ALGINATE PRODUCTION BY *AZOTOBACTER* SP. AND

ITS APPLICATION IN ENZYME IMMOBILIZATION

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A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Master of Science in Biotechnology

Suranaree University of Technology

Academic Year 2008

การผลิตอัลจิเนทโดยเชื้อ *AZOTOBACTER* SP. และ
การประยุกต์ใช้ในการตรึงเอนไซม์

นางสาวอัญญาณี พรหมปภากร

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

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อัญญาณี พรหมปภากร : การผลิตอัลจิเนทโดยเชื้อ *AZOTOBACTER* SP. และการประยุกต์ใช้ในการตรึงเอนไซม์ (ALGINATE PRODUCTION BY *AZOTOBACTER* SP. AND ITS APPLICATION IN ENZYME IMMOBILIZATION) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.โชคชัย วนภู, 104 หน้า

อัลจิเนทสามารถสกัดได้จากผนังเซลล์ของสาหร่ายทะเลสีน้ำตาลแต่ปริมาณการลดลงของสาหร่ายและความกังวลทางด้านความปลอดภัยในการสกัดที่ทำให้เกิดการสะสมของโลหะหนักจากสารเคมีที่ทำให้น้ำทะเลเป็นพิษ รวมไปถึงราคาที่สูงในกระบวนการสกัดรวมไปถึง การทำให้บริสุทธิ์ เป็นเหตุผลหลักที่ทำให้เกิดความสนใจที่จะหันมาผลิตอัลจิเนทจากเชื้อแบคทีเรียแทน อัลจิเนทจากเชื้อ *Azotobacter* sp.จึงกลายเป็นผลิตภัณฑ์ที่ใช้กันอย่างแพร่หลาย เพราะปลอดภัยต่อสิ่งแวดล้อม ไม่เป็นเชื้อแบคทีเรียก่อโรค และมีโครงสร้างคล้ายกับอัลจิเนทที่ผลิตได้จากสาหร่าย วัตถุประสงค์ของการศึกษาครั้งนี้ เพื่อหาสภาวะที่เหมาะสมต่อการผลิตอัลจิเนทและประยุกต์ในการตรึงเอนไซม์บีต้าอะไมเลส ในการดำเนินงานครั้งนี้จะทำการศึกษาหาแหล่งคาร์บอน ค่าพีเอช แหล่งไนโตรเจน และอุณหภูมิที่เหมาะสมในอาหาร LG medium ปริมาตร 100 มิลลิลิตร ในการทดลองระดับฟลาสก์พบว่า *Azotobacter* sp. จะให้ปริมาณการผลิตอัลจิเนทสูงที่สุดประมาณ 5-6 กรัมต่อลิตร ที่ค่าพีเอช 6.5-7 เมื่อเลี้ยงที่อุณหภูมิ 30 องศาเซลเซียล น้ำตาลซูโครสเป็นแหล่งคาร์บอน ความเข้มข้น 1% โดยปริมาตรและไม่มีการเติมแหล่งไนโตรเจนลงไปใน LG medium จากนั้นศึกษาการผลิตอัลจิเนทในถังหมักขนาด 2 ลิตรที่มีปริมาตรอาหาร 1.5 ลิตร โดยใช้สภาวะที่เหมาะสมต่อการผลิตอัลจิเนทที่ได้จากการศึกษาในขั้นต้นเพื่อหาสภาวะที่เหมาะสมของอัตราการให้อากาศและความเร็วของใบกวน โดยพบว่าอัลจิเนทที่ผลิตได้กับการเจริญของเซลล์จะมีความสัมพันธ์ที่เกี่ยวข้องกัน การเจริญของเซลล์และการผลิตอัลจิเนทจะให้ค่าสูงที่สุดที่ความเร็วรอบของใบกวนเป็น 500 รอบต่อนาที ที่อัตราการให้อากาศ 2.5 ปริมาตรของอากาศต่อปริมาตรของน้ำหนักต่อนาทีใน LG medium (μ = 0.295 ต่อชั่วโมงและY_{ps} of 0.503 กรัมของอัลจิเนทต่อกรัมของน้ำตาลซูโครส) ภายใน 24 ชั่วโมงแรกและจะมีค่าลดลงอย่างช้าๆ และความหนืดของอัลจิเนทที่ผลิตได้จะมีค่าเพิ่มขึ้นตามเวลา ซึ่งแสดงให้เห็นว่าอัลจิเนทที่ผลิตได้ค่าพฤติกรรมของของไหลเป็นแบบนันนิวโตเนียนเนื่องจากความหนืดเพิ่มขึ้น(จาก77.52 เซนติพอยต์ ถึง 252.5 เซนติพอยต์) เมื่ออัตราเฉือนเพิ่มขึ้น (1.29 ถึง 24.81 เซนติพอยต์ต่อนาที)

กรดอินทรีย์ทั้ง 9 ชนิด เช่น กรดซัลซินิก กรดฟูมาริก กรดโพพิโอนิก กรดไฟติก กรดมาลิก กรดอะดิปิก กรดอะมิโนเบนโซอิก กรดแลคติก และกรดทาร์ทาริกจะถูกนำมาใช้ในการเพิ่มประสิทธิภาพของอัลจิเนทที่ผลิตได้ พบว่าความหนืดของอัลจิเนทที่ผลิตได้ในถังหมักขนาด 2 ลิตรเพิ่มขึ้น เมื่อเติมกรดซัลซินิก ความเข้มข้น 0.15% โดยปริมาตร (ความหนืด = 432.52 เซนติพอยต์,

μ = 0.297 ต่อชั่วโมง, Y_{ps} = 0.505 กรัมของอัลจิเนทต่อกรัมของน้ำตาลซูโครส) ลงไป นอกจากนี้ได้พัฒนาการผลิตอัลจิเนท จาก *Azotobacter* sp.ในถังหมักขนาด 5 ลิตร ซึ่งพบว่าอัลจิเนทที่ผลิตได้มีค่าน้อยกว่าในถังหมักขนาด 2 ลิตร ประมาณ 2.5 เท่า (ความหนืด = 168.78 เซนติพอยต์, μ = 0.221 ต่อชั่วโมง, Y_{ps} = 0.397 กรัมของอัลจิเนทต่อกรัมของน้ำตาลซูโครส) ดังนั้นในการผลิตเชิงอุตสาหกรรมเพื่อให้ได้ปริมาณมาก จึงต้องมีการศึกษาเพิ่มเติม ลักษณะสัณฐานวิทยาของอัลจิเนทและเชื้อ *Azotobacter* sp. ถูกศึกษาภายใต้กล้อง SEM พบว่าเซลล์มีรูปร่างเป็นแท่งขนาดประมาณ 1 ไมโครเมตร และอัลจิเนทที่ถูกนำไปละลายและทำการอบแห้งที่ได้จาก *Azotobacter* sp และอัลจิเนทที่ผลิตได้จากสาหร่ายนั้นจะมีลักษณะเป็นโครงร่างตาข่ายเหมือนกัน น้ำหนักโมเลกุลของอัลจิเนทที่ผลิตได้จาก *Azotobacter* sp. และ สาหร่ายนั้นจะถูกวิเคราะห์ด้วยเครื่อง HPLC พบว่าขนาดน้ำหนักโมเลกุลของอัลจิเนททั้งสองแหล่งที่มาเป็น 2.87 x 10^3 and 2.88 x 10^3 Da ตามลำดับ อัลจิเนททั้งสองแหล่งที่มาจะถูกนำมาใช้ในการตรึงเอนไซม์บีต้าอะไมเลสเพื่อเปรียบเทียบกับการใช้บีต้าอะไมเลสแบบไม่ถูกตรึงในอัลจิเนท พบว่าประสิทธิภาพในการนำกลับมาใช้ใหม่ของบีต้าอะไมเลสที่ถูกตรึงในอัลจิเนทในการย่อยแป้งนั้นจะลดลงจาก 36.4 และ 42.4 ตามในรอบที่ 8 ลำดับ ขณะที่บีต้าอะไมเลสที่ไม่ถูกตรึงในอัลจิเนทนั้นจะมีประสิทธิภาพในการย่อยแป้งค่อนข้างคงที่เนื่องมาจากบีต้าอะไมเลสบางส่วนที่ถูกตรึงไว้หลุดหายออกไปจากอัลจิเนท

