medical applications: Physico-chemical parameters and production technique. **Biomaterials**. 15: 474-477.

Guisan, M. J. 2006. Immobilization of Enzymes and Cells Second Edition. **Institute of Catalysis**, *CSIC*.

Grant, G.T., Morris, E.R., Rees, D.A., Smith, P.J.C. and Thom, D. (1973). Biological interactions between polysaccharide and divalent cations: The egg-box model. **Federation of European Biochemical Societies Letter.** 32: 195-198.

Haug, A. (1964). **Composition and properties of alginates,** Thesis, Norwegian Institute of Technology, Trondheim.

Iyer, P. V. and Ananthanarayan, L. 2008. Enzyme stability and stabilization-aqueous and non-aqueous environment. **Process Biochemistry**. 10: 1019-1032.

Ikoma, T., Yoshioka, T., Nakamura, S., Hanagata, N., Abe, T., Sakane, M., Ochiai, N. and Tanaka, J. (2008). Rigid hydroxyapatite-alginate beads for sustained release of paclitaxel. **Engineering Materials**. 361-363, 535-538.

Kourkoutas, Y., Douma, M., Koutinas, A. A., Kanellaki, M., Banat, I. M. and Marchant, R. (2002). Continuous winemaking fermentation using quince-immobilized yeast at room and low temperatures. **Process Biochemistry** 39: 143-148.

Littlecott, G.W. (1982). Food gels-the role of alginates. **Journal of Food Technology of Australian.** 34: 412-418.

Liu, D. M., (1996). Fabrication and characterization of porous hydroxyapatite granules. **Biomaterials.** 17: 1955-1957.

Mishra P., Kar R. (2003). Treatment of grapefruit juice for bitterness removal by amberlite IR 120 and amberlite IR 400 and alginate entrapped naringinase enzyme. **Journal of Food Science and technology.** 68: 1229-1233.

Meena, K. and Raja, T. K. 2006. Immobilization of *Saccharomyces cerevisiae* cells by gel entrapment using various metal alginates. **World Journal of Microbiology and Biotechnology**. 22: 651-652.

Monmaturapoj N. (2008). Nano-size Hydroxyapatite powders preparation by wet-chemical precipitation route**. Journal of Materials and Minerals**. 18: 15-20.

Mizushima Y., Ikoma T., Tanaka J., Hoshi K., Ishihara T., Ogawa Y., et al. (2006). Injectable porous hydroxyapatite microparticles as a new carrier for protein and lipophilic drugs. **Journal of Controlled Release.** 110: 260-503.

Marquez, L.D.S., B.V. Cabral, F.F. Freitas, V.L. Cardoso, E.J. Ribeiro. (2008). Optimization of invertase immobilization by adsorption in ionic exchange resin for sucrose hydrolysis. **Journal of Molecular Catalysis Enzymatic.** 51: 86-92.

Navratil M., Gemeiner P., Klein J., Sturdik E., Malovikova A., Nahalka J., Vikartovska A., Domeny Z., Smogrovicova D. (2002). Properties of hydrogel materials used for entrapment of microbial cells in production of fermented beverages. **Cells Blood Substitute Immobilize Biotechnology**. 30: 199-218.

Nazzaro, F., Orlando, P., Fratianni, F., Coppola, R. (2012) Microencapsulation in food science and biotechnology. **Current Opinion in Biotechnology**. 23: 182-186.

Onsoien, E. (1996) **Carbohydrate Research**. 14: 26.

Otterlei, M., Ostgaard, K., Skjak-Braek, G., Smidsrod, O., Soon-Shiong, P. and Espevik, T. (1991). Induction of cytokine production from human monocytes stimulated with alginate. **Journal of Immunotherapy**. 10: 286-291.

Porath, J. and Axen, R. (1976). **Immobilization of enzymes to agar, agarose and Sephadex supports**, in *Methods* in *Enzymology*, vol. XLIV (Mosbach, K., ed.), Academic, New york, p.19-45.

Steinbuchel, A., Vandamme, E.J. and De Baets, S. (2001). **Biopolymer**. Wiley-vch verlag GmbH, Weinheim, Germany, pp.179-180.

Smidsrid, O. (1973). **Some physical properties of alginates in solution and in the gel state**, Thesis, Norwegian Institute of Technology, Trondheim.

Skjak-Braek, G., Grasdalem, H. and Larsen, L. (1986). Monomer sequence and acetylation pattern in some bacterial alginates. **Carbohydrate Research**. 154: 239-250.

Steinbuchel, A., Vandamme, E.J. and De Baets, S. (2001). **Biopolymer**. Wiley-vch verlag GmbH, Weinheim, Germany, pp.179-180.

Smidsrid, O. and Draget, K.I. (1996). Chemistry and physical properties of alginates. **Europe Journal of Carbohydrate.** 14: 6-13.

Skjak-Braek, G., and Moe, S.T. (1992). **Alginate gels**. US Patent 5,144,016.

Sanchez, M.P., J.F. Huidobro, I. Mato, S. Munigategui and M.T. Sancho. (2001). Evolution of invertase activity in honey over two years. **Journal of Agricultural and Food Chemistry.** 49: 416-422.

Sutherland, I.W. and Ellwood, D.C. (1979) **Microbial exopolysaccharide industrial polymers of current and future potential.** London, England: Cambridge University Press Ltd.

Smidsrid, O. and Skjak-Braek, G. (1990). Alginate as immobilization matrix for cells. **Trends of Bioteechnology**. 8: 71-78.

Soon-Shiong, P., Feldman, E., Nelson, R., Heints, R., Yao, Q., Yao, T., Zheng, N., Merideth, G., Skjak-Braek, G., Espevik, T., Smidsrod, O. and Sandford, P. (1993) Long-term reversal of diabetes by the infection of immunoprotected islets. **Proceedings of the National Academy of Sciences**. 90: 5843-5847.

Sime, W. (1990). **Alginates, in: Food Gels** (Harris, P., Ed.), London: Elsevier, 53-78.

Sheldon, R. A., Schoevaart, R., Langen, L. M. V. 2006. Cross-Linked Enzyme Aggregates. **Methods in Biotechnology**. 22: 31-45.

Sanchez, M.P., J.F. Huidobro, I. Mato, S. Munigategui and M.T. Sancho. (2001). Evolution of invertase activity in honey over two years. **Journal of Agricultural and Food Chemistry.** 49: 416-422.

Smidsrid, O. (1973). **Some physical properties of alginates in solution and in the gel state**, Thesis, Norwegian Institute of Technology, Trondheim. P: 35-83.

Takahashai, T., Kamitakahara, M., Kawachi, G. and Loku, K. (2008). Preparation of spherical porous granules composed of rod-shaped hydroxyapatite and evaluation of their protein adsorption properties. **Key Engineering Materials**. 361-363, 83-86.

Woodward, J. (1985). **Immobilized enzymes: adsorption and covalent coupling,** in *Immobilized Cells and Enzymes*: A Practical Approach (Woodward, J., ed.), IRL, Oxford, 3-17.

Vieille, C. and Zeikus, G. J. 2001. Hyperthermophilic enzymes: sources, uses, and molecular mechanisms for thermostability. **Microbiology and Molecular Biology Reviews.** 65: 1-43.

Velten, F., Laue, C. and Schrezenmeir, J. (1999). The effect of alginate and hyaluroante on the viability and function of immunoisolated neonatal rat islets. **Biomaterials**. 20: 2161-2167.

Wadiack ,D. T. and Carbonell, R. G. 1975. Kinetic behavior of microencapsulated galactosidase. **Biotechnology Engineering**. 17: 1157-1181.

Won, K., Kim, S., Kim, K., Park, H. W., Moon, S. J. 2005. Optimization of lipase entrapment in Ca-alginate gel beads. **Process Biochemistry**. 40:2149–2154.

Zhang, X., Chen, J. and Zhou, (1989). Porous hydroxyapatite granules: their synthesis, applications and characterization. **Clinical Materials**. 4: 319-327.

CHAPTER III

STABILITY IMPROVEMENT OF ALGINATE BEADS USING NATURAL RUBBER/HYDROXYAPATITE NANOCOMPOSITE FOR ENZYME IMMOBILIZATION

3.1 Abstract

In nanocomposite beads CA/LX and CA/HA/LX, preparation the suitable concentrations of LX adding were found at 2.5%, 5% and 10%. It was demonstrated that at the beads of LX concentration of 15% and 20% were not used because they floated. The viscosity was related with temperature, when the temperature increased, the viscosity decreased. However, the viscosities of 10% LX slightly increased as the temperature increased due to LX heat-sensitization. LX concentration at 10% showed the highest strength. Furthermore, the HA material improved strength of the bead and provided the crack character. A single crack was in CA/LX and a double crack was found in CA/HA/LX. Bead swelling was decreased at 10% LX because the LX's hydrophobic character obstructed the swelling of defective CA/LX and CA/HA/LX beads. The morphological and molecular structure analysis of CA/LX and CA/HA/LX were depicted by SEM micrography. Outstanding shape of CA/LX was flat and irregular; the morphology of CA/HA/LX showed highly gaps, porous structure and flocculated shape. TEM technique was utilized to observed in-situ HA crystal inside

beads' structure. HA was the main material to induce flocculate shape in CA/HA/LX bead. FT-IR technique was utilized to observe the LX concentration increased from 2.5% to 10%, the wave number at 1325–1380 cm^{-1} shifted. This was because of the CH_2 character band in LX.

From the result of immobilized invertase enzyme activity, when various temperatures of gel forming state the LX clogging effect was found at 40°C and 50°C. In comparison between CA/LX and CA/HA/LX, LX was clogged the bead' pore structure on high temperature and provide non enzyme activity but had no effect on CA/HA/LX because HA in CA/HA/LX expanded pores inside the beads. Thus LX was determined the rate of relative enzyme activity for CA/LX and CA/HA/LX. The highest enzyme activity was found in CA/HA/LX. Due to the fact that HA constructed its structure and expanded pores inside a bead. The result of reused immobilized invertase enzyme in CA/HA and CA/HA/LX decreased by 40-50 % after the 8^{th} cycle. Whereas CA/LX with 10% LX, the enzyme activity decreased more than 30% from the first to the second test and decreased to completely lost activity by the 5^{th} test.

Keyword: nanocomposite beads; Alginate; Hydroxyapatite; Latex; immobilization

3.2 Introduction

Alginate is a family of unbranched binary copolymer comprising a backbone of (1-4)-linked β-D-mannuronic acid (M) and C-5 epimer, α-L-guluronic acid (G) residues (Draget et al., 2006). Alginate has been used for a variety of industrial purposes, such as stabilizers, thickeners and gelling agents in food production,

bioreactor and pharmaceutical applications. Furthermore, it has been applied in immobilize of enzyme into the food products (Kraekoopt et al., 2006), drug delivery (Hari et al., 1996), and immobilize the fertilizer in plant production (Pan et al., 2008). However, the most attractive application of alginate is the calcium-induced gela tion resulting from specific and strong interactions between calcium ions and guluronate residues in alginate (Grant et al., 1973). But the original alginate gel beads are unstable, soft and cannot control porous inside the beads. Due to objective were increase stability and development of pore size in the beads.

This research focused on the development of alginate bead structure by using hydroxyapatite (HA). Because HA is often combined with biocompatible polymers to remodel the natural structure of bead and to immobilize controlled drug or enzyme releasing (Paital Dahotre, 2009). There are several studies regarding the preparation of polymer (sodium alginate) composite scaffolds using HA of variable crystallinity and amount to increase porous (Rajkumar and Meenakshisundaram, 2011). And one important material to improve strength and stability is natural rubber or latex (LX). From special property such as high strength, temperature resistant and highly elastic character of LX are suitable for use to improve the stability of alginate gel beads.

The goals of the research were to control the bead structure for using in enzyme immobilization. In the steps of gel solution preparation, pH, temperature and LX concentrations were varied to develop the beads structure and enzyme immobilization.

3.3 Materials and Methods

3.3.1 Materials

3.3.1.1 Sodium alginate (SA): SA was obtained from the brown algae (Fluka). Calcium alginate (CA) is a water-insoluble, gelatinous substance that can be created by adding aqueous SA into aqueous calcium chloride.

3.3.1.2 Hydroxyapatite (HA): By adding diammonium phosphate ($(NH4)_2HPO_4$), (Univar) to calcium nitrate ($Ca(NO_3)_2.4H_2O$) (Carlo Erba), than HA can be achieved. Both diammonium phosphate and calcium nitrate are of AR grade.

3.3.1.3 Latex (LX): Natural LX solution was obtained from Suranaree University of Technology Farm.

3.3.1.4 Invertase: Enzyme invertase was β-D-fructofuranoside fructohydrolase, EC 3.2.1.26 produced from baker's yeast, *Saccharomces cerevisiae*, (Fluka).

3.3.2 Methods

3.3.2.1 Preparation of the Alginate/Hydroxyapatite and various concentration additions of latex nanocomposite beads

The nanocomposite beads were prepared by mixing with diammonium phosphate and calcium nitrate which contained SA and LX by the following the procedure below. Five grams of SA was dissolved in 36 mL distilled water and stirred at 1000 rpm, 40°C. After SA solution was completely mixed with 0%, 2.5%, 5%, 10%, 15% and 20% of LX. The diammonium phosphate solution (0.6 g of diammonium phosphate with 12.5 mL water) was added into SA and LX solution. The mixture was further stirred for 1 h to ensure the homogeneity of the solution. Subsequently, the CA bead were formed by dropping aqueous solution of

SA and diammonium phosphate using syringe pump and drop in to 500 ml of 6% calcium nitrate aqueous solution at 30˚C and stirred at 50 rpm with a 1 mm inner diameter needle in hypodermic syringe. The pH of the system was maintained by using 28% ammonium hydroxide and 2 M sulfuric acid. The milky white nanocomposite bead formed immediately and was left for 16 h to further crosslinking and facilitating in the growth of HA nano-particles in the beads. After that, the nanocomposite beads were filtered with a 100 mesh screen and rinsed three times with distilled water to removed the excess Ca^{2+} as well as other impurities on the surface of the beads (Zhang et al., 2010). The condition of CA/LX and CA/HA/LX nanocomposite beads was shown in Table1. The first group consisted of CA and LX in 2.5%, 5%, 10%, 15% and 20% concentration using pure CA or 0% LX as the control condition. The second group consisted of CA/HA and LX in 2.5%, 5%, 10%, 15% and 20% concentration using CA/HA or CA/HA/LX 0% as the condition of control.

Table 1. Preparation conditions of CA/LX and CA/HA/LX

Latex (LX) concentration	**Type of bead gel**	
	CA	**CA/HA 5%**
0%	CA/LX 0%	CA/HA/LX 0%
2.5%	CA/LX 2.5%	CA/HA/LX 2.5%
5%	CA/LX 5%	CA/HA/LX 5%
10%	CA/LX 10%	CA/HA/LX 10%
15%	CA/LX 15%	CA/HA/LX 15%
20%	CA/LX 20%	CA/HA/LX 20%

3.3.2.2 Study optimum pH and temperature on nanocomposite beads

The nanocomposite beads were prepared according to 3.3.2.1 in beads forming step. Calcium nitrate solution was adjusted to the temperature at 4˚C, 30˚C and 50˚C and adjusted to the pH at 4, 7 and 10 using 28% ammonium hydroxide and 2 M of sulfuric acid. The CA, LX and HA formed into nano-composite bead structure completely in 16 h. After that, the nanocomposite beads were filtered with a 100 mesh screen, rinsed three times with distilled water to remove the excess Ca^{2+} and other impurities on the surface of the beads. After that the adjusted pH and temperature nanocomposite beads were analyzed in the next experimented (Zhang et al., 2009).