ผลการวิจัยในครั้งนี้พบว่า อัลจิเนทสามารถผลิตได้จากแบคทีเรียและมีคุณภาพทัดเทียมกับที่ผลิตจากสาหร่าย แต่การผลิตเชิงพาณิชย์ เพื่อให้ได้ปริมาณมากจึงจำเป็นจะต้องวิจัยการเพิ่มกำลังการผลิตเพิ่มเติม

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา จันฺฑาณ์ พ่ีหฺมปฆฆฆ
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ANYANEE PROMPAPHAGORN : ALGINATE PRODUCTION BY *AZOTOBACTER* SP. AND ITS APPLICATION IN EMZYME IMMOBILIZATION. THESIS ADVISOR : ASST. PROF. CHOKCHAI WANAPU, Ph.D., 104 PP.

ALGINATE PRODUCTION/ *AZOTOBACTER* SP.

Commercial alginate is extracted from the cell wall of brown seaweed. However, the decreasing of seaweed, the safety concerning extraction (that are potential accumulators of the heavy metal from reagent present in polluted seawater) and high cost for extraction-purification processes were the main reasons for the present interest towards the microbial production of alginate. Alginate from *Azotobacter* sp. may become a major commercial product because of environmental safety, non-pathogen bacteria and was similar in structure to the algae alginate. The aim of this research was to optimize conditions in alginate production and apply β-amylase immobilization. The optimization of carbon sources, pH, nitrogen sources and temperature for alginate production were conducted in LG medium. In shake flask experiment, *Azotobacter* sp. produced the highest alginate (5-6 g/L) at pH 6.5-7 when incubated at 30°C in LG medium with 1% w/v sucrose and without nitrogen source. The optimum condition in shake flask experiments was also conducted in 2L fermenter for studying the optimum aeration rate and agitation speed. It was found that the alginate production was growth-associated. Growth and alginate production were highest at 500 rpm of agitation speed with 2.5 vvm of aeration in LG medium (μ

= 0.295 h^{-1}, Y$_{ps}$ = 0.503 g of alginate/g of sugar) within the first 24 hours and gradually decreased. The viscosity of alginate was increased as time passed which exhibited non-Newtonian behavior because viscosity increased (77.52 to 252.5 cP) with the shear rate increased (1.29 to 24.81cP·s^{-1}).

Nine organic acids such as succinic acid, fumaric acid, propionic acid, phytic acid, malic acid, adipic acid, 4-aminobenzoic acid, lactic acid and tartaric acid were used in increasing the efficiency of alginate. Visocosity of alginate produced in 2L fermenter was increased after adding 0.15% w/v of succinic acid (viscosity = 432.52 cP, μ = 0.297 h^{-1}, Y$_{ps}$ = 0.505 g of alginate/g of sucrose). The alginate production from *Azotobacter* sp. was scaled up to 5L fermenter. The production in 5L fermenter was lower than in 2L fermenter about 2.5 times (viscosity = 186.67 cP, μ = 0.221 h^{-1}, Y$_{ps}$ = 0.397 g of alginate/g of sucrose). The morphological characteristic of *Azotobacter* sp. and alginate were studied under SEM. The morphology of *Azotobacter* sp. cells had a rod shape with the size of about 1 μm. Dry form of both alginate from *Azotobacter* sp. and algae alginate had the same crosslinked-structure. The Molecular weights (MW) of alginate from *Azotobacter* sp. and algae were 2.87 x 10^3 and 2.88 x 10^3 Da, respectively when detected by HPLC. Both of the alginate were used for immobilizing β-amylase and compared with free enzymes. It was found that the efficiency of reusing of β-amylase immobilized bead (*Azotobacter* alginate and seaweed alginate) for starch hydrolysis decreased by 36.4% and 42.4%, respectively at 8th cycle while β-amylase enzyme would hydrolyze starch constantly due to the fact that some parts of the mobilized β-amylase were removed from alginate.

The study found that the alginate could be produced from bacteria and had the

same quality as seaweed. However, it is necessary to do further research to increase

more productivity as the commercial production would require large quantity.

School of Biotechnology   Student's Signature_____

Academic Year 2008   Advisor's Signature_____

ACKNOWLEDGEMENT

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LIST OF ABBREVIATIONS

$^{\circ}C$	=	degree celcius
g	=	gram
g/L	=	gram per liter
g/g	=	gram per gram
h	=	hour
h^{-1}	=	reciprocal hour
mg	=	milligram
NaOH	=	sodium hydroxide
HCl	=	hydrochoric acid
HPLC	=	high performance liquid chromatography
ITS	=	internal transcribed spacer
L	=	liter
μm	=	micrometer
N	=	normal
mL/min	=	milliliter per minutes
g/mol	=	gram per molarity
mM	=	millimolar
M	=	molarity
nm	=	nanometer
rpm	=	round per minutes
SEM	=	scanning electron microscope

LIST OF ABBREVIATIONS (Continued)

K	=	kelvin
DO	=	dissoloved oxygen
kDa	=	kilodalton
cfu/mL	=	colonies forming unit per milliliter
s^{-1}	=	per second
$cP \cdot s^{-1}$	=	centipoint per second
μm	=	micrometer
cP	=	centipoint

CHAPTER I

INTRODUCTION

1.1 Significance of the study

Alginate, sometimes shortened to "algin", is a main representative of a family of polysaccharides that neither show branching nor repeating blocks or unit pattern. Alginate is a linear copolymer composed of two monomeric units, β-D-mannuronic acid and C5-epimer-α-L-guluronic acids, its unique and random structural pattern has attracted a lot of scientific and commercial interest over the past decade. Due to properties of alginate, it has various industrial application examples as viscosifiers, stabilizing, thickening, emulsifying, gelling agent or film forming agents or water-binding agents in food, and pharmaceutical. These products are also used in textile printing, paper industries, manufacturing of ceramics and production of welding rods and water-treatment (Onsien, 1996). Alginate was firstly discovered in the late 19th century by an English chemist, E.C.C. Standford that is quiet abundant in nature since they occur as a structure component in marine brown algae (*Laminaria, Phaeophyceae* and *Macrocystis*) (Gasesa, 1998). Microbial alginate was discovered, more than 80 years later, by Linker and Jones (1964), when they were isolating and partially characterizing the exopolysaccharide from a mucoid strain of *Pseudomonas aeruginosa* isolated from sputum of a cystic fibrosis patient. Two years later, Gorin and Spencer (1966) demonstrated that acetylated alginate can also be produced by the soil bacterium *Azotobacter vinelandii*. The decreasing availability of seaweed

(traditional sources not only of alginate but also of agar and carrageen production), the

safety concern for using extracts that are potential accumulators of the heavy metal cations Hg^{2+}, Cd^{2+}, Cr^{3+}, Cu^{2+}, etc. present in polluted seawaters, and increasing costs for their extraction-purification processes are the main reasons for the present interest towards the microbial production of exopolysaccharide (EPS). Therefore, alginates from *Azotobacter* sp. may become major commercial products (Rehm and Valla, 1997) because of environment safety, non-pathogenic bacteria, (Rehm and Valla, 1997) and similar to the algae alginate structure. The sequences of L-guluronic acid residues or G-block was showed similar to its algae (Skjak-Bræk et al., 1986) and it has advantages if implemented at commercial level because the process can be manipulated to obtain a product with specific characteristics. From a commercial point of view the most important characteristics of alginates are their ability to form viscous solution and gels (Parente et al., 1998). Their intrinsic apparentwhich determines their ability to induce apparentin solution mainly depends on the molecular weight of the polymer, and secondary, on its composition, whereas the capability of forming strong brittle gels reactions with Ca^{2+} ions is due to the long sequences of homopolymeric regions of L-guluronic acid residues along the chain (Smidsrod and Draget, 1996).

1.2 Research objectives

1.2.1 To find the optimum conditions for alginate production by *Azotobacter* sp. in Lab scale.

1.2.2 To find methods for increasing the efficiency of alginate production with

Addition of some organic acids.

1.2.3 To apply bacterial alginate in enzyme immobilization.

1.3 Research hypothesis

Azotobacter sp. could produce alginate in the same quality as algae alginate and it could be used in β-amylase enzyme immobilization.

1.4 Scope and limitation of the study

The optimization of alginate production conditions such as pH, temperature, sucrose concentration, nitrogen source, nitrogen concentration and oxygen concentration for alginate production by *Azotobacter* sp. were investigated in 2L fermenter with working volume 1.5L and increased alginate productivity in 5L fermenter with working volume 3L. Alginates were tested efficiency in β-amylase immobilization.

1.5 Expected results

The high efficient bacterial alginate could be obtained from *Azotobacter* sp. and shows high efficiency in β-amylase immobilization.

CHAPTER II

LITERATURE REVIEW

2.1 Alginate

2.1.1 Chemical structure

Alginate is composed of the uronic acid, β-D-mannuronate (M-block) and its C-5 epimer α-L-guluronate (G-block) residues arranged in irregular block along the linear chain (Fig 2.1). These monomers can be organized in block of consecutive G-residues (G-blocks), consecutive M-residues (M-blocks), alternating M and G-residues (MG-blocks) or randomly organized blocks (Fig 2.2). Alginate is currently extracted from the cell wall of the brown seaweeds such as *Laminaria digitata*, *L. hyperborean*, *Macrocystis pyrifera* (Saude and Junter, 2002) and *Phaeophyceae* where they exist as the mixed calcium-sodium-potassium salt of alginic acid (Chementi et al., 1995). The relative amounts of which vary greatly between alginate from different species of algae, or between the different regions in the same algae (Sabra, 1998). The present source of commercial alginate is the giant brown kelp *M. pyrifera*. Because only a few species of brown algae are suitable for commercial alginate production in respect to abundance, location and uniform quality, there is a present interest in an alternative bacterial alginate. Several bacteria such as the nitrogen fixing aerobe *Azotobacter* sp. (Gorin and Spencer, 1966) and the opportunistic pathogen *Pseudomonas* (Evans and Linker, 1973; Govan et al., 1981;

Cote and Krull, 1988) are the only prokaryotic sources that also produce alginate and the structures of the blocks of monomer

residues are similar in alginate production by seaweeds and those synthesized by *Azotobacter vinelandii* (Steinbuchel et al., 2001). *P. aeruginosa* was first reported to produce alginate so most of knowledge of the genetics of alginate biosynthesis originates from studies of *P. aeroginosa*, mainly because of the medical relevance of this organism as an opportunistic human pathogen, particularly for patients suffering from cystic fibrosis (CF) (May and Chakrabarty, 1994). Alginate plays an important role as a virulence factor during the infectious process (Gacesa and Russell, 1990). The reason for this appear to be related to the alginate-mediated mode of biofilm growth, which causes resistant colonization of the lung, *A. vinelandii* and *P. aeroginosa* produce alginate as an extracellular polysaccharide (EPS) in vegetatively growing cells, whereas in *A. vinelandii* alginate is also involved in the differentiation process leading to a so called "cyst" (Sadoff, 1975; Steinbuchel et al., 2001). In addition, bacterial alginates are normally *O*-acetylated on the 2 and/or 3 position(s) of the β-D-mannuronate residues. Consequently, bacteria produce a range of alginate with different block structures and degrees of *O*-acetylation (Steinbuchel et al., 2001). The high molecular mass of bacterial alginate and the negative charge ensure that the polysaccharide is highly hydrated and viscous. It is well established that alginates from *P. aeroginosa* do not contain poly-guluronate blocks (Sherbrock-Cox et al., 1984; Skjak-Braek et al., 1986) while those from *A. vinelandii* may do so. The block structure and degree of *O*-acetylation, as well as the molecular weight, determine the physico-chemical properties of alginate (Moe et al., 1995).

In view of the commercial exploitation of alginate, the apparent viscosity and gel-forming capacities are the most important characteristics of this polymer. There in turn are affected largely by the block structure and chain length of the polymer (Sabra, 1998). Extension *O*-acetylation of alginate increases the water-binding capacity of the polysaccharide, which may be significant in enhancing survival under desiccating conditions. Additionally, the strength of alginate gels was found to be dependent on the number of cross-links formed between chains, on the type of cross-linking ions and on the length of blocks between the links (Steinbuchel et al., 2001).
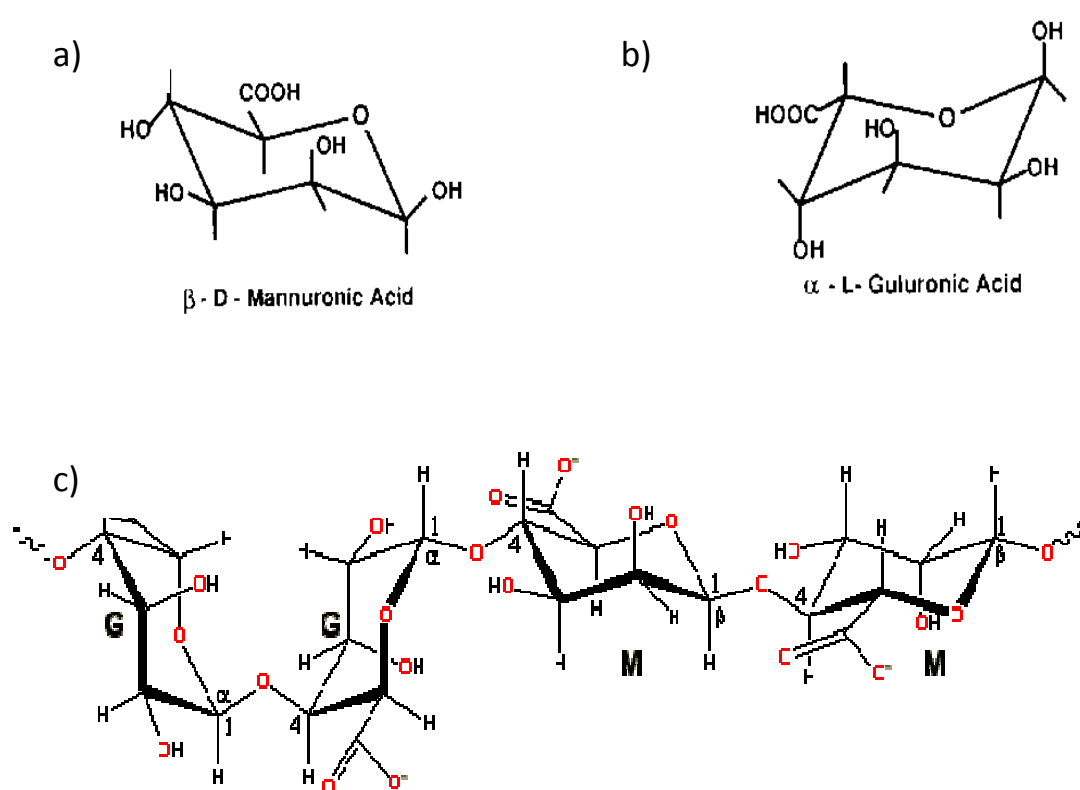
Figure 2.1 Structure of a) β-D-mannuronic acid or M-block, b) α-L-guluronic acid or