3.3.2.3 Viscosity

The apparent viscosities of alginate solution in various LX concentrations were analyzed with viscometer (Well-Brookfield LVT, series 82198) and rheometer (TA instrument series AR-G2, USA). Sample was prepared at room temperature (25˚C). Operating condition was prepared according to Rehm and Valla (1997).

3.3.2.4 Texture profile analysis

CA, CA/HA and CA/HA/LX beads were analyzed using texture analyzer (TA-XT_2 Stable Micro System, UK) by placing all samples in a single layer and compressed them using a 2-inch diameter stainless steel cylinder. The pre-test speed is 2.0 mm/sec. The test and the post-test speeds were 1.0 mm/sec. Samples were 95% compressed, held for 1 second, released and compressed again to complete the two-cycle compression test (Bourne, 1982). Samples were analyzed for their hardness, adhesiveness and cohesiveness.

3.3.2.5 Stability of beads structure in controlled condition fermenter tank

The percentage loss of beads type CA/LX and CA/HA/LX in various LX concentrations was studied using 2 L of distilled water working volume in 5 L-fermenter tank. Then agitating the solution at 1,000 rpm and time holding of 4 h at 25 °C. A hundred beads were used in each batch. After finishing filtering with a 100 mesh screen, specific fully beads were counted and converted to percent of beads remaining.

3.3.2.6 Measurement of beads' swelling

The 2.5 g beads type CA/LX and CA/HA/LX in various LX concentrations were dried at 40˚C for 24 h. After that, the sample was soaked in 400 mL of distilled water at room temperature for 16 h. The swollen beads were separated from the unabsorbed fluids by filtering through 100-mesh screen. The beads were blotted to remove excess fluid and weighed immediately. The equilibrium water uptake or swelling (EWU) was calculated using the following equation.

$$\% \text{ EWU} = [(W_s - W_d)/W_s] \times 100$$

Where, W_s is the weight of the swollen beads and W_d is the weight of the dry beads. EWU is calculated as grams of water per gram of sample (Zhang et al., 2009).

3.3.2.7 Morphology structure study

Morphological investigations of CA beads, CA/LX and CA/HA/LX in various LX concentrations were observed by using scanning electron microscope (SEM). The samples were dried properly and coated with gold before observing under

SEM. The SEM pictures have been taken at different magnifications by mounting on metal stubs were observed by transmission electron microscope (TEM). The materials were diluted in water solution and spreaded on a support grid coated with plastic. The specimen appeared as a picture when viewing it in the TEM (Tu, 2005).

3.3.2.8 FT-IR Analysis

The samples were manually grounded in an agate mortar for approximately 2 micron for infrared analysis. FT-IR spectroscopy attenuated total reflectance (ATR). The total reflection was performed using a Tensor 27 (Bruker) infrared spectroscopy which was equipped with a globar source. The spectrum was obtained in the range of 4,000-800 cm^{-1} range. The spectrum was recorded with a spectral resolution of 1 cm^{-1} and 128 scans. Data processing was performed by OPUS version 6.5 (Bruker Optic GmbH, Germany).

3.3.2.9 Enzyme immobilization

Entrapment of the enzyme in nanocomposite beads was carried out by extruding through a pasteur pipette (1mm diameter). Invertase enzyme (1.57 unit/mg) was dissatisfied in 1 ml DI water (invertase produced from baker's yeast, *S. cerevisiae,* Fluka). Then the invertase solution was added in SA/diammonium phosphate solution state before forming state of CA/LX and CA/HA/LX in various LX concentrations according to 3.3.2.1.

The nanocomposite beads were filtered with 100 mesh screen and rinsed three times with 0.5 M of acetate buffer to remove the enzyme on the surface. The nanocomposite beads were kept in 0.5 M of acetate buffer at 4˚C before using in the next experiment (Konsoula et al., 2006; Chang and Juang, 2005).

3.3.2.10 Determination of enzyme activity

The invertase activity was analyzed by measuring the reducing sugar in sucrose solution. One mL of immobilized invertase beads or 1 mL free invertase solution (1.57 unit/mg) was added to 100 mL of sucrose solution (100 g/L in acetate buffer, pH 4.5) and then incubated at 50˚C. The reducing sugar concentration was measured according to DNS (3,5-dinitrosalicylic acid) colorimetric method. The samples were boiled for 10 min to develop the red-brown color characteristic. Density of sample was determined at 540 nm after cooling (Miller, 1959).

3.3.2.11 Reusability of immobilized enzyme

The immobilized invertase enzyme activity was analyzed by measuring the reducing sugar in sucrose solution. One mL of immobilized invertase beads or 1 mL free invertase solution (1.57 unit/mg) was added to 100 mL of sucrose solution (100 g/L in acetate buffer, pH 4.5) and then incubated at 50˚C. After that, the beads were stored in 0.5 M acetate buffer (pH 4.5) at 4˚C. The immobilized beads from each preparation were taken in duplicate at the gap of 2 days for 8 times and were then analyzed for the remaining enzyme activity.

3.4 Results and Discussion

3.4.1 Preparation of the alginate/hydroxyapatite and latex nanocomposite beads

The beads were formed by dropping aqueous solution of SA and diammonium phosphate using hypodermic syringe and dropped into 500 ml of 6% calcium nitrate aqueous solution with a 1 mm inner diameter needle in hypodermic syringe (Figure 11). All beads were separated into two groups, CA/LX and CA/HA/LX. The CA/LX group in various concentrations of LX showed that the

pure CA beads were more transparent than the ones added with LX (Figure 12 a1). The bead with 2.5% LX (Figure 12 a2), began opaque and the beads' color changed to translucent and more translucent at the higher LX concentration (Figure 12 a2-a6). At 15% and 20% LX concentration, the CA beads could not entirely capture the LX (Figure 12 a5 and a6). Some part of the LX solution would leak out of the beads into calcium nitrate aqueous solution at the bottom (the milky white color could be observed in solution). Moreover, the specific gravity effect of the LX caused the beads float on water. Therefore, at 15% and 20% LX were not used in the next experiment. In the second group, CA/HA/LX and the same various concentrations at of LX were found that opaque of beads presented in CA/HA (Figure 12 b1). Beads were more opaque when compared with the pure CA. The off-white CA/HA occurred due to HA. However, the color of CA was not the same of CA/HA. At 2.5% LX, CA/HA/LX beads turned opaque. The color of the beads changed to milky white (Figure 12 b2). The phenomenon 15% and 20% LX at CA beads were shown again (Figure 12 b5 and b6). Therefore, at 15% and 20% LX concentration would be used.

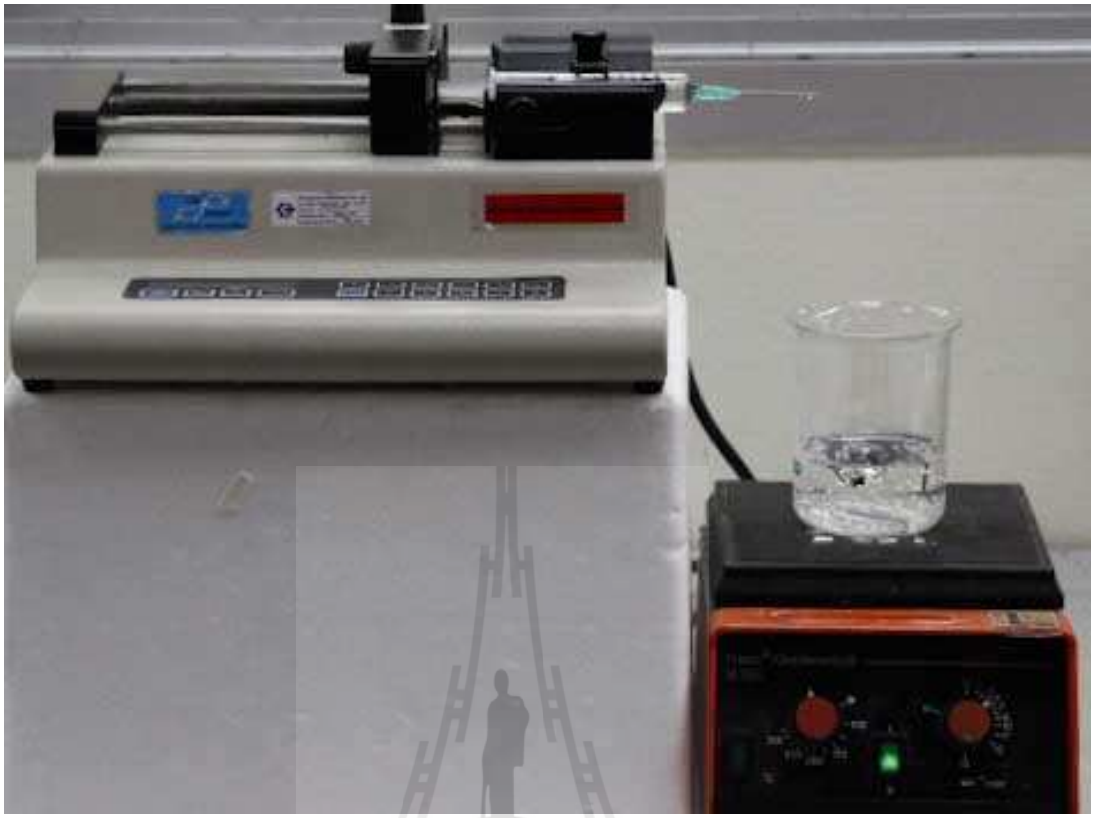


Figure 11. Equipment for gel forming using hypodermic syringe.

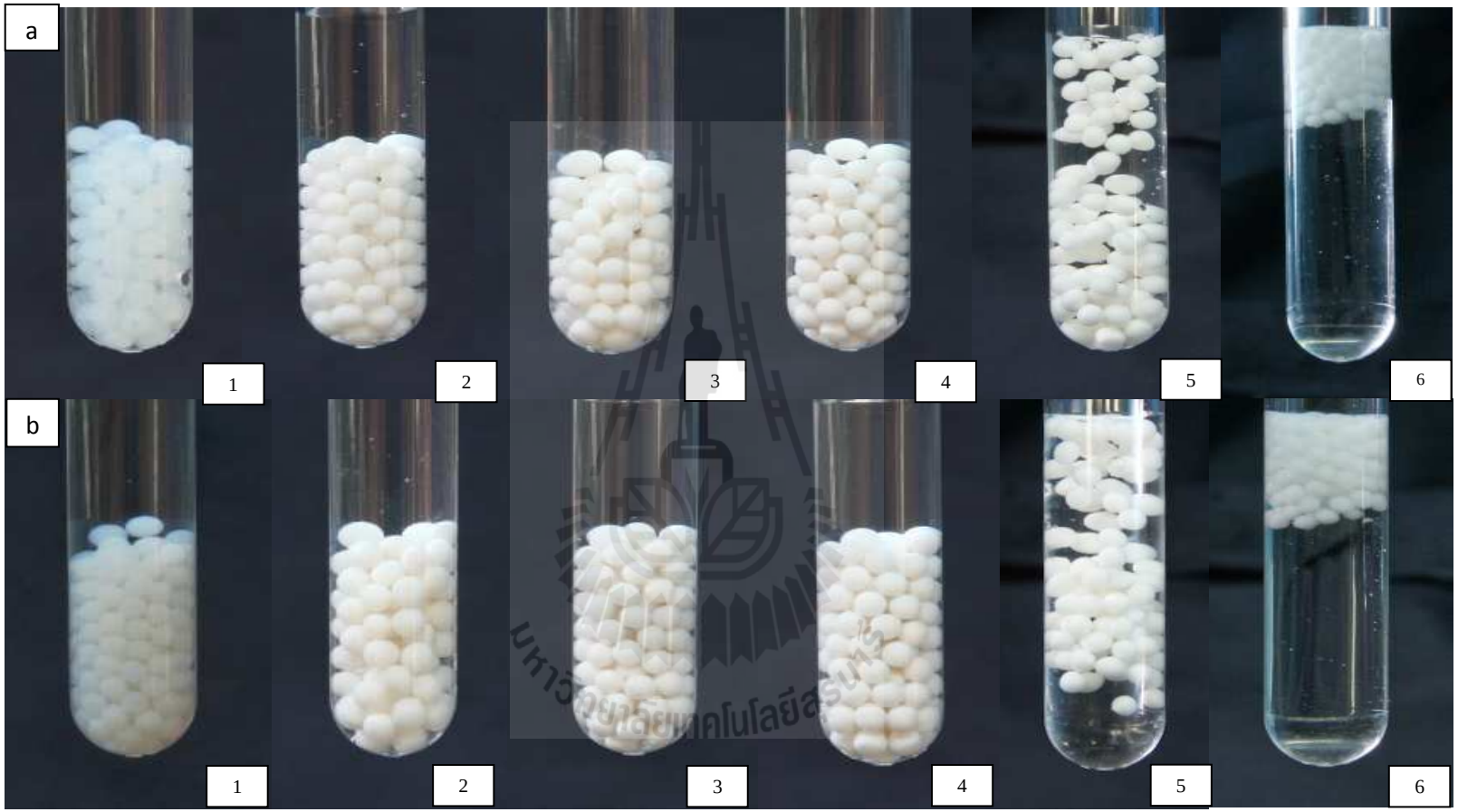


Figure 12. Structure and physical characteristic of beads various LX concentrations. (a) CA/LX at 0, 2.5, 5, 10, 15 and 20 % LX concentrations respectively. (b) CA/HA/LX LX at 0, 2.5, 5, 10, 15 and 20 % concentrations at respectively.

3.4.2 Viscosity

SA and SA/HA samples were employed in order to investigate the temperature influence on viscosities of the sample. The viscosity at 4°C, 25°C and 50°C were shown in Figure 13. After mixing 2.5 % of SA in distilled water, the molecules of SA were not completely flexible. Rotation around the glycosidic linkages in the G-block regions was somewhat hindered and resulted in a stiffening of the chain. Solutions of stiff macromolecules were highly viscous. When the temperature of all samples was increased, the liquid phase molecules moved around freely, but were weak and transiently bounded to their nearest adjacent molecules (Parente et al., 1998). As the temperature increased, there was more energy available to disrupt the intermolecular forces. The average speed of the molecules in liquid increased, but the viscosity decreased. This result did not show behavior of latex heat-sensitization (Livonniere, 1993). The LX heat-sensitization behavior decreased in solutions viscosity as temperature increased. Lower viscosity of SA-HA compared to SA solution was shown in Figure 13b. It was because HA crystal particle in SA-HA solution increased viscosity. However, the viscosity was slightly increased at 10% LX addition because of the LX's elastic character.