G-block and c) alginate (Avella et al., 2007).
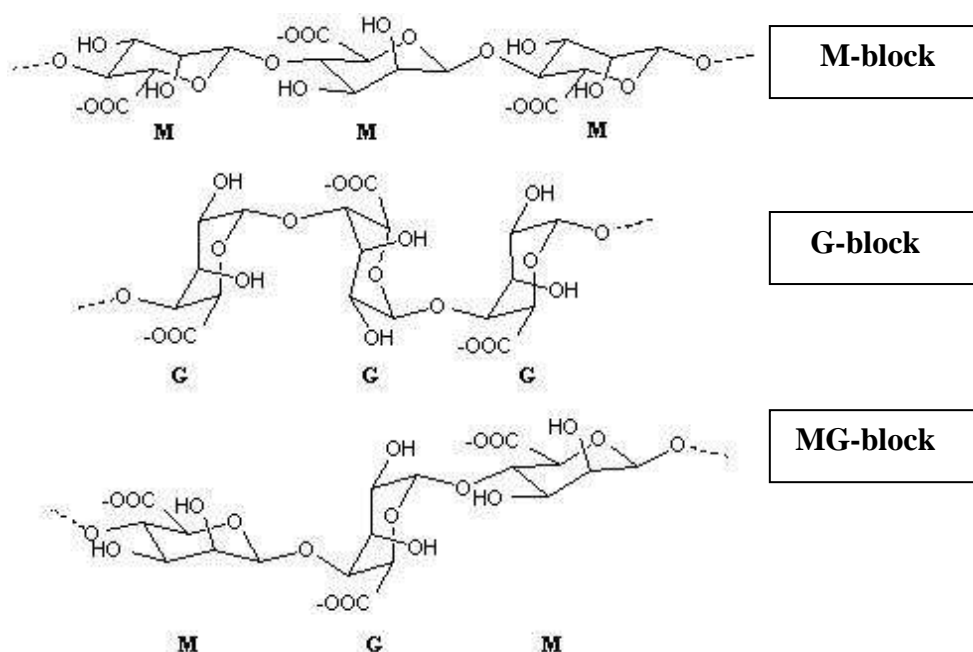
Figure 2.2 Block polymers structure of alginate (Avella et al., 2007).

2.1.2 Biosynthetic pathway of the alginate

The biosynthesis of alginate in the brown algae was first studied by Lin and Hassid (1966), a cell-free system from *Fucus gardnerii* was detected the enzyme activities necessary for the synthesis of mannuronan. A similar pathway has since been found in *A. vinelandii* and *P. aeruginosa*. In Fig 2.3 shows the principle enzymes involved in alginate biosynthesis and the activity of all enzymes (Pindar and Bucke, 1975).

The alginate biosynthesis starts from fructose-6-phosphate in the cytosol. six-carbon growth substrates are oxidized via the Entner-Doudoroff pathway, and that the resultant pyruvate [1 mol (mol of hexose)$^{-1}$] is ultimately channeled into alginate biosynthesis (Lynn and Sokatch, 1984). The pyruvate derived from the oxidation of hexoses is fed into alginate biosynthesis via the formation of oxaloacetate and subsequent gluconeogenesis. The initial steps in the alginate biosynthesis are related

to general carbohydrate metabolism, and the intermediate GDP-mannose serves not only as a precursor for alginate biosynthesis but also for lipopolysaccharide (LPS) biosynthesis (Goldberg et al., 1993). Accordingly, the GDP-mannose dehydrogenase exhibits a key role in the biosynthesis of alginate. However, the alginate biosynthesis enzyme phosphomannose isomerase/guanosine-di-phosphomannose pyrophospho-rylase (PMI-GMP) is a bifunctional protein catalyzing the initial and third steps of alginate synthesis. The PMI reaction pulls the fructose-6-phosphate out of the metabolic pool, leading to the first intermediate, mannose-6-phosphate. Phosphomannosemutase (PMM) then catalyzes the second step, resulting in the formation of mannose-1-phosphate. The GMP activity of PMI-GMP then, with concomitant GTP hydrolysis, converts mannose-1-phosphate to GDP-mannose. The enzyme favors the reverse reaction, but because of the efficient removal of the GDP-mannose in the next step, the entire pathway proceeds efficiently in the direction of alginate synthesis. The almost irreversible oxidation of GDP-mannose to GDP-mannuronic acid involves the enzyme guanosine-diphosphomannose dehydrogenase, and the reaction product is the immediate precursor for polymerization.
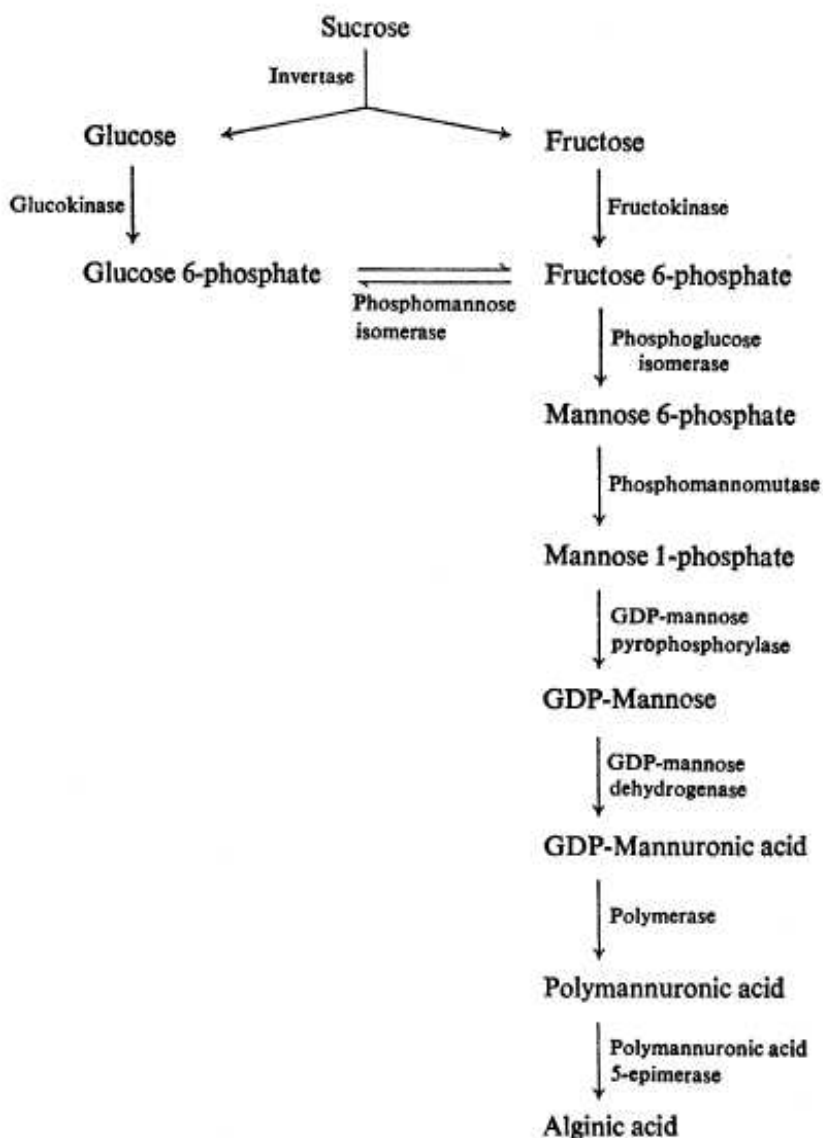
Figure 2.3 Biosynthesis pathway of alginic acid in *A. vinelandii* (Pindar and Bucke, 1975).

2.1.3 Physical properties

2.1.3.1 Solubility

There are three essential parameters determining and limiting the solubility of alginates in water. (i) The pH of the solvent is important because it will determine the presence of electrostatic charges on the uronic 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. (iii) In the latter case, the "hardness" of the water (i.e., the content of Ca^{2+} ions) is most likely to be the main problem (Steinbuchel et al., 2001).

Potentiometric titration (Haug, 1964) revealed that the dissociation constants for mannuronic acid monomers were 3.38 and 3.65, respectively. The pK_a value of the alginate polymer differs only slightly from those of the monomeric residues. An abrupt decrease in pH below the pK_a value causes a precipitation of alginic acid molecules, whereas a slow and controlled release of protons may result in the formation of an "alginic acid gel". Precipitation of alginic acid has been studied extensively (Haug, 1964; Haug and Larsen, 1963; Myklestad and Haug, 1966; Haug et al., 1967), and addition of acid to an alginate solution leads to a precipitation within a relatively narrow pH arrange. This range depends not only on the molecular weight of the alginate but also on the chemical composition and sequence. Alginate containing more of the "alternating" structure (MG-blocks) will precipitate at lower pH values compared with the alginates containing more homogeneous block structures (poly-M and poly-G). The presence of homopolymeric blocks seems to favor precipitation by the formation of crystalline regions stabilized by hydrogen bonds. By increasing the degree of alternating "disorder" in the alginate chain, as in alginates isolated from *Ascophyllum nodosum*, the formation of these crystalline regions is not formed as easily. A certain alginate fraction from *A. nodosum* is soluble at a pH as low as 1.4 (Myklestad and Haug, 1966). Because of this relatively limited solubility of alginates at low pH, the esterified propylene glycol alginate (PGA) is applied as a food stabilizer under acidic conditions (Steinbuchel et al., 2001).

Any change of ionic strength in an alginate solution generally will have a profound effect, especially on polymer chain extension and solution viscosity. At high ionic strengths, the solubility also will be affected. Alginate may be precipitated and fractionated to give a precipitate enriched with mannurinate residues by high concentrations of inorganic salts like potassium chloride (Haug and Smidsrod, 1967). Salting-out effects like this exhibit large hysteresis of the sense that less than 0.1M salt is necessary to slow down the kinetics of the dissolution process and limit the solubility (Haug, 1959). The gradient in the chemical potential of water between the bulk solvent and the solvent in the alginate particle, resulting from very high counter-ion concentration in the particle, is most probably the drive of the dissolution process of alginate in water. This drive becomes severely reduced when attempts are made to dissolve alginate in aqueous solvent already containing ions. If alginates are to be applied at high salt concentrations, the polymer should first be fully hydrated in pure water followed by addition of salt under shear.

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 3mM free calcium ions. Alginate can be solubilized at $[Ca^{2+}]$ above 3 mM by the addition of complexing agents, such as polyphosphates or citrate, before addition of the alginate powder.

2.1.3.2 Selective ion binding

The basis for the gelling properties of alginates is their specific ion-binding characteristics (Haug, 1964 and 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

markedly 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 (Fig 2.4).

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 gel has a markedly higher affinity toward Ca^{2+} ions than has the Na-alginate 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.
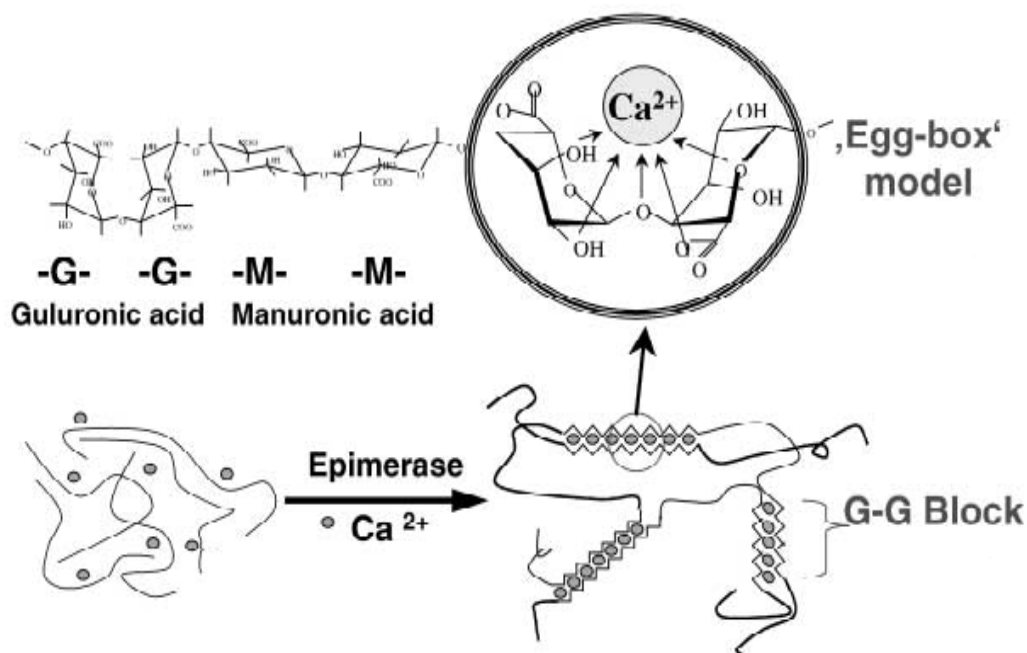
Figure 2.4   The calcium ion-dependent epimerization process and the formation of the so called "egg-box model" (Steinbuchel et al., 2001).

2.1.3.3   Gel formation and ionic cross-linking

A very rapid and irreversible binding reaction of multivalent cations is typical for alginate; a direct mixing of these two components therefore rarely produces homogeneous gel. The result of such mixing is likely to be a dispersion of gel lumps ("fish-eyes"). The only possible exception is the mixing of a low molecular weight alginate with low amounts of cross-linking ion at high shear. The ability to control the introduction of the cross-linking ions hence becomes essential.

A controlled introduction of cross-linking ions is made possible by the two fundamental methods for preparing an alginate gel; the diffusion method and the internal setting method. 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 (bio-) active agent (Smidsrod and Skjak-Braek, 1990; Funduennanu, 1999). High-speed setting is 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).

2.1.4 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.