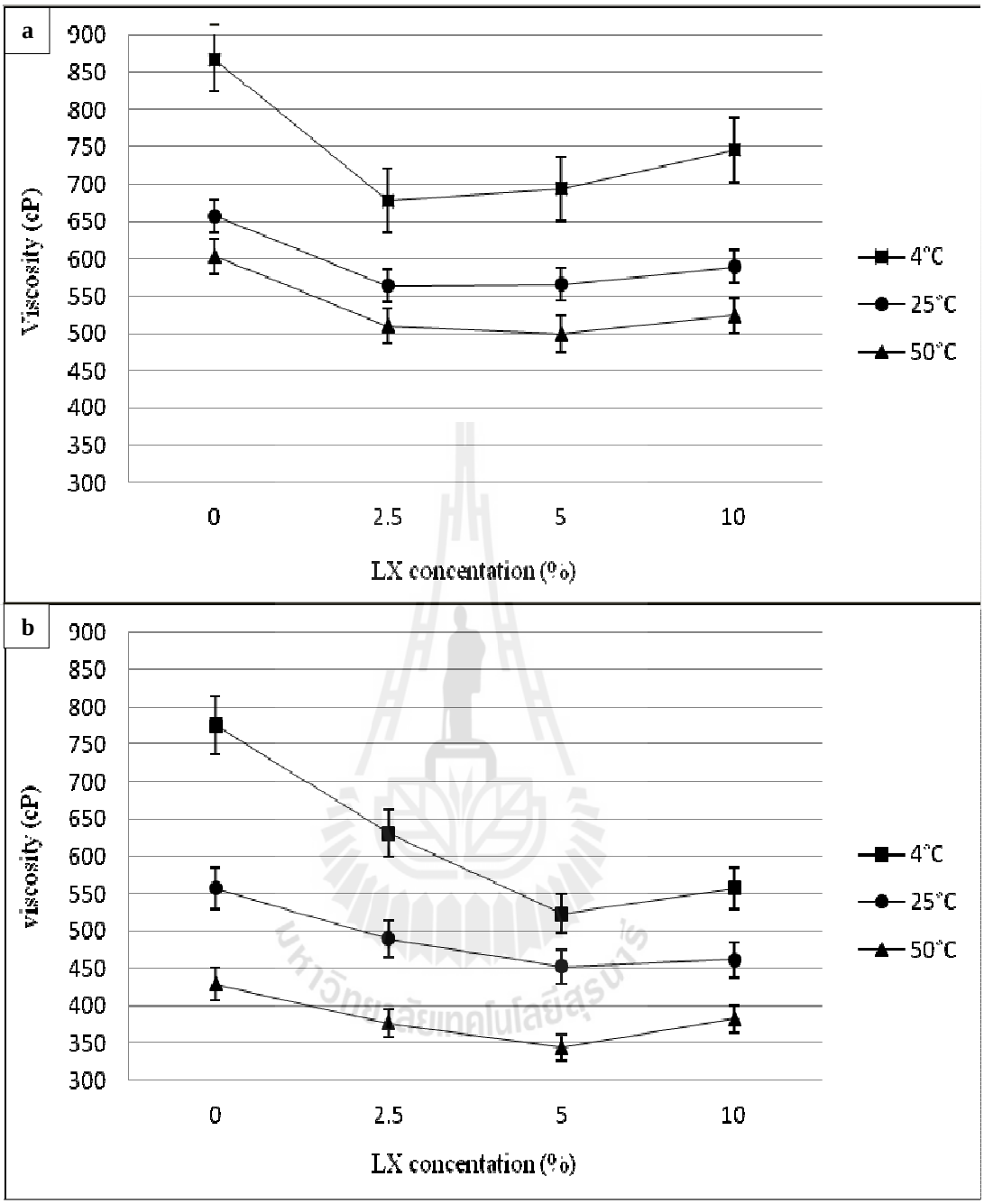


Figure 13. Viscosity of polymer solutions at various LX concentrations at different gel forming as temperature increased. (a) SA solution with LX, (b) SA/HA solution with LX.

3.4.3 Texture profile analysis

CA, CA/HA and CA/HA/LX beads were analyzed for their hardness by using texture analyzer. The resistance and durability of the beads were also studied by clacking points. Figure 14 showed the evolution of maximal mechanical force required to compress the beads. This test of the compressed resistance was chose to evaluate the CA and CA/HA in various LX concentrations. In Figure 14a, the CA beads limpness was shown in sudden peak drop after beads cracked. In addition, the CA bead did not have any resistance after being cracked. The resistance and durability were improved after adding LX at 0%, 2.5%, 5% and 10% to CA/LX, the strength of the beads was 600, 685, 720 and 970 N. It could be explained by the higher network density and elastic behavior in the beads induced by LX. Furthermore, the stronger beads shown in LX addition more than pure CA beads (Orive et al., 2003). Also the previous result, all the CA/LX prepared under various LX additions had good mechanical resistance after beads formation (Figure 14b). The resistance and durability were presented at 806, 834, 897 and 997 N due to addition of HA into CA/HA/LX. Double crack characters were demonstrated in CA/HA/LX. The first crack was relatively induced by the CA/HA complex at outer skin-membrane. The second crack, which most likely reflected the strength and elastic behavior of inner CA/HA/LX, consisted of many small peaks that indicated the series of micro-cracks due to the rigid and brittle character of the beads' structure (Avella et al., 2007).

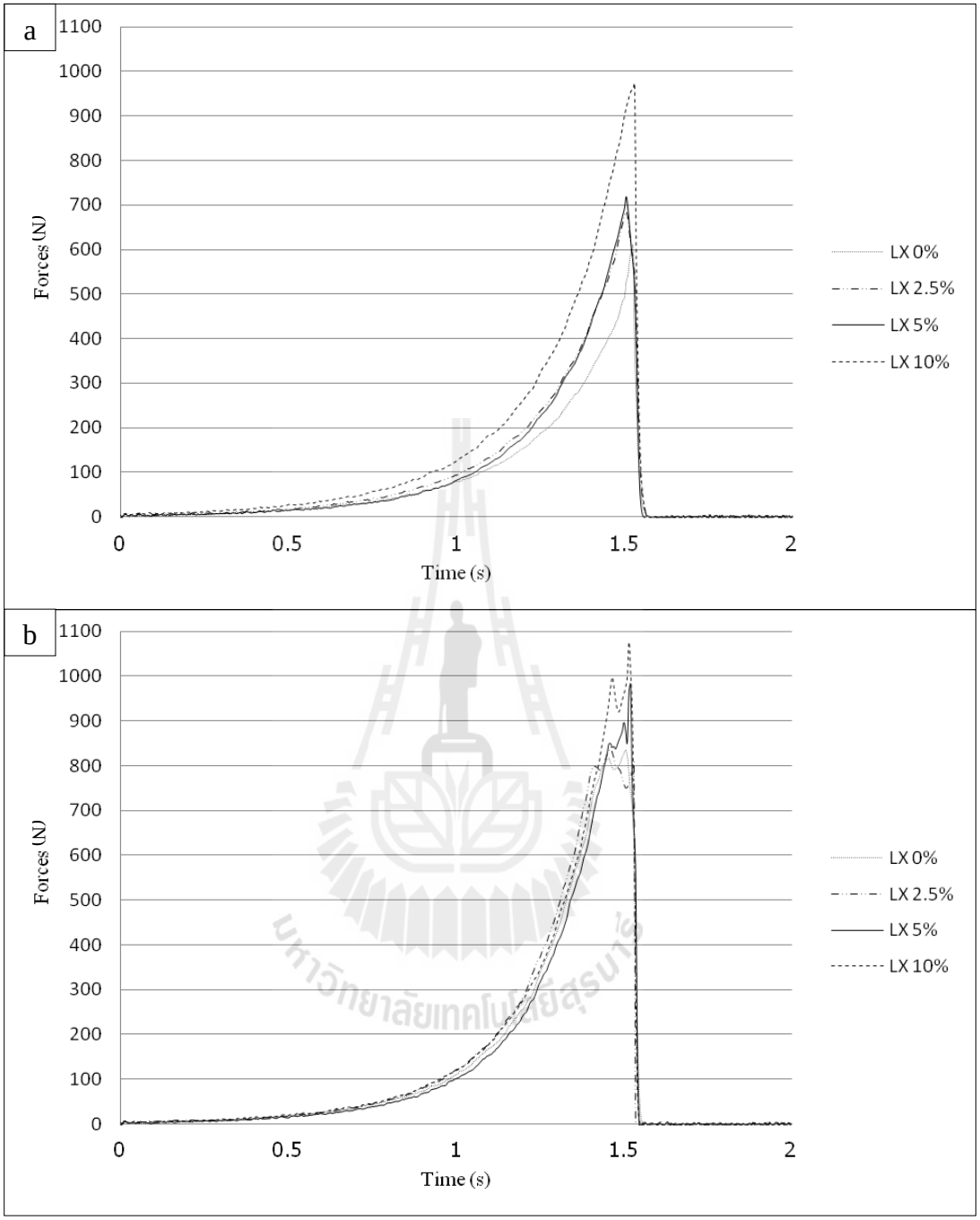


Figure 14. Mechanical resistance of CA and CA/HA in various LX concentrations (a) CA/LX and (b) CA/HA/LX.

3.4.4 Stability of beads' structure in controlled conditions fermenter

The beads remain were studied in 5L fermenter tank (Figure 15). The beads comparisons of various conditions were demonstrated in Figure 16. Beads remained approximately 5-10% in pure CA and CA/HA. However, the effect of HA structure inside the beads was shown in percentage CA/HA of the remained beads more than the pure CA condition. Strength of CA/LX and CA/HA/LX beads increased after adding 2.5% LX, the remaining beads increased from pure CA by 15-25% in the same condition. The reason of bead' strength increasing was from LX concentration. At LX 5%, the strength of CA/LX and CA/HA/LX beads was greater than at 2.5% LX concentration. For the 10% LX in of CA/LX and CA/HA/LX, the remaining of beads showed the highest bead' strength which was approximately 85-90% remained beads. From the previous result, the CA/LX beads had greater strength than CA/HA/LX beads. The highest percentage of beads remained were presented in comparison between CA/HA/LX and CA/LX in various concentrations (Figure16).

Figure 15. Stability of beads structure testing in fermenter condition.

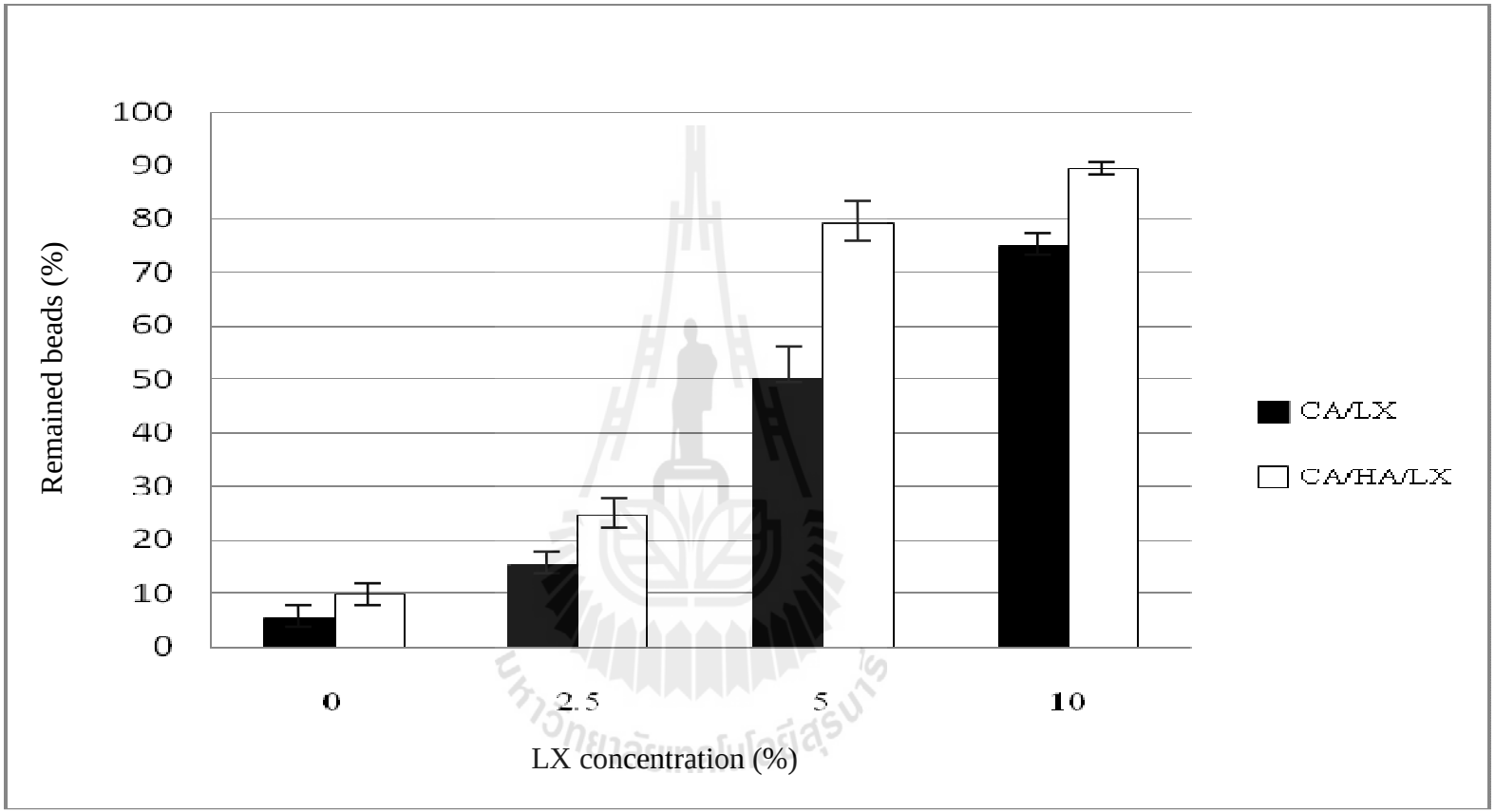


Figure 16. Remaining of beads type CA/LX and CA/HA/LX in various LX concentrations at 1,000 rpm agitate and holding time 4 hour at 25 °C.

3.4.5 Measurement of beads swelling

Variation of swelling of the CA and CA/HA beads with various LX concentrations showed in Figure 17. The sample beads were submerged in distilled water at 25°C the beads swelling. Then, the beads' weight before and after swelling was investigated. Overall samples showed the decreasing in the beads swelling when LX was increased.

As the highest 91.5% percent swelling was found in the pure CA bead. After adding HA and LX, the inner pores of the beads were clogged. As the result, the LX's hydrophobic property was the main cause of beads decreasing swelling. Generally, LX was hydrophobic because they were polystyrene-based. Therefore, the use of high LX concentration was contrary to the water absorption of the beads (Warren et al., 2005).

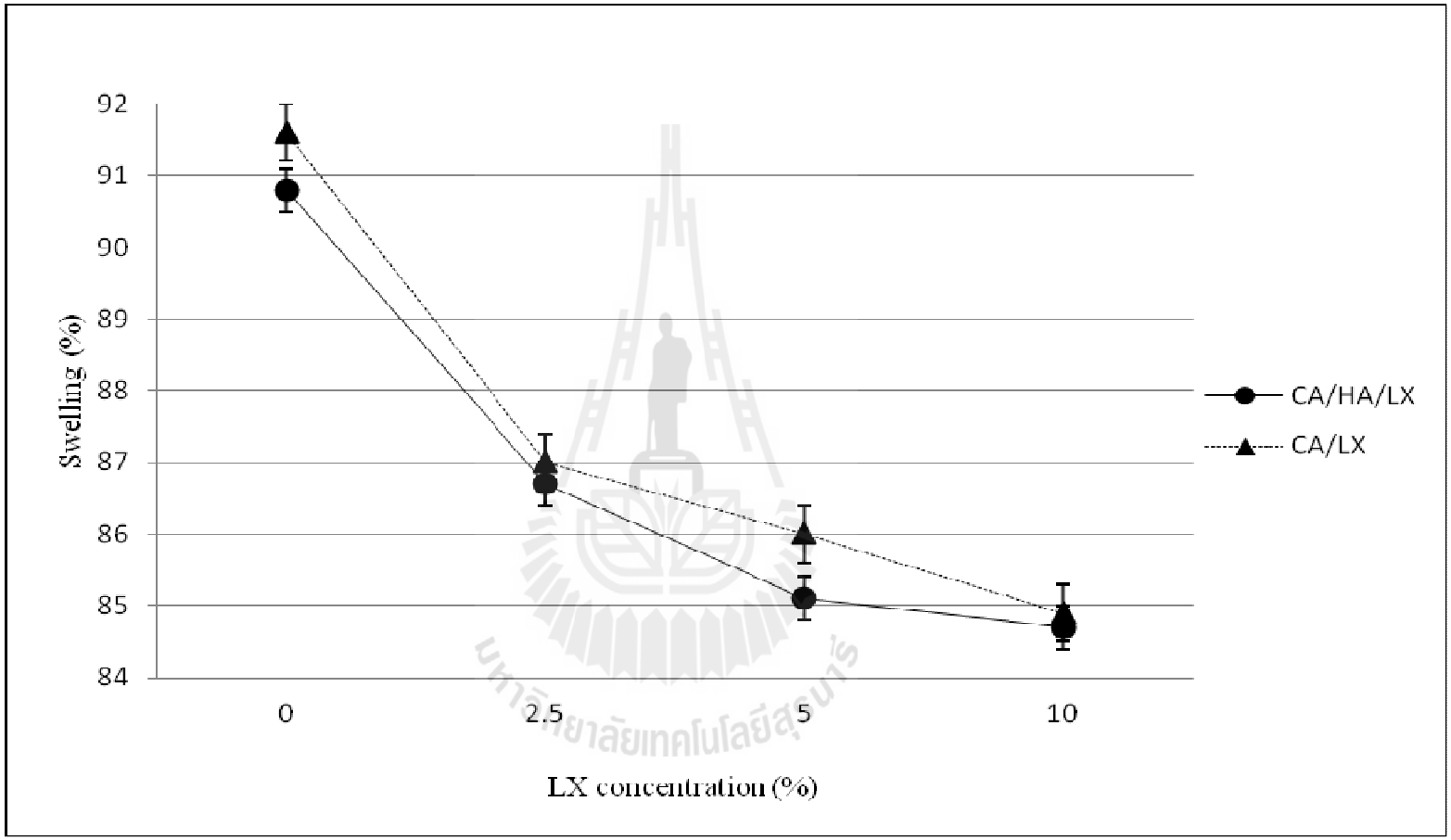


Figure 17. Swelling [%] of CA and CA/HA beads in various LX concentrations.

The swelling of CA/LX and CA/HA/LX were depended on the LX concentrations. As LX increased, the beads swelling decreased. The main cause of the beads swelling was due to CA. The CA occurred due to proton-catalyzed hydrolysis. Moreover, the proton-catalyzed hydrolysis depended on pH and temperature. Figure 18a displayed several of pH investigated at pH 4, 7 and 10. The most swelling beads were found at pH 4 and the least swelling at pH 10. Such result involved the introduced hydrogen ions (Gorka et al., 2009).

Due to the fact that CA was a hydrophilic and water soluble anionic polysaccharides, gelation took place when divalent cations (usually Ca^{2+}) interacted ionically with blocks of guluronic acid residues resulting in three-dimensional network formation which was described by egg-box model in aqueous media. The ion-exchange (mainly Ca^{2+}) bounded with COO^- groups mainly in the polymannuronate sequences and enhanced the swelling gel (Gheorghe et al., 1999). Therefore, the hydrogen ions mainly supported the swelling of the beads. For this reason, the swelling decreased as LX concentration increased. Because LX was hydrophobic, and beads were formed by ionic bonds between COO^- in alginate and Ca^{2+} (Zhang et al., 2009). Due to the LX that contained no carboxyl group, the cross linking density of beads decreased. The water absorption ability of the beads decreased. The bead swelling behavior was investigated at temperature of 4°C, 25°C and 50°C. As shown in Figure 18b, the maximum swelling was found at 25°C.

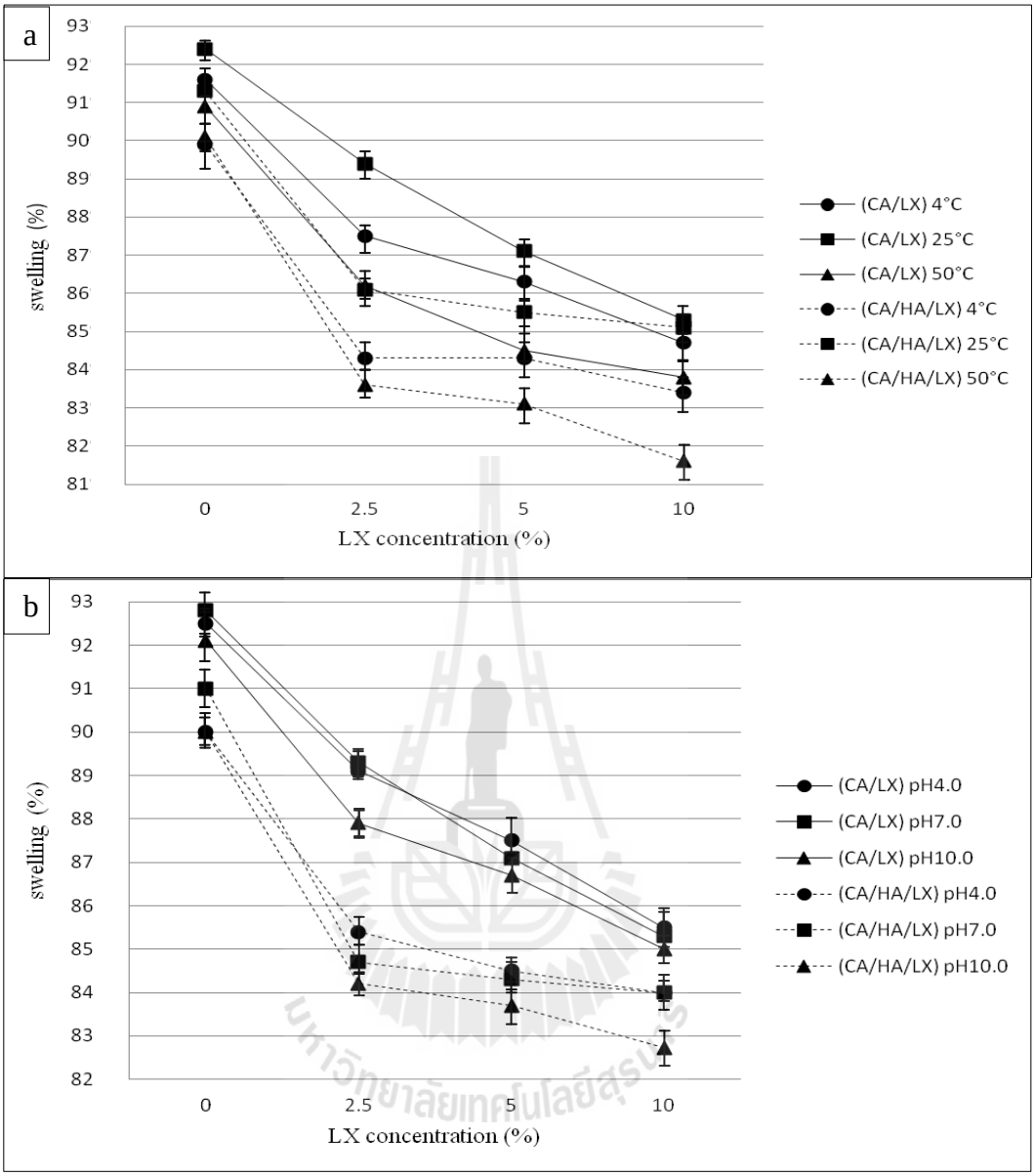


Figure 18. Swelling of CA and CA/HA beads in various LX concentrations. (a) CA and CA/HA beads in various LX concentrations and pH conditions. (b) CA and CA/HA beads in various LX concentrations and various temperatures in gel forming condition.

The swelling of CA/LX and CA/HA/LX were depended on temperature and pH. The temperature in gel forming stage at 4°C, 25°C and 50°C showed in Figure 19a. The prominent swelling due to temperature effect was presented at 25°C for both CA/LX and CA/HA/LX at 88.5% and 87% swelling. The optimum pH was shown in pH 4.0 and pH 7.0 at 90% and 87% swelling in both CA/LX and CA/HA/LX (Figure 19b). Therefore, temperature had greater influence than pH for beads' swelling.

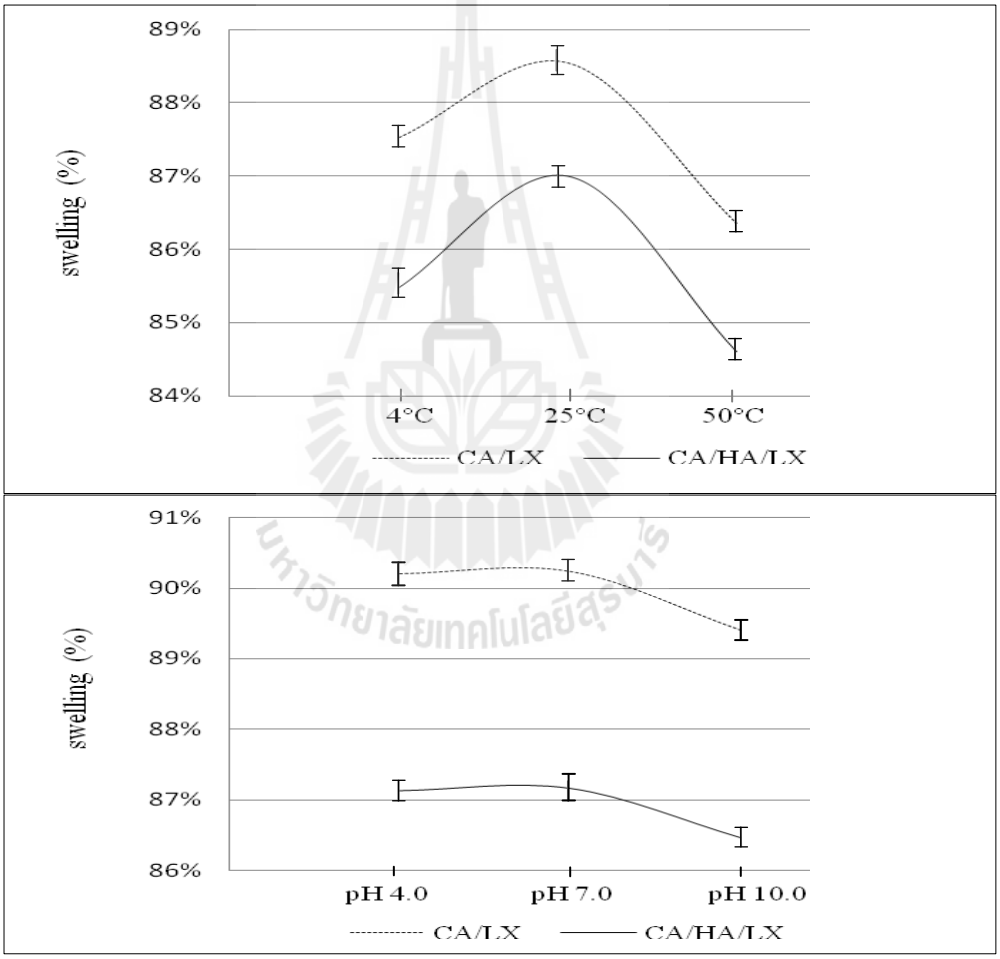


Figure 19. Optimum swelling of CA/LX and CA/HA/LX beads in various LX concentrations depending on pH and temperature at gel forming stage.

3.4.6 Scanning Electron Micrographs (SEM)

Morphological investigation of pure CA, CA/LX and CA/HA/LX beads in various LX concentrations was observed by using scanning electron microscope (SEM). Scanning electron micrographs of dry pure calcium–alginate beads were demonstrated in Figure 20. In morphological comparison, the size of the wet beads was approximately 2 mm. The dried beads were shrunk at a half of their diameter. In the case of observing the surface and internal morphologies, pure calcium alginate beads were freeze-dried and then subjected to SEM (Figure 20a and 20b). Normally, calcium cross-linked alginate had hydrogel characteristic where the alginate monomers were 3-dimensionally networked by ionic bonds with calcium ions containing a large amount of water. After freeze-drying, the inner structure of the highly porous CA illustrated the hydrogel characteristic of the CA bead. However, the surface was highly densed, which was attributed to the cross-linking within Ca ions.

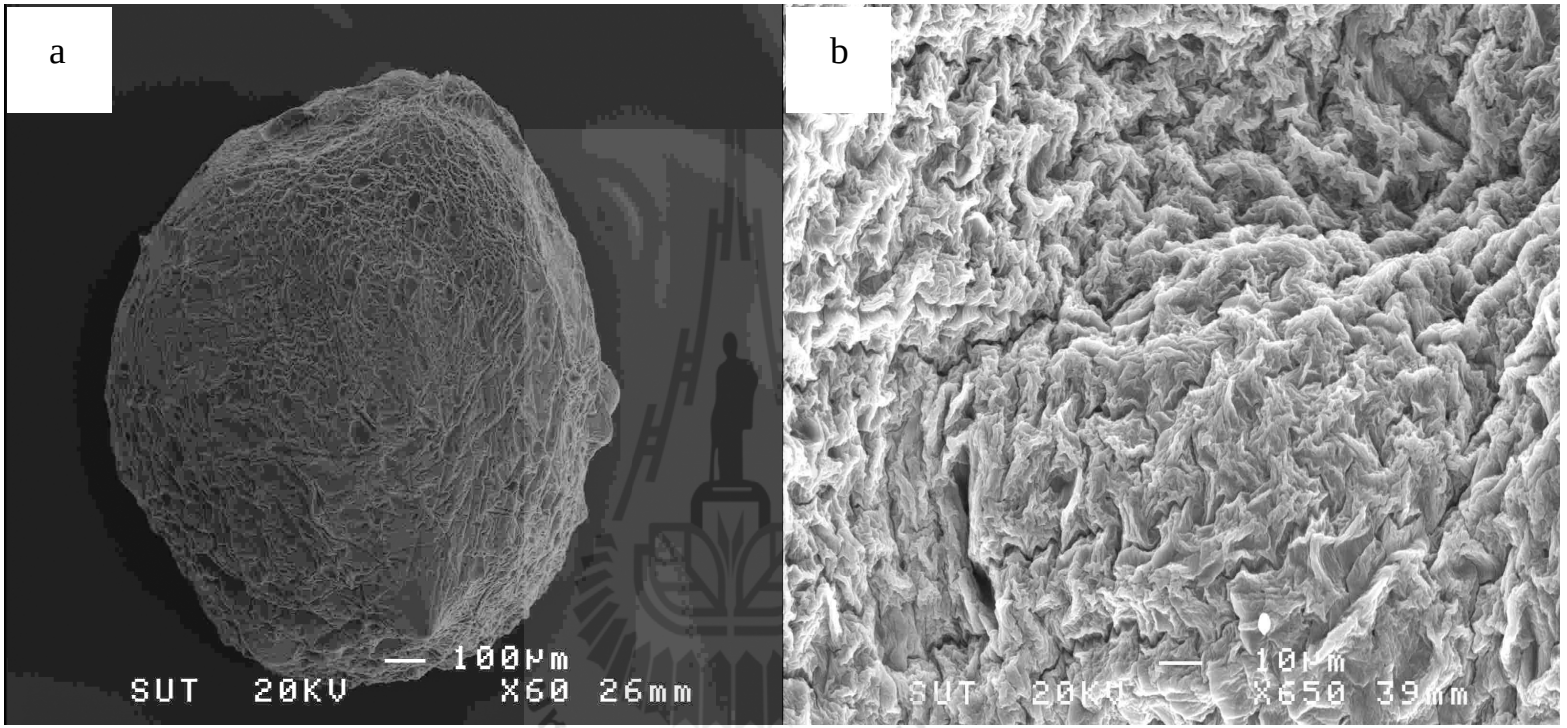


Figure 20. SEM micrographs of pure calcium–alginate beads with freeze dried method (a) dehydration of the bead's surface, (b) surface shrunk at a half of alginate beads.