2.1.4.1 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 cells to the production of monoclonal antibodies from hybridoma cells (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).

2.1.4.2 Textile industrial

The quantitatively most important technical application of alginates is as a shear-thinning viscosifyer 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 (Sutherland and Ellwood, 1979). 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).

2.1.4.3 Paper industrial

The main use 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 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. Alginates are also excellent film former and improve ink holdout and printability. The quantity of alginate used is usually 5-10 percent of the weight of starch in the size (Steinbuchel et al., 2001).

Alginate is also used in starch adhesives for making corrugated boards because it stabilizes the apparentof the adhesive and allows control of its rate of penetration. One percent sodium alginate based on the weight of starch used, is usually sufficient (Steinbuchel et al., 2001).

2.1.4.4 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).

2.1.4.5 Binders for fish feed

The worldwide growth in aquaculture has led to the use of crude alginate as a binder in salmon and other fish 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.

2.1.4.6 Release agents

The poor adhesion of films of alginate to many surface, 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 plastic. Sodium alginate also makes a good coating for anti-tack paper, which is used as a release agent in the manufacture of synthetic resin decorative boards. Films of calcium alginate, formed *in situ* on a paper have been used to separate decorative laminates after they have been formed in a hot-pressing system.

2.1.4.7 Medicine and Pharmacy

Alginates have been used for decade as helping agents in various human-healths 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 sodium alginate 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 (Smidsrød 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 Parkinsome'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). Tab 1 shows some biomedical applications of alginate-encapsulated cells.

Table 2.1   Some potential biomedical application of alginate-encapsulated cells

Cell type	Treatment	Reference
Adrenal coromaffin cells	Parkinson's disease	Aebischer et al., 1993
Hepatocytes	Liver failure	Aebischer et al., 1993
Paratyroid cells	Hypocalcemia	Aebischer et al., 1993
Langerhans islets (β-cells)	Diabetes	Soon-Shiong et al.,1993
Genetically altered cells	Cancer	Read et al., 2000

2.1.4.8 Foods

Alginate is used mainly in food industry, which currently consumes about 50% of alginate produced. It is used as food additives to improve, modify and stabilize the texture of foods. This is valid for such properties as apparentenhancement, gel-forming ability and stabilization of aqueous mixtures, dispersions and emulsions. Alginates can interact readily with positively charged amino acid residues of denatures proteins which are utilized in pet foods and reformed meat. Cottrell and Kovacs (1980); Sime (1990) and Lttlecott (1982) have given numerous descriptions and formulations on alginates in food applications. A general review on this topic is given by McHugh (1987).

Special focus perhaps should be placed on restructured food based on Ca-alginate gels because of its simplicity (gelling being independent upon temperature) and because it is a steadily growing alginate application. Restructuring of foods is based on binding together a flaked, sectioned, chunked or milled foodstuff to make it resemble the original. Many alginate-based restructured products are already on the market, as is exemplified by meat products (both for human consumption and as pet food), onion rings, pimento olive filling, crabsticks and cocktail berries.

For applications in jams, jellies, fruit filling, etc., the synergetic gelling between alginates high in guluronate and highly esterified pectin may be utilized (Toft et al., 1986). The alginate/pectin system can give thermoreversible gels in contrast to the purely ionically cross-linked alginate gels. This gel structure is almost independent of sugar content, in contrast to pectin gels and therefore may be used in low calorie products.

The only alginate derivative used in food is propylene glycol alginate (PGA). Steiner (1947) first prepared PGA, and Steiner and Mcneely (1950) improved the process. PGA is produced by a partial esterification of the carboxylic groups on the uronic acid residues by reaction with propylene oxide. The main product gives stable solutions under acidic conditions where the unmodified alginate would precipitate. It is now used to stabilized acid emulsions (such as in French dressings) acid fruit drinks and juices. PGA also is used to stabilize beer foam (Neidleman, 1991).

In the U.S, ammonium, calcium, potassium and sodium alginate are included in a list of stabilizers that are generally recognized as safe (GRAS). Propylene glycol alginate is approved as a food additive (used as an emulsifier, stabilizer or thickener)

and in several industrial applications (used as a coating for fresh citrus fruit as an inert pesticide adjuvant and as a component of paper and paperboard in contact with aqueous and fatty foods). In Europe, alginic acid and its salts and propylene glycol are all listed as EC (Emergency contraception) approved additives other than colors and sweeteners.

2.2 **Bacterial alginate formation**

2.2.1 *Azotobacter* **sp. on alginate production**

Azotobacter sp. is a gram-negative bacterium, which grows in aerobic environments and fixes atmospheric nitrogen (Fig 2.5). *Azotobacter* plays a remarkable role, being broadly dispersed in different environments, such as soil, water and sediments. In addition it is bacterium with a broad metabolic diversity, This feature enables it to degrade numerous highly resistant substrates (Chan 1986; Wu et al., 1987; Moreno et al., 1990), to increase plant yield (Jackson et al., 1964; Rovira, 1965; Denarie and Blanchere, 1966) due to the increase of fixed nitrogen content in soil (Gouri and Jagasnnatathan, 1995; Maltseva et al., 1995), to the Spencer, 1966; Vermani et al., 1996). Alginate formation was partially growth-associated with a marked increased in the alginate biosynthesis after the end of growth (Parente et al., 2000; Deavin et al., (1977).
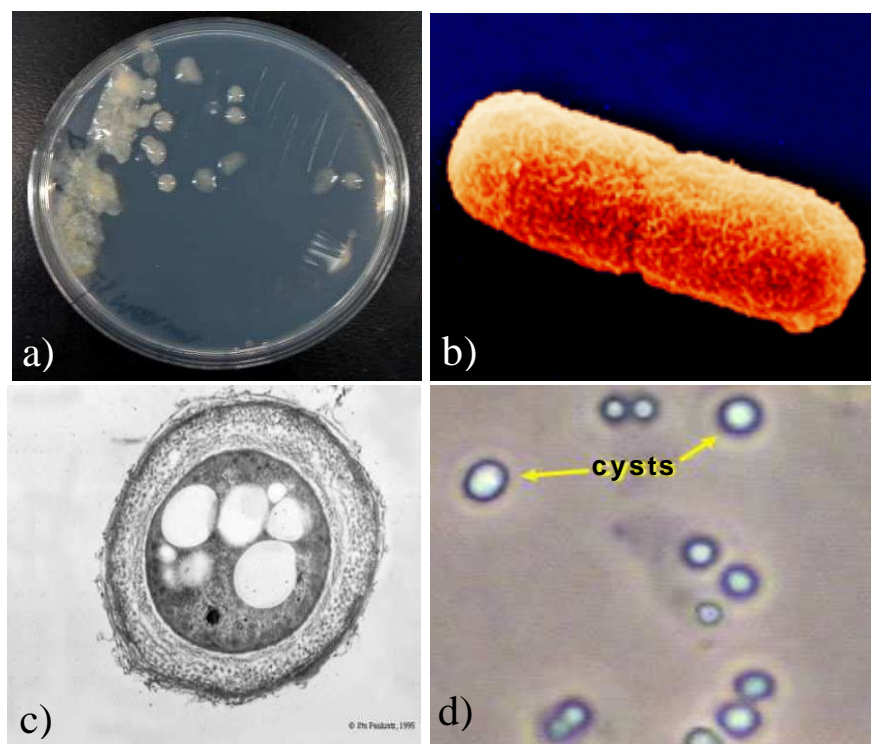
Figure 2.5 *Azotobacter* cell; a) colonies of *Azotobacter* sp. (www, 2000); b) vegetative cell of *Azotobacter* sp.; c) and d) *Azotobacter* cyst (www, 2005)