CA/LX composite scaffolds with different LX concentrations were shown in Figure 21. The pure calcium–alginate structure was highly porous as shown in Figure 21a. The composite exhibited a looser and coarser framework influencing the cross linking agent on the porosity. (Copolymer complex with alginate and latex structure can be seen in Figure 21b, 21c and 21d by adding LX at 2.5%, 5% and 10%, respectively). The morphology and arrangement of the uniform microstructure was unaffected by the presence of LX. Figure 21b-21d presented the flat and irregular shape of particles between 0.5 µm and 5µm in diameter. However, the particles layer increased as the LX content increased up to 10%. Eventually, the pore's structure was clogged.

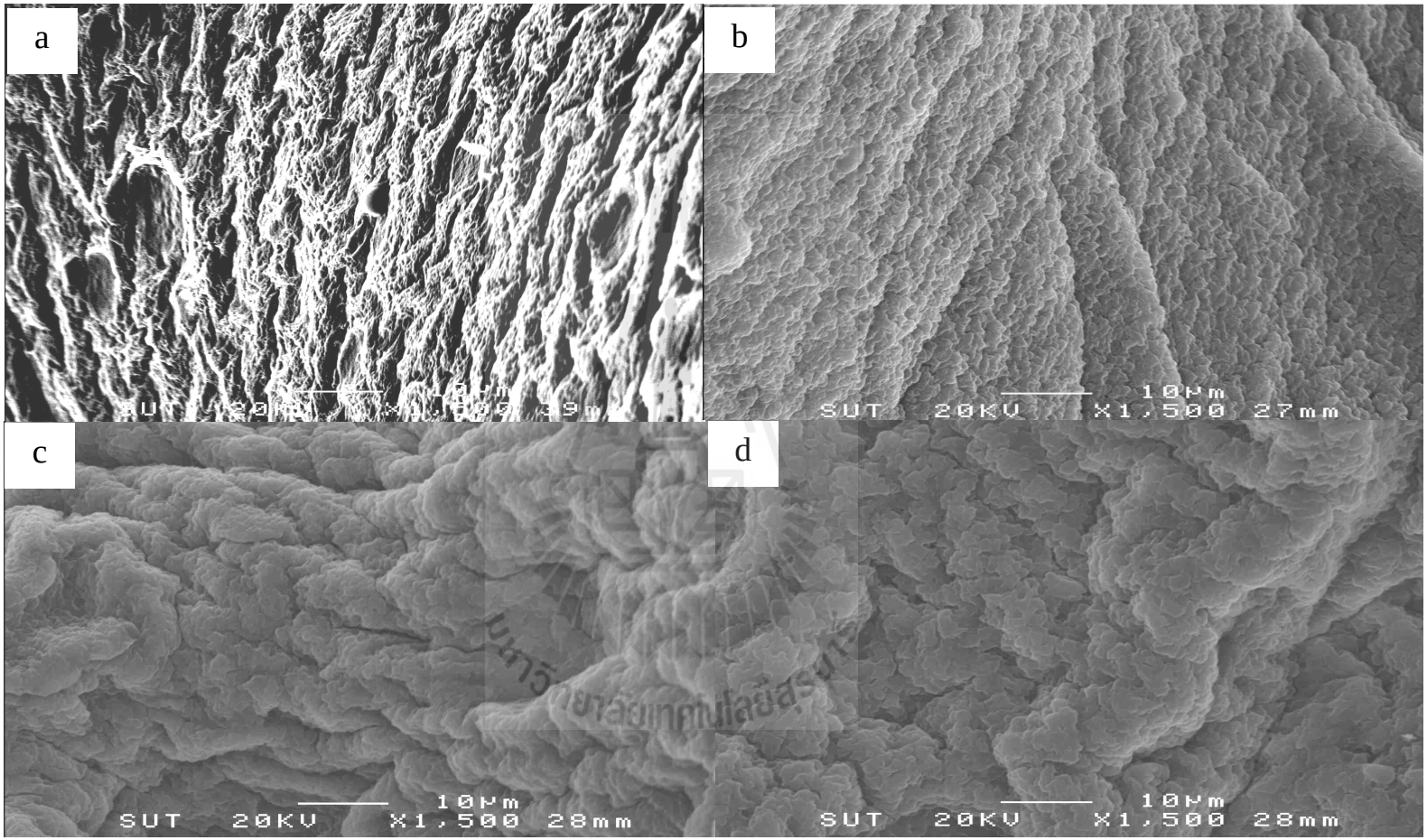


Figure 21. Morphology of surface beads CA/LX beads surface in different LX concentration (a) CA/LX 0%,(b) CA/LX 2.5%, (c) CA/LX 5% and (d) CA/LX 10%.

SEM micrograph of the CA/HA/LX composite showed widely gaps and porous structure. Figure 22 depicts SEM microscopy samples that were compared in various LX concentrations. Figure 22(a) depicts widely gaps and irregular arrangement flat particles' size from 1-10 μm in CA/HA composite. The arrangement and shapes were changed on the composite structure in the CA/HA and various LX concentrations. Figure 22(b), the particle shape of CA/HA/LX in 2.5% LX was changed, notably traditional particles shapes were flat. In CA/HA/LX composite, particles' shapes were agglomerate and changed to flocculate shape (Descamps, et al., 2009). By increasing LX concentration up to 10%, the flocculate particles became more densed (Figure 22d).

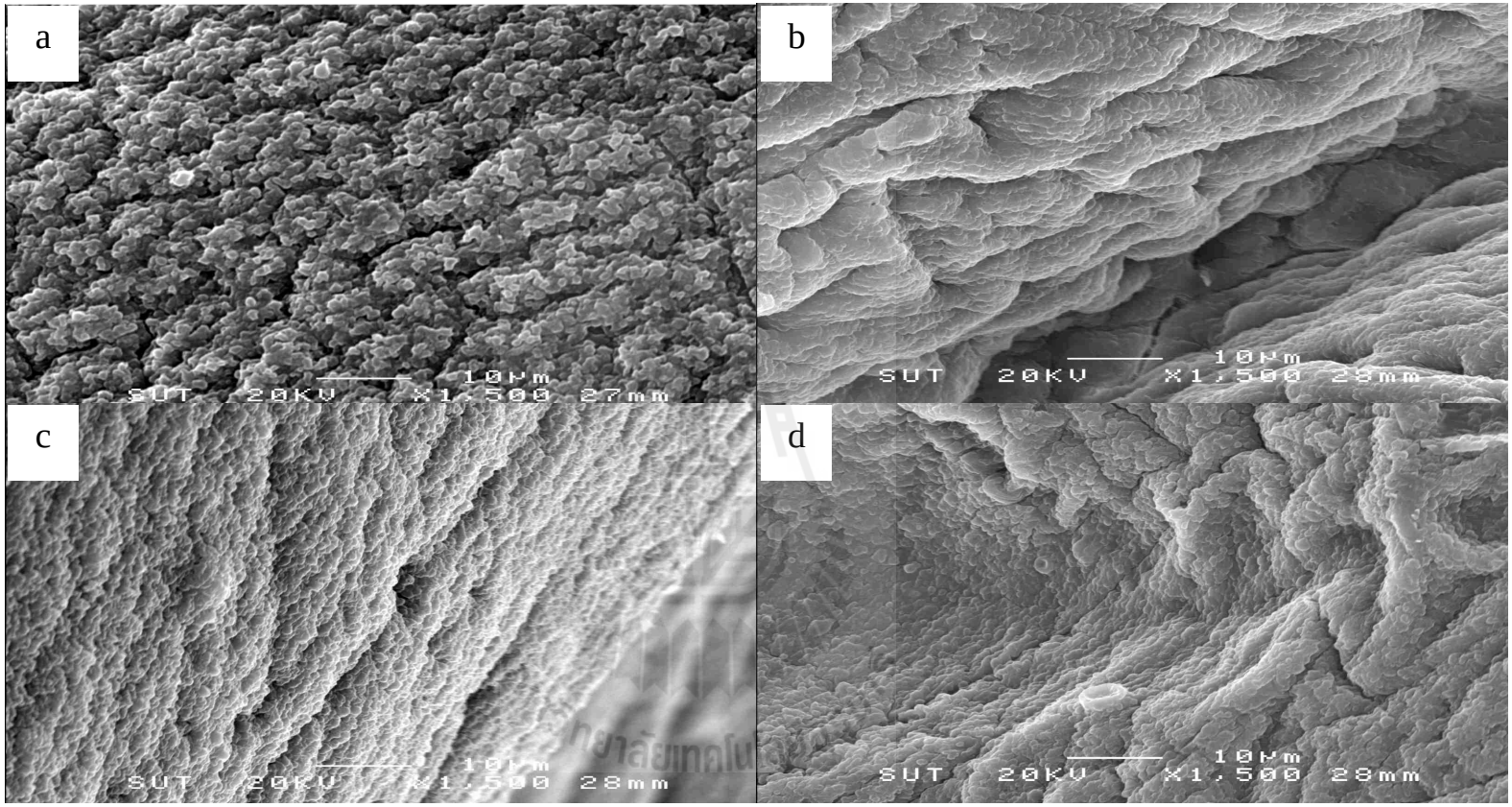


Figure 22. Morphology of surface beads CA/HA/LX in different LX concentrations (a) CA/HA/LX 0%, (b) CA/HA/LX 2.5%,(c) CA/HA/LX 5% and (d) CA/HA/LX 10%.

3.4.7 Transmission electron microscope (TEM)

The morphology of HA crystals was displayed in Figure 23a. Pure HA aqueous solution showed a needle-like structure with irregular sizes from 10–60 nm in length and approximately 5 nm in width. The pure sodium–alginate structure was demonstrated in Figure 23b. Noticeable of the HA crystal was spread around in the CA/HA (Figure 23c). The result of CA/HA structure was formed the strong ionic interactions between alginate and Ca^{2+} ions forming specific stereo-chemical arrangement and charge distribution of reactive groups in alginate-Ca complexes. Ca^{2+} ions enhanced the HA crystal by the high spatial charge density and possibility of hydrogen-bond to the surface of phosphate groups (Descamps et al., 2009).

Alginate was a kind of natural polysaccharide and was composed of β-D-manunronic and α-L-guluronic acids joined by 1-4 linkages and a strong thermostable gel. The calcium alginate was formed in the presence of calcium ion (Steeland and Walker 1991). The "egg-box model", in which each calcium ion was located into electronegative cavities formed by four guluronate residues. Two from each chain were used to explain the interactions of Ca^{2+} with alginate (Dorozhkin and Matthias 2002). Meanwhile, phosphate from HA was a tetrahedral structure. It was believed that the peculiar morphology, size and crystallographic orientation of biominerals existing from electrostatic, structural, and stereo-chemical matchings between inorganic and organic materials (Zhang, et al., 1989). The stereo-chemical geometry and charge distribution in alginate-Ca complexes were supposed to endow alginate with the capability to control the crystallization process (Descamps et al., 2009). Afterwards, phosphate ion was accumulated at the calcium complexes, and a solid phase of calcium phosphate initiated from an otherwise stable solution. Mineral nuclei that formed inside gradually grew longer. However, the size of HA crystal was

not greater than nanomolecule. And the shape of HA crystal particles are needle shape (Fathi and Hanifi, 2007). Therefore, the amorphous phases were attributed to alginate, transient amorphous calcium phosphates and poorly crystalline HA. Figure 23d showed the structure of the CA with 10% LX. The morphology was irregular quadrate shape, and the average size was 40–80 nm. Figure 23e showed crystal's characters of HA in CA-(10%)LX were needle and knotty-like shapes.

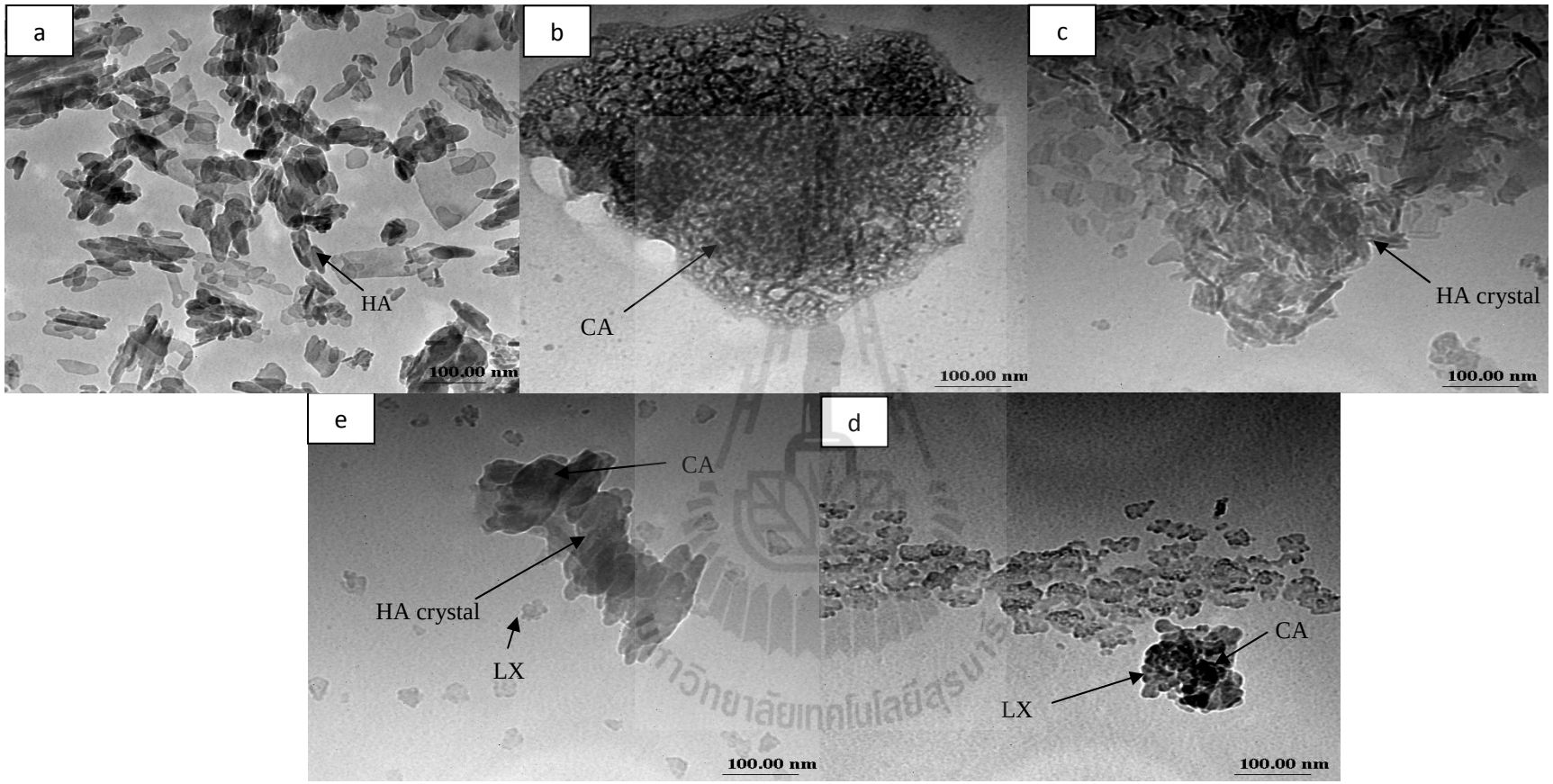


Figure 23. TEM micrographs of material and nano-composite. Material structures were presented in figure (a) HA, (b) CA, (c) CA/HA, (d) CA/(10%)LX, (e) CA/HA/(10%)LX.

3.4.8 FT-IR Analysis

FT-IR spectra of alginate (CA), pure HA, and LX composites showed in Figure 24. The absorption band at 3570 cm^{-1} was assigned to a hydroxyl group in CA, HA and LX. In CA, the absorption band at 1628 cm^{-1} was the wavelength of carbonyl (C-O) bond. The absorption band at 1413 and 1037 cm^{-1} in alginate was the wavelength of stretching bands of carboxyl (–COOH) and (C–O) (Won et al., 2005).

The HA was obtained by mixing $(NH_4)_2HPO_4$ and Ca $(NO_3)_2.4H_2O$ and showed in Figure 24. For the FT-IR analysis, the absorption band of PO_4^{3-} groups were identified at 1082, 1030 and 964 cm^{-1}. The three bands were different vibrational modes of PO_4 (Yamaguchi et al. 2001). The bands appearing at wavenumber values of 1660, 1422, and 876 cm^{-1} exposed the presence of carbonate ions in the resultant, (Cho et al., 2009) indicative of the precipitated HA containing carbonate ions (Bonfield and Hing 2002). No carbonate source was introduced into the starting materials, and all samples were prepared in an atmospheric environment. It was reasonable to infer that the carbonate ions incorporated into HA must arise from carbon dioxide gas.

The FTIR LX spectrum was found to be the absorbent band of hydroxyl group at 3360 cm^{-1} in double bond stretched and the C–H valency vibrated. The strong asymmetrical and symmetrical stretching of methylene group (CH_2) vibrated was presented at 2930 and 2860 cm^{-1}(Joglekar et al., 2011). The peak at 1717 cm^{-1} corresponded to the acetate group. The band at 1544 cm^{-1} was presented a few proteins in LX, The absorptions at 1447 cm^{-1} and 1380 cm^{-1} were attributed to the bending vibrations of methene and the vibrations of methyl in LX. The primary of alcohol was presented at 1045 cm^{-1} vibrations of C-O. The characteristic's peaks of

LX could be observed at 836 and 1664 cm^{-1} corresponding to the stretching and bending of the C=C of isoprene unit (Wang et al., 2009).

The spectra comparison between CA and LX showed in Figure 25. The band at 1628 cm^{-1} corresponded to carbonyl (C-O) bond. The bands at 1413 and 1037 cm^{-1} in alginate also showed outstanding bands in CA/LX complex. The bands from carboxyl (–COOH) and (C–O) group had stronger CA personality. The absorption band of LX at 2960 - 2857 cm^{-1} was corresponded to CH_2 bonds in LX. By 2.5% LX addition in CA/LX composite, the absorption band peak of $CH_2$was 1325–1380 cm^{-1}. The spectrum band of Lx was prominent band shifts toward higher wave numbers. That proved the LX had been incorporated into the bead composites.

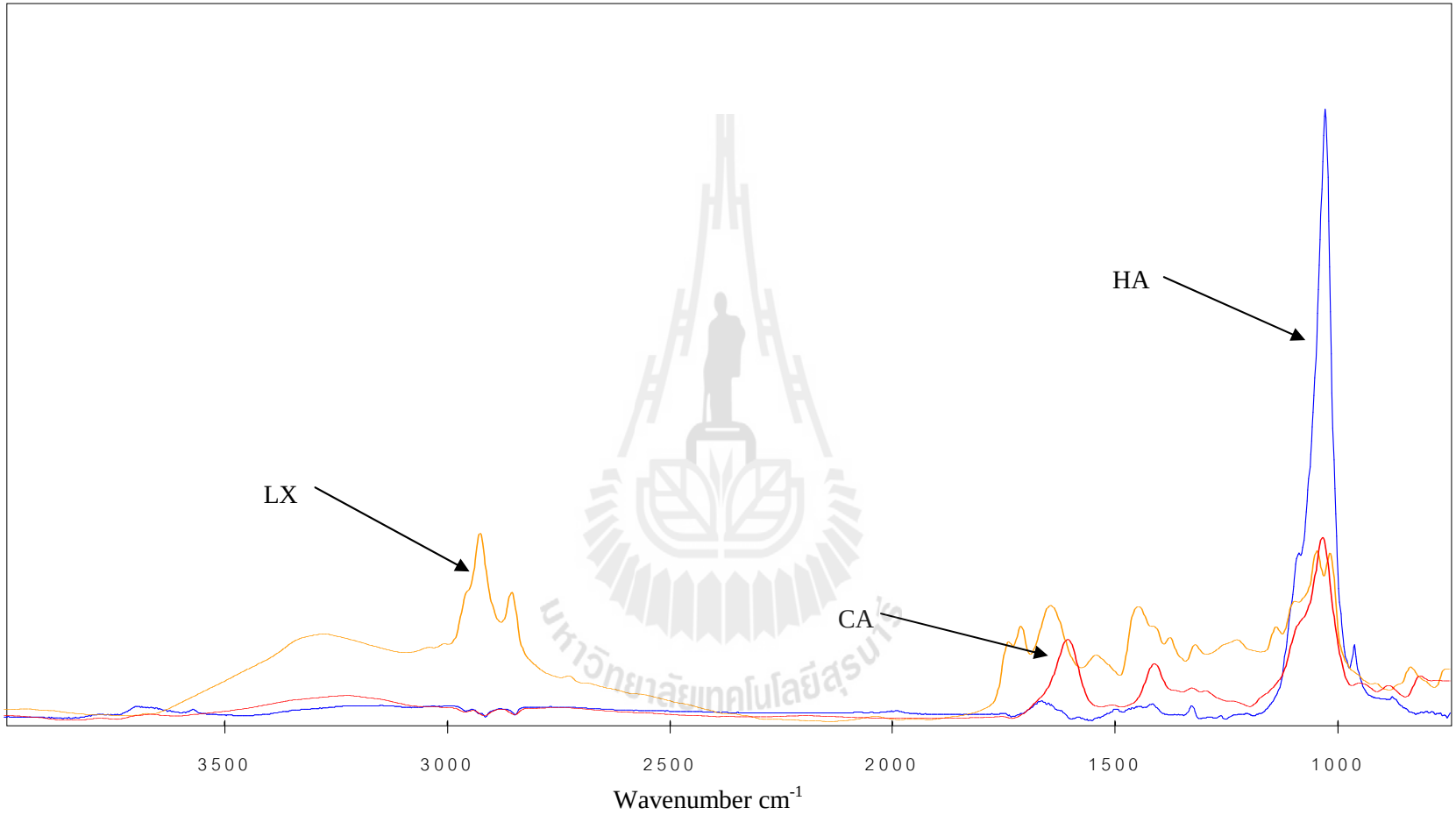


Figure 24. Spectrums of FT-IR analyzing the character week of CA, HA and LX in spectra covering the 4000-800 cm^{-1} range.

These typical bands from CA, HA and LX were observed in the CA/HA/LX composites. The wider bands at 1082, 1030 and 964 cm^{-1} were from PO_4 which was personality of HA. The 10% LX in CA/HA/LX composite corresponded to the bands between 2960-2857 cm^{-1} was attributed to CH_2 bonds in LX. The result of CA/LX after mixed with LX, the band of LX was prominent band shifts toward higher wave numbers. That proved the LX had been incorporated into the bead composites.

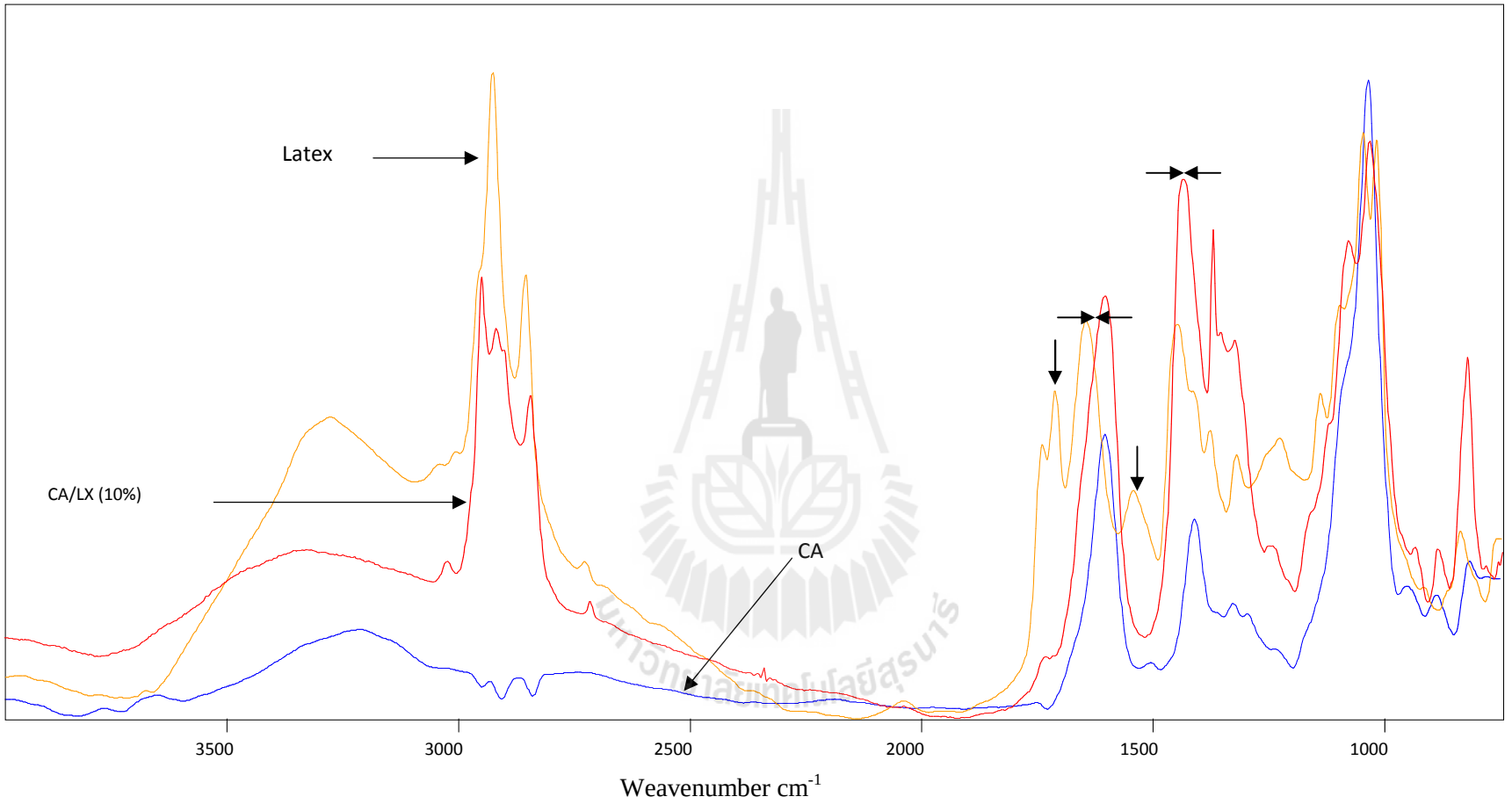


Figure 25. Spectrums of the FT-IR analyzing of CA, LX and CA/LX (10%) in spectra between 4000-800 cm^{-1}. ( ) increase of peak, ( ) shifts wave numbers.

3.4.9 Optimization conditions of immobilized invertase enzyme

The optimization conditions of relative activities of invertase enzyme were described in 3.3.2.10. The activities of enzyme in each LX concentrations were stabled at 10, 48, 130 and 200 h in 0%, 205%, 5%, and 10%, respectively, as shown in Figure 26. The enzyme activities were demonstrated in gel forming stage at temperature of 4, 10, 20, 30, 40 and 50°C. However at 50°C, there was no enzyme activity.

In comparison between CA/LX and CA/HA/LX, it was found that at the 2.5% LX concentration, the result showed no activity in gel forming stage at 50°C (Figure 26b). Because the LX was inflated and clogged the bead pours structure at 50°C in gel forming structure stage (Robb et al., 2007). After that inflate effect from LX was sealing the pore in bead and interrupted or blocked the substrate flowing into the bead. At 40°C of 5% LX gel forming stage, the enzyme activities were decreased. And 2.5% LX of gel forming stage at 50°C, there was not enzyme activity (Figure 26c). Activities of enzyme was shown in 2.5% of LX addition and then activities was decreased when increase LX at 5%. And 10% LX (Figure 26d) at 40°C and 50°C in gel forming stage, there were no enzyme activity.

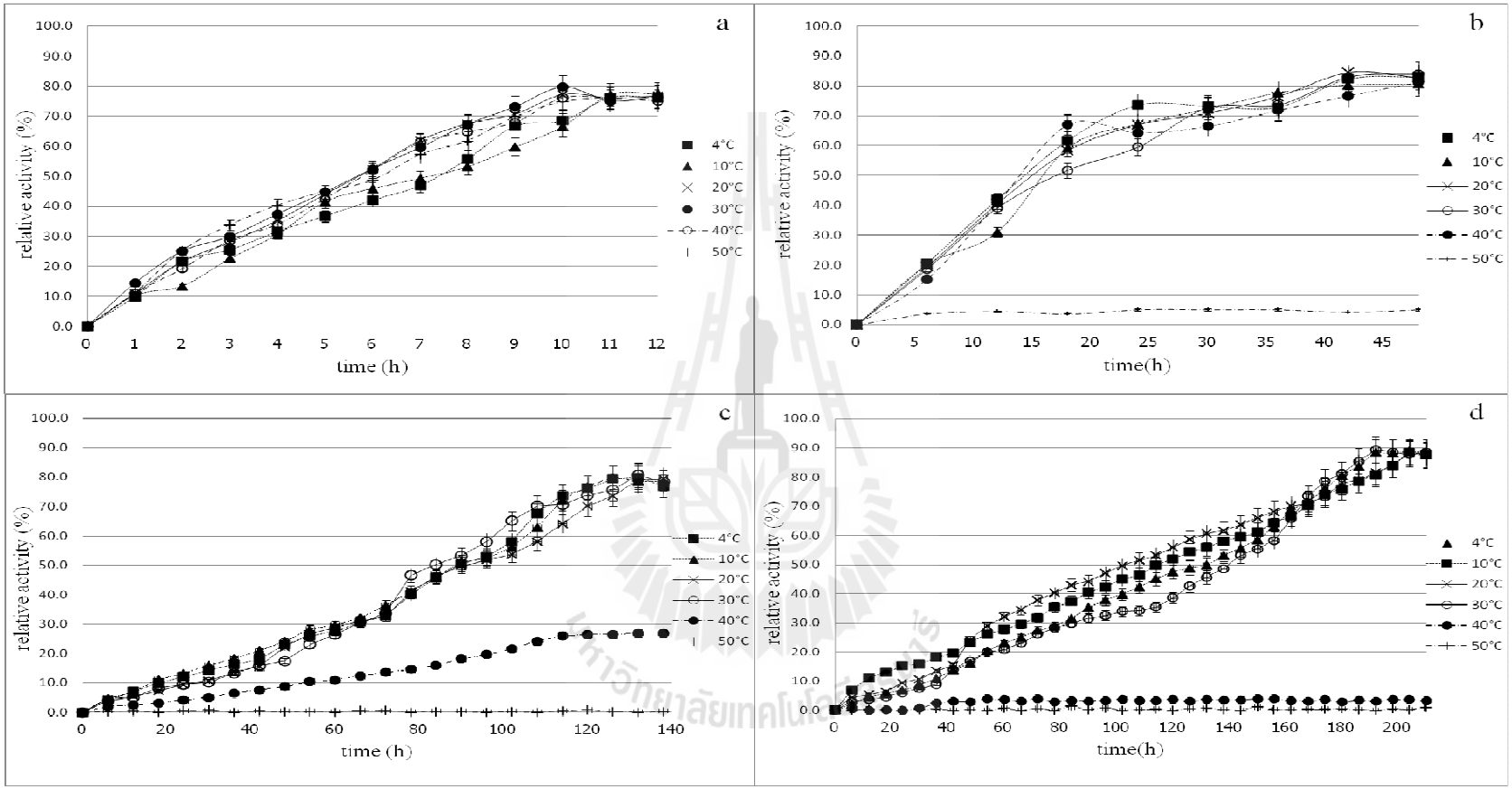


Figure 26. Invertase enzyme was investigated form begging the end of activity (a) CA (b) CA with 2.5% LX concentration (c) CA with 5% LX concentration (d) CA with 10% LX concentration.