2.2.2 Life cycle of *Azotobacter* sp.

The life cycle is diagrammatically represented in Fig 2.6. Unlike the endospore of a *Bacillus* species, the cyst is formed by the rounding up the entire cell. *Azotobacter* cysts are not resistant to extremes of temperature but are resistant to desiccation and to some deleterious chemical and physical agent (Socolowsky and Wyss, 1962). As is usually the case with sporulation or encystment, the developmental events are set in motion by a nutritional shift-down; encystment in *A. vinelandii* (the organism used for most of the developmental studies). This encystment is considerably accelerated by the addition of β-hydroxybutyric acid (PHB; Sadoff et al., 1971), which serves as a precursor of the electron-transparent

poly-β-hydroxybutyrate granules that are characteristic of the cyst. The cells remove their flagella, stop nitrogen fixation, gradually become rounded and finally become optically refractile. The cyst is surrounded by thickened, multilayered outer coat called "the exine" consisting of lipoprotein and lipopolysaccharide. A number of unique lipids have been shown to be synthesized and form part of the cyst structure (Reusch et al., 1981). Germination occurs when the cysts are placed in the presence of an exogenous carbon source such as glucose. This immediately induces respiration, macromolecular synthesis, and the conversion of the cyst to the vegetative cell.

The cellular differentiation cycle leading to encystment and germination in this strain is presented in Fig 2.6. *Azotobacter* vegetative cells undergoing division have a typical "peanut" shape. Some strains are motile by means of peritrichous flagella. Upon induction of encystment, the cells lose motility become spherical, their walls become thickened over a period of hours, and the developing cysts become optically refractile. The cyst consists of a central body that is encased in an inner coat-the intine and an outer coat-the exine-separated from the intine by the intine space.
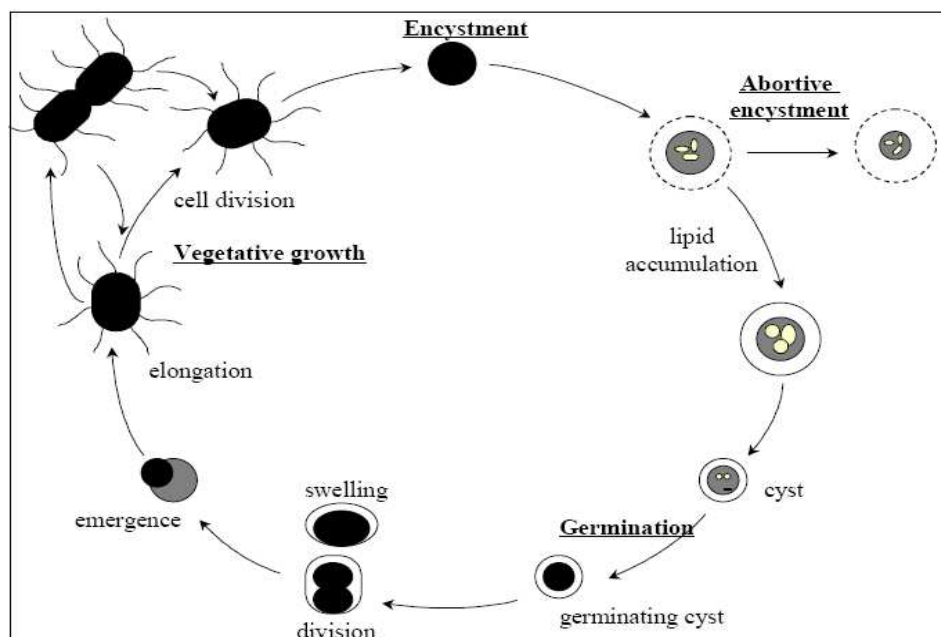
Figure 2.6 Schematic diagram of the cycle of *A. vinelandii* taken from Fyfe and

Govan (1983).

Cyst germination is a slow process during which the central body swells and occupies the intine volume, the growth of the cyst within the exine causes a ring type fracture of the outer cyst coat, and then the dividing cell emerges leaving the non utilized exine components as an empty cup like structure (Sadoff, 1975).

Azotobacter cells grow well in media in which sucrose is the carbon source but the cells from few cysts. Cyst formation can be induced on nitrogen free agar medium that is supplemented with 0.2% butyl alcohol as the sole carbon source. It was also reported that substrates, which enhance PHB production in cells, promotes the encystment of *Azotobacter*. β-Hydroxybutyrate, the monomer of PHB, a polymer known to accumulate prior to encystment, was found to induce the encystment in *Azotobacter* (Lin and Sadoff, 1968).

2.3 Effects of conditions culture on alginate production by

Azotobacter sp.

The rate of growth of microorganisms is influenced by both internal and external or environment factors. The internal factors may be due to the genetic constitution of the microorganism, the age of the inoculums (Clementi et al., 1999). In several instances, slow or fast growing strains or races of microbial cultures are obtained by exposure to several physiology or chemical mutagenic agents. The concentrations of media constituents were varied to get maximum yield of alginate as following;

2.3.1 Carbon sources

Azotobacter sp. grows in nitrogen-free medium and with limited phosphate (Horan et al., 1981; Sabra, 1998) which contains fermentable carbohydrate to supply energy and to synthesis different compounds (Gonzalez-Lopez et al., 1991; Revillas et al., 2000). Sources of carbon included monosaccharide, disaccharide and trisaccharied.

Most strains of the different *Azotobacter* sp. use the following substances as sole carbon and energy source; fructose, glucose, sucrose, ethanol, acetyl methyl carbinol, acetate, fumarate, pyruvate, α-oxoglutarate, gluconate, succinate. The ability of other organic substances to support the growth of different *Azotobacter* species has been exhaustively investigated by Thompson and Skerman (1979). For alginate production, the media used usually contains glucose (Belder, 1993), sucrose as carbon source (Kang et al., 1993).

Emtiazi et al., (2004) showed that *Azotobacter* AC2 produced maximum alginate (7.5 mg/mL) in media with sucrose as the only carbon source while *Azotobacter chroococum* 1723 was able to produced exopolysaccharide greater than 5 mg/mL when used lactose as carbon source.

Saude and Junter (2002) revealed that polysaccharide was produced in after organism had entered the stationary phase. This confirms other results showing that alginate production by *A. vinelandii* is partially associated to growth (Horan et al., 1981; Chen et al., 1985; Brivonese and Sutherland, 1989; Pena et al., 1997; Annison, 1986). The amount of polysaccharide recovered ranged between 0.8 and 1.4 g/L, with a mean of 1.0 ± 0.2 g/L corresponding to a production yield of 89 ± 6 mg of alginate/g of sucrose. These values are low compared to those reported for diverse *A. vinelandii* strains, in particular highly mucoid natural isolates (Brivonese and Sutherland, 1989) or mutants (Horan et al., 1981; Chen et al., 1985). The weight-average molecular weight of the polysaccharide recovered after incubation for 140 h was equal to 168 ± 58 kDa. This molecular weight value is close to the molecular weight value obtained from Clementi (1999). But much lower than those given by Pena et al., (1997) ranging from 1400 to 2000 kDa after sucrose fermentation for 72 h in shakes flask experiment.