The results of relative enzyme activity of CA/HA/LX were stabled at 7, 8, 10 and 17 h as shown in Figure 27. The difference between CA/LX and CA/HA/LX was the LX clogged. However, the influence of LX has no effect on CA/HA/LX. Because HA in CA/HA/LX expanded pore inside the bead, and the LX infraction was in inadequate to seal expanded pores. The LX concentration determined the rate of relative enzyme activity.

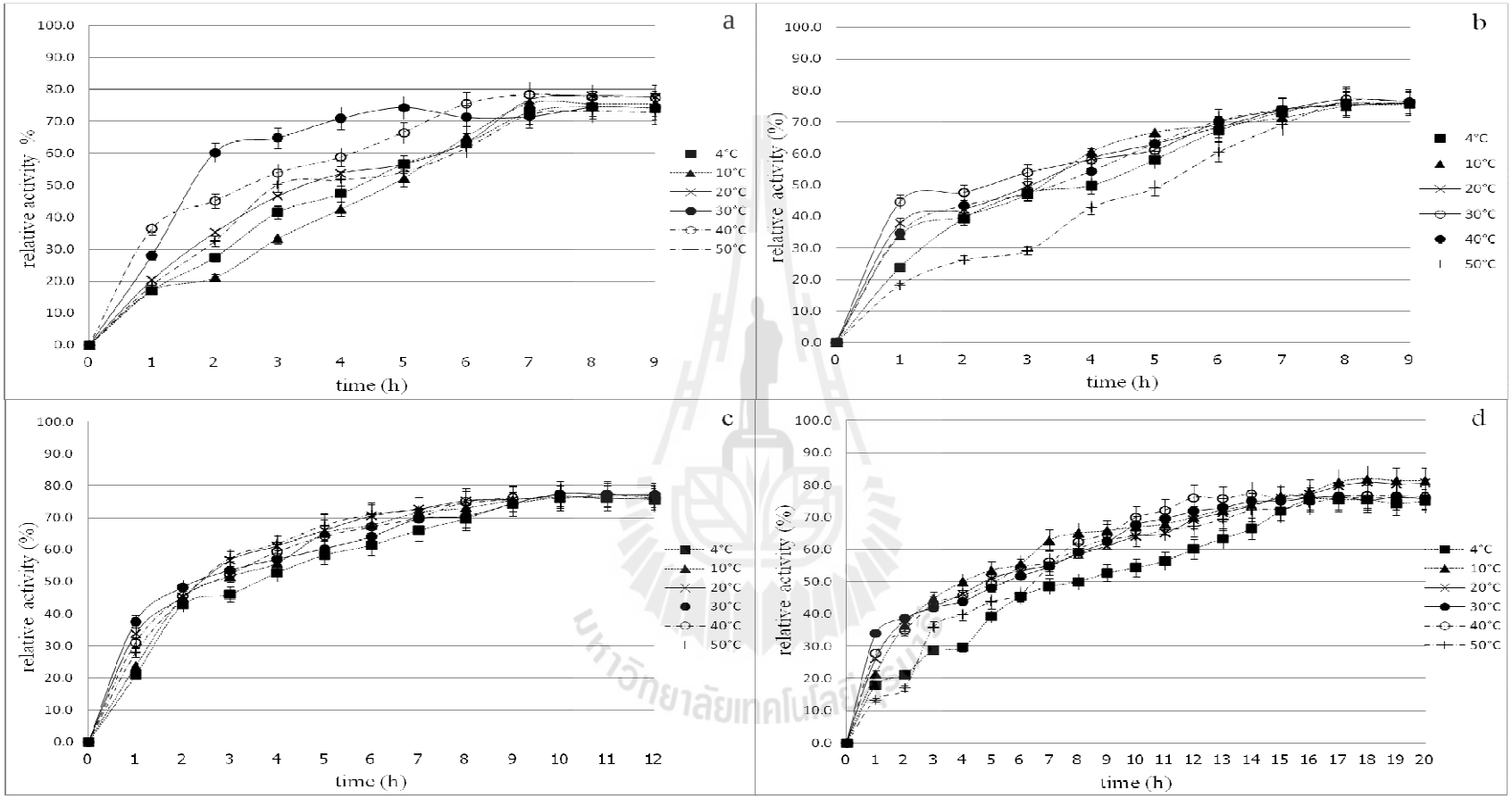


Figure 27. Invertase enzyme were investigated form begging the end of activity (a) CA/HA (b) CA/HA with 2.5% LX concentration (c) CA/HA with 5% LX concentration (d) CA/HA with10% LX concentration.

3.4.10 Initial velocity

The initial velocity of CA/LX and CA/HA/LX in various LX concentrations was investigated and studied of these structures. Initial velocity of the invertase enzyme in various temperatures on gel forming stage at 4°C, 10°C, 20°C, 40°C and 50°C showed in Figure 28. All samples of CA/HA/LX had higher initial velocity than those of CA/LX group. Furthermore the initial velocity of CA/HA was higher than CA. Due to the fact that HA constructed its structure and expand pores inside a bead, the initial velocity of CA/HA was higher than CA. For the same phenomenon, the initial velocity of CA/HA/LX was higher than CA/LX. After having tested at various temperatures in gel forming stage, the optimum initial velocity was found at 30 °C. In comparison, the initial velocity at 4°C in gel formation stage was lower than at 30 °C because the greater structure of the bead was presented at low temperature. In addition, the initial velocity at high temperature at 30°C was at the optimum. Finally the initial velocity at 40°C and 50°C decreased due to LX inflation.

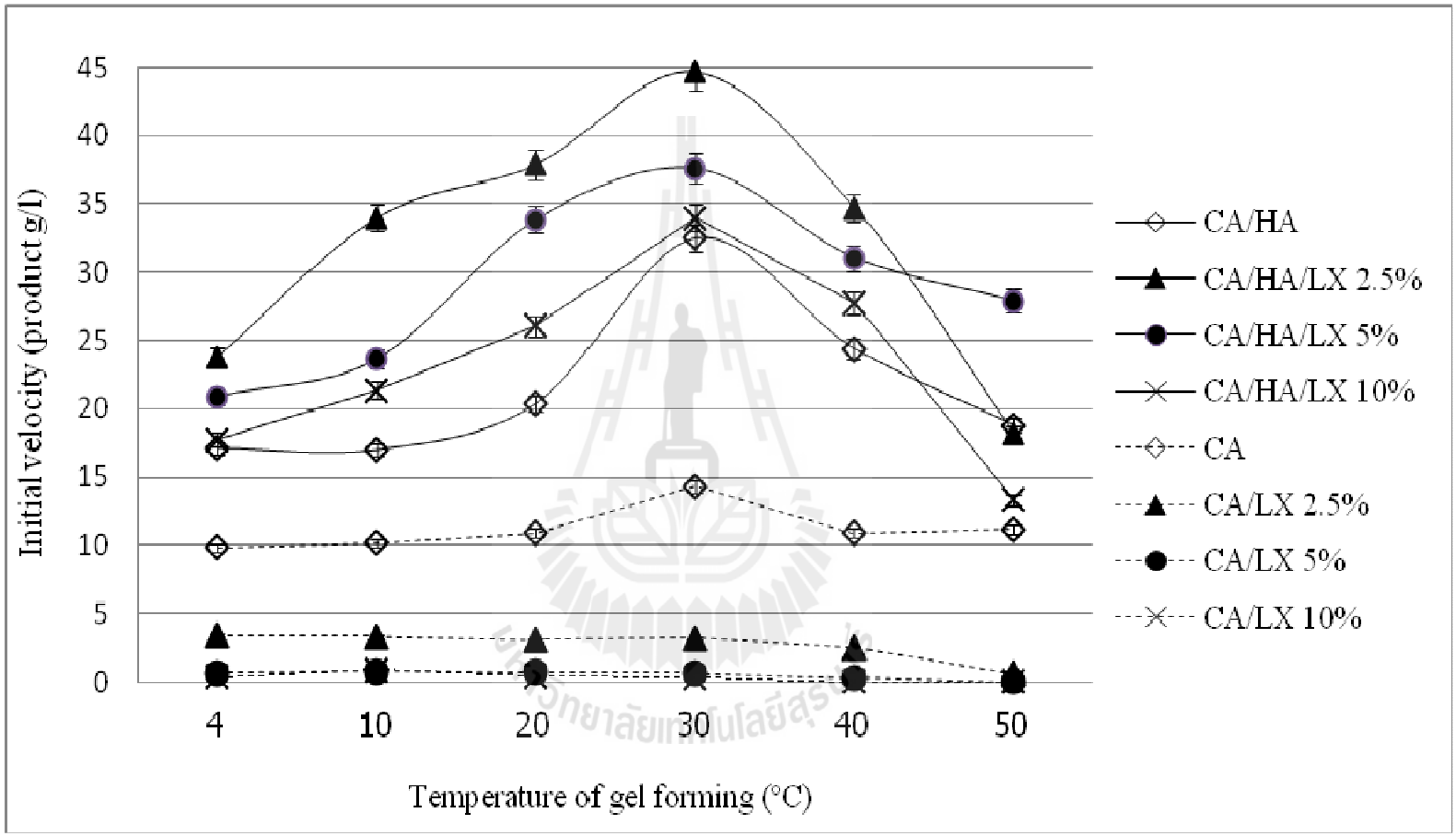


Figure 28. The graph of initial velocity at various temperatures in gel forming stage.

3.4.11 Reusability of enzyme

The invertase enzyme was immobilized by CA/LX and CA/HA/LX. Then, it was stored in 0.02 M acetate buffer (pH 4.8) at 4^{o}C for 2 days. After that it was studied for bead stability. Figure 29 showed the percentage of relative enzyme activity as a function of reuse time for CA, CA/LX and CA/HA/LX in various concentration of LX contained invertase enzyme compared to the free enzyme. The result showed that efficiency of enzyme activities in sucrose solution was decreased as number of reused cycle increase. The invertase enzyme activity of CA and CA/HA/LX beads immobilized were decreased by 40-50 % after the 8th cycle. For CA/LX with 10% LX, the enzyme activity decreased more than 30% from the first to the second test and decrease to nearly zero activity by the 5th test. Because the influenced by LX and alginate concentration, mechanical treatment of the beads and physical shaking produces beads (Pilkington et al., 1998). Even though, the best of enzyme activity was presented in free enzyme but immobilization enzyme offers more advantages (Norton and Vuillemard 1994; Mitsura et al., 1980). These factors led to a subsequent increase in bioprocess efficiency (Nazzaro et al., 2012).

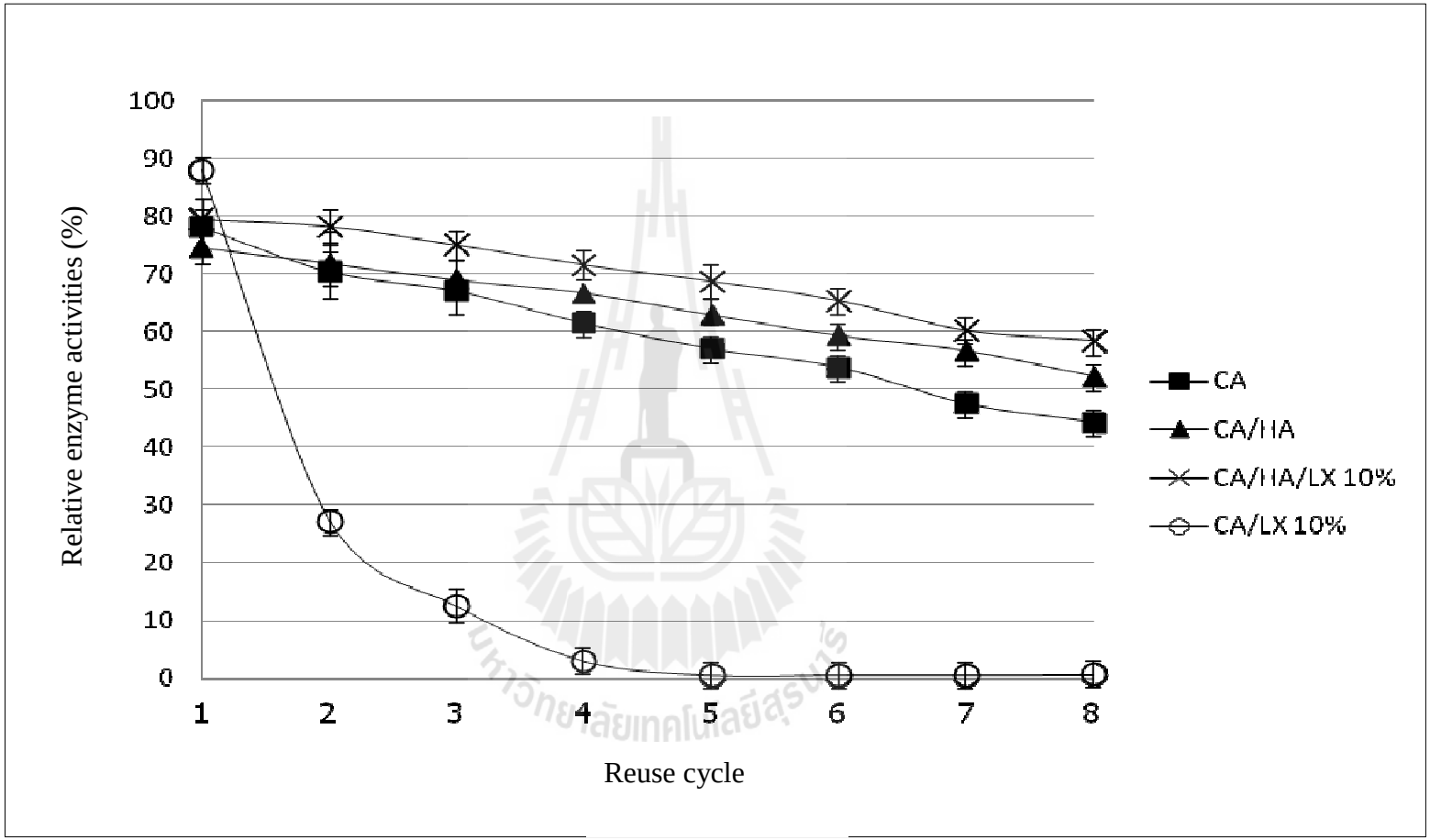


Figure 29. Relative invertase activities of reused immobilized CA and CA/HA at 0 and 10% LX concentration in 8 cycles. The slope of invertase activity in every condition was decrease after used to 8 cycles.

3.5 References

Avella, M., Pace, E.D., Immirzi, B. and Impallomeni, G. (2007). Addition of glycerol plasticizer to seaweeds derived alginates: Influence of microstructure on chemical-physical properties. **Carbohydrate Polymer**. 31: 165-172

Bonfield W, and Hing K.A. (2002). A comparative study on the in vivo behavior of hydroxyapatite and silicon substituted hydroxyapatite granules. **Journal of Minerals & Materials Characterization & Engineering**. 13(12): 1199-1206.

Bourne, M.C. (1982). Food Texture and Viscosity: Concept and Measurement. **Academic** Press. New York, N.Y.

Chang, M.Y. and Juang, R.S. (2005) Activities, stabilities, and reaction kinetics of three free and chitosan-clay composite immobilized enzymes. **Enzyme Microbial Technology**. 36: 75-82.

Descamps M., Hornez J.C., Leriche A. (2009). Manufacture of hydroxyapatite beads for medical applications. **Journal of the European Ceramic Society**. 29: 369-375.

Draget, K. I., Ostgaard, K., Smidsrod, O. (2006) Homogeneous alginate gel: a technical approach. **Carbohydrate Polymers**. 14:159-178.

Dorozhkin S.V. and Matthias Epplel. (2002). Biological and medical significance of calcium phosphates. **Chemie International Edition**. Volume 41, 17: 3130-3146.

Fathi, M.H. and Hanifi, A. (2007). Evaluation and characterization of nanostructure hydroxyapatite powder prepared by simple sol-gel method. **Materials Letters.** 18: 3978-3983.

Funduenanu, G., Nastruzzi, C., Carpov, A., Desbrieres, J. and Rinaudo, M. (1999) Physico-chemical characterization of Ca-algiante microparticles produced by different methods. **Biomaterials.** 20: 1427-1435.

Geant, G.T., Morris, E. R., Rees, D. A., Smith, P.J.C., & Thom, D. (1973). Biological interactions between polysaccharides and divalent cations-egg-box model. **FEBS Letters**. 32:195-198.

Gorka Orive , María De Castro , Hyun-Joon Kong , Rosa Ma Hernández , Sara Ponce, David J. Mooney, José Luis Pedraz. (2009). Bioactive cell-hydrogel microcapsules for cell-based drug delivery. **Journal of Controlled Release**. 135: 203-210.

Hari, P.R., Chandy, T., Sharma, C.P. (1996). Chitosan/calcium alginate microcapsules for intestinal delivery of nitrofurantoin. **Journal Microencapsul**. 13:319-329.

Joglekar S., Kodam K., Dhaygude M., Hudlikar M. (2011). Novel route for rapid biosynthesis of lead nanoparticles using aqueous extract of *Jatropha curcas* L. latex. **Materials Letters**. 65: 3170-3172.

Khelendra A., Gurbhinder S., Devendra P.,and Satya P. (2011). Synthesis and characterization of hydroxyapatite powder by sol-gel method for biomedical application. **Journal of Minerals & Materials Characterization & Engineering**. 8: 727-734.

Kohjiya S., Katoh A., Suda T., Shimanuki J., Ikeda Y. (2006) Visualisation of carbon black networks in rubbery matrix by skeletonisation of 3D-TEM image. **Polymer.** 47(10): 3298-301.

Konsoula, Z., Kyriakides, M.L. (2006) Starch hydrolysis by the action of an entrapped in alginate capsules α-amylase from *Bacillus subtitis***. Enzyme Microbial Technology.** 41:3 3-349.

Krasaekoopt, W., BBhandri, B., and Deeth, H. (2006). Survival of probiotics encapsulated in chitosan-coated alginate beads in yoghurt from UHT-and conventionally treated. **Lebensmittel-Wissenschaft & Technologie.** 39: 177-183.

Livonniere De, H. (1993). Natural rubber latex protein reduction with an emphasis on enzyme treatment. **Clinical Review Allergy.** 11: 309-323.

Mitsura, W. Kato, J.; Chibata, I. (1980). Continuous production of ethanol using immobilized growing yeast cells**. Microbial Biotechnology**. 10: 275-278.

Norton S., and Vuillemard J.C. (1994). Food bioconversions and metabolite production using immobilized cell technology. **Critical Review Biotechnology**. 14: 193-224.

Orive G., M. De Castro, S. Ponce, et al., (2005). Long-term expression of aerythropoietin from myoblasts immobilized in biocompatible and neovascularized microcapsules. **Molecular Therapy**. 12: 283-289.

Pilkington H., Margaritis A., Mensour N.A., and Russell I. (1998). Fundamentals of immobilized yeast cells for continuous beer fermentation. **Journal of Industrial Brewer**. 104: 19-3.

Paital, S. R. and Dahotre, N. B. (2009). Calcium phosphate coatings for bio-implant applications: materials. **Material Science Engineering**. 66:1-70.

Pan, G., Kurumada, K.I., Yamada, Y. (2008). Applicatopn of hydrogel for the removal of pollutant phenol in water. **Journal of the Chinese Institute of Chemical Engineers**. 39:361-366.

Parente, E.,Crudele, M.A., Aquino, M. and Clementi, F. (1998). Alginates production by *Azotobacter vinelandii* DSM576 in batch fermentation. **Journal of Industrial Microbiology Biotechnology.** 20: 171-176.

Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. **Analytical Chemistry**. 31: 426-429.

Rajkumar, M. and Meenakshisundaram, N. (2011). Development of nanocomposites based on hydroxyapatite/sodium alginate: synthesis and characterisation. **Material Characteristic**. 62:469-479.

Rehm B.H. and Valla, S. (1997). Bacterial alginates: Biosynthesis and applications. **Apply Microbiology Biotechnology**. 48: 281-288.

Robb, F., Antranikian, G., Grogan, D., Driessen, A. 2007. Thermophiles Biology and Technology at High Temperatures CRC. p. 368.

Sethuraj MR, and Mathew NM. (1992). Natural rubber: Biology, Cultivation and Technology**. Amsterdam. Elsevier.** p. 80.

Smidsrid, O. and Skjak-Braek, G. (1990). Alginate as immobilization matrix for cells. **Trends of Bioteechnology**. 8: 71-78.

Steel, D.M. and Walker, J.M. 1991. Thermostable Proteins. **Life Chemistry Reports**. 108: 293-298.

Tu J., Bolla S., Barr J., Miedema J., Lia X., Jasti B. (2005). Alginate microparticles prepared by spray–coagulation method: Preparation, drug loading and release characterization. **International Journal of Pharmaceutics**. 303: 171-181.

Warren J. Grigsby, Christopher J. Ferguson, Robert A. Franich, Gregory T. Russell. (2005). Evaluation of latex adhesives containing hydrophobic cores and poly (vinyl acetate) shells: potential to improve poly (vinyl acetate) performance. **International Journal of Adhesion and Adhesives**. 25: 121-137.

Wang, J. S., Chen, S. B., & Fang, Y. (2009). Viscosity study of interactions between sodium alginate and CTAB in dilute solutions at different pH values. **Carbohydrate Polymers**. 75: 333-337.

Won, K., Kim, S., Kim, K., Park, H. W., Moon, S. J. 2005. Optimization of lipase entrapment in Ca-alginate gel beads. **Process Biochemistry**. 40: 2149-2154.

Yamaguchi I, Tokuchi K, Fukuzaki H, Koyama Y, Takakuda K, Monma H, Tanaka J. (2001). Preparation and microstructure analysis of chitosan/ hydroxyapatite nanocomposites. **Japan Biomedicine Materials Research**. 55(1): 20-7.

Zhang J., Wanga Q., Wang A. (2009). In situ generation of sodium alginate/ hydroxyapatite nanocomposite beads as drug-controlled release matrices. **Acta Biomaterialia**. 6(2): 445-454.

CHAPTER IV

CONCLUSIONS

The optimum LX concentration in the gel bead preparation consisted of CA/LX and CA/HA/LX in various LX concentrations including 2.5%, 5%, 10%, 15% and 20%. It was found that at LX concentration of 15% and 20% the beads were not used because they floated on the water, and some part of the LX leaked out. Viscosities of alginate solutions prior to beads forming were investigated. The relationship between viscosity and temperature showed that as the temperature increased, the viscosity decreased. However, at 10% LX the viscosities slightly increased as the temperature increased due to LX heat-sensitization.

The texture ability to withstand compression depended on the LX concentration. As the ability to withstand compression, the beads remaining also increase. At 10% LX concentration, the ability to withstand compression was at the maximum. After adding HA in CA/LX, the ability to withstand compression improved 5 times. Furthermore, the differences of bead cracking were presented in both CA/LX and CA/HA/LX. A single crack was found in CA/LX and a double crack was found in CA/HA/LX. Due to the hydrophobic property of LX, the swelling of CA/LX and CA/HA/LX decreased. The optimum pH and temperature were found to be pH 4 and 25°C at 90% swelling.

The difference of structure between CA/LX and CA/HA/LX were demonstrated using SEM and TEM analysis. The morphology of CA/LX was flat and irregular in shape. Looking from the top view, the dimension of the object was

between 0.5 µm and 5 µm. The morphology of CA/HA/LX showed highly gaps, porous structure and flocculated shape. TEM technique was used to observed in-situ HA crystal inside beads' structure. HA was the main material to induce flocculate shape in CA/HA/LX bead.

FT-IR technique was carried out to observe the change in functional groups after mixing CA, HA and LX in various concentrations. The spectra of each sample were unchanged. However, certain peaks decreased as LX concentration increased. As LX concentration increased from 2.5% to 10%, the wave number at 1325-1380 cm^{-1} shifted. This was because of the CH_2 character band in LX. This proving was confirmed that LX adding had influence on nano-composite beads structure.

Determination of enzyme activity, the invertase enzyme was immobilized in CA/LX and CA/HA/LX. At various temperatures of gel forming state, the LX clogged beads' structure at 40°C and 50°C. In comparison between CA/LX and CA/HA/LX, LX had no influence on CA/HA/LX. It was because HA in CA/HA/LX expanded pores inside the beads, and the LX expansion was inadequate to seal the expanded pores. In addition, LX determined the rate of relative enzyme activity for CA/LX and CA/HA/LX.

When compared initial velocity, CA/HA/LX was higher than CA/LX. Due to the fact that HA constructed its structure and expanded pores inside a bead, the initial velocity of CA/HA/LX was higher than CA/LX. The result of reused immobilized invertase enzyme in CA/HA and CA/HA/LX decreased by 40-50 % after the 8^{th} cycle. For CA/LX with 10% LX, the enzyme activity decreased more than 30% from the first to the second test and decreased to nearly zero activity by the 5^{th} test.

The strength and porous properties of CA/LX and CA/HA/LX were depended on LX concentration. The LX had ability to improved prolong enzyme activity and

increased strength of the bead’s structure in the same time. It is very interesting to study in the future, to apply CA/LX and CA/HA/LX in medicine or fertilizer.

APPENDICS

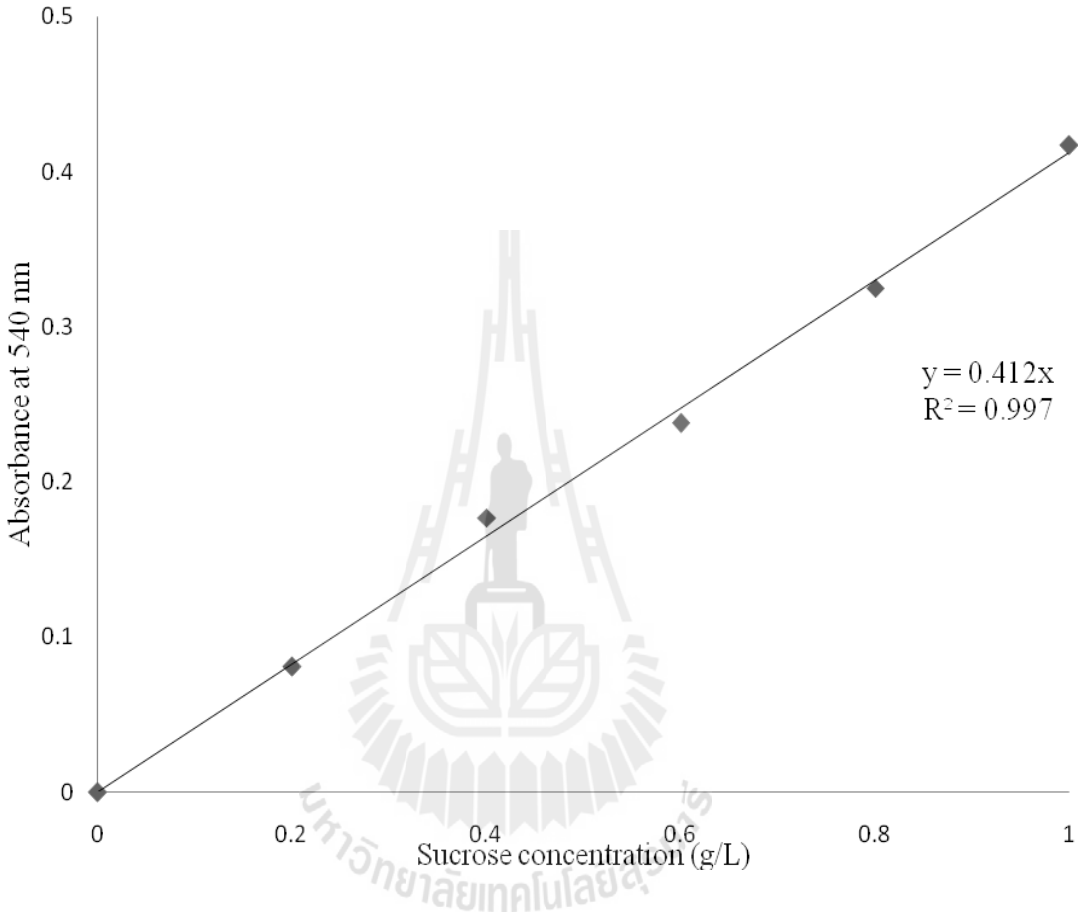


Figure 1. standard curve of sucrose concentration analysis using DNS method.

BIOGRAPHY

The author's name Apiwat Satsum. He was born on 18th June 1983 in Chainat, Thailand. He studied in Food Science and Technology, Rajamangala University of technology Suvarnabhumi. He graduated the Bachelor's of Food Science and Technology in 2005. After graduation, in 2007, he was Master's in School of Biotechnology at Suranaree University of Technology. He conducted the research in the topic of Stability improvement of alginate beads using hydroxyapatite/natural rubber nanocomposite beads for enzyme immobilization. The result of this project has been presented as poster and oral presentation at the TSB 2010 International Conference on Biotechnology of Healthy Living (The 22nd Annual Meeting of the Tai Society for Biotechnology) October 20-22, 2010, Prince of Songkla University, Trang Campus, Thailand. And The 3rd SUT Grade Conference 2010, November 21-23, 2010, Suranaree University of Technology, Nakhon Ratchasima, Thailand. During master degree enrollment, he has been an assistant researcher by Assistant Professor Dr. Chokchai Wanaphu in school of biotechnology.