2.3.2 Initial pH

Hydrogen ion concentration has a significant influence on industrial fermentation due to much its importance in controlling bacterial growth, fermentation rates and product formation. The variation in growth rate related to the pH presents an optimum value and extreme limits. *Azotobacter* species are known to growth over a wide range of pHs. The initial pH of 7.0 was found to be optimum for both cell

growth and polysaccharide production by commercial polysaccharide-producing bacteria (Prasertsan et al., 2008) and decreased when pH dropped to 5.8 (Vermani et al., 1997). It was reported that pH had more influence on polysaccharide production than on cell growth (Pace, 1981) and the specific pH affected directly the synthesis of those enzymes responsible for polysaccharide production (Lawson and Sutherland, 1978) and the molecular weight of the polysaccharide produced (Jeanes, 1977). Embuscado et al., (1994) reported that when bacterial cells are exposed to pH beyond their optimum range, maintenance energy is used for pH control. This reduces the energy available for biopolymer production, thus the bacterial ability to produce the biopolymer is reduced. The media pH also affects the permeability of the bacterial cell membrane thus affecting the biochemical activities of the cell required for biopolymer production (Embuscado et al., 1994). This was within the optimum pH range (6.0-7.5) for synthesis of polysaccharides (Lawson and Sutherland, 1978). Beside of substance uptake is dependent on the external pH and adequate control of pH value is essential both in batch and in continuous culture for alginate synthesis (Sutherland et al., 1979).

2.3.3 Temperature

Incubation temperature is often a critical factor in polysaccharide biosynthesis. All commercial polysaccharide-producing microorganisms are mesophiles (Lawson and Sutherland, 1978). The optimum temperature for polysaccharide production depends on the type of microorganism (Thompson and Skerman, 1979).

The optimum temperature for growth and exopolysaccharide synthesis has been found to be 30°C. This is usually used for exopolysaccharide production from *A. vinelandii* (Vermani et al., 1997) while Clementi et al., (1995) showed that the highest

alginate production was obtained when incubate *A. vinelandii* at temperature of 35°C and on shaking speeds of 250-300 rpm. Such values of temperature and shaking speeds were almost equal to these regarded as optimal for other mutant strains of *A. vinelandii* by Chen et al., (1985); Brivonese and Sutherland (1989). Alginate-like polymers from *A. vinelandii* DSM 576 in aqueous dispersion exhibited the typical pseudoplastic behavior that was described by the well known Ostwald-de Waele model when the polysaccharide concentration, shear rates and temperature respectively ranged from 0.3-1.5% w/v, 1.1-1400 s^{-1} and from 278.16-308.16 K (Clementi et al., 1999).

2.3.4 Nitrogen sources

The *Azotobacters* in general is nitrogen fixers. Racine et al., (1991) showed that biopolymer production was enhanced when nitrogen supply was limited and the carbon source was in excess. From the resulted, concluded biopolymer production decreased as the nitrogen concentration increased. Embuscado et al., (1994) suggested that in different organic nitrogen sources *A. chroococum* 1723 produces biopolymer that differ in rheological quality. Similar observations were made by Kennedy et al., (1992). Emtiazi et al., (2004) showed that addition of vitamin, different nitrogen sources (Ammonium salts, yeast extract and peptone) did not effect exopolymer production in *Azotobacter* spp. In continuous cultures, nitrogen limitation was preferable for exopolysaccharide production in *A. vinelandii* (Jarman et al., 1979). Nitrogen fixation and respiration should conflict with PHB production since all three reactions are sinks for reducing power. Thus nitrogen-fixing cells have a very low PHB content and the additions of organic nitrogen spares the need for nitrogen fixation and the respiratory protection of the oxygen labile nitrogenase complex and

thus allow the reducing power and Acetyl-CoA derived from active sugar metabolism to be used for PHB production (Page et al., 1997). Similar result was obtained by Pal et al., (1998) using an *A. chroococum* strain.

2.3.5 Agitation

Since the intensity of agitation influences the transport of nutrients into cells, increased agitation may increase microbial productivity, due to better mixing and the elimination of the so-called "dead zone." Sabra (1999) observed as both alginate and biomass concentrations increased with increasing the agitation speed till 600 rpm but beyond this value both alginate and biomasses decreased sharply till 1,000 rpm. By excess turbulence, it is most likely that the decreased biomass and alginate production may be due to damaging cell membranes and limited mass transfer in localized zones (Toma et al., 1991). The growth of *A. vindlanii* under diazotrophic conditions was always accompanied by the presence of alginate capsule around the cell. However, this layer was easily removed upon shaking and hence no mass transfer resistance in sample take from the agitated fermenter occurred (Peters et al., 1989; Lobas et al., 1992). Additionally, at higher agitation intensity, the alginate capsular material was rich in gulronic acid forming harder gel resistant to dissolution. Moreover, this capsule layer was not decreased in thickness with the increase in agitation speed (Sabra, 1999). Increased agitation or shaking speed was frequently used by many authors for optimizing the aeration rate for alginate production by *A. vindlanii* (Jarman et al., 1978; Jarman, 1979; Annison and Couperwhite, 1984; Brivonese and Sutherland, 1989; Clementi et al., 1995; Pena et al., 1997; Parente et al., 1998).

2.3.6 Oxygen consumption

Azotobacter sp. is a nitrogen-fixing bacterium. The biological fixation of dinitrogen depends on the activity of the highly oxygen-sensitive nitrogenase enzyme complex (Linkerhagner and Oelze., 1997). For the survival of these bacteria under aerated conditions, one of the priorities of their entire metabolism is to protect the active nitrogenase from being damaged by oxygen. Protection of this enzyme from oxygen has been proposed to occur in *Azotobacters* mainly through two mechanisms: (i) high respiratory activity that removes oxygen already at the cell surface and (ii) reversible conversion of the enzyme into a protected inactivated state (Linkerhagner and Oelze, 1995; Liu et al., 1995). The first mechanism is believed to explain the function of nitrogenase when cell growth diazotrophically in the presence of O_2. The second mechanism is considered to be used to protect the reversibly inactivated enzyme from O_2 damage when the respiratory protection becomes overburdened such as with a sudden increase in the ambient O_2 concentration (Kuhla and Oelze, 1988; Linkerhagner and Oelze, 1997) or under conditions of phosphate concentration limitation (Tsai et al., 1979 and Horan et al., 1981) and nitrogen-limited (Chen et al., 1985) or rich media (Brivonese and Sutherland, 1989). However, very little information on the kinetics of fermentation at controlled pH and on the effect of dissolved oxygen concentration (DO) on alginate yields is available (Horan et al., 1981 and Pena et al., 1997).

With few exceptions, there are general agreements among the investigations that the oxygen supply and stirrer speed are of critical important for alginate production by *Azotobacter* sp.

Parente et al., (1998) demonstrated that the effect of dissolved oxygen concentration in batch fermentation by *A vinelandii* DSM576 under nitrogen and phosphate-rich medium. The results showed that growth was faster at higher dissolved oxygen concentration (DO), but maximum biomass concentration was lower. At 10% DO, alginate was not produced. Alginate production was faster at 5% and 2% DO but higher alginate concentrations and yields were obtained without DO control. These results was consistent with those obtained by Pena and Galindo (1996) who found that specific growth rate was significantly reduced at DO 0.5% (0.06 h^{-1}) compared to DO 5% (0.22 h^{-1}). Beside of Horan et al., (1981) found that the growth of *A. vinelandii* DSM576 in batch fermentation showed a similar result: in fact, growth rate increased and biomass yield significantly decreased at DO $\geq$ 5%. From the previous studies, concluded that alginate production was strongly influenced by DO. Normally maximum alginate concentration was obtained in fermentation without DO control but studies on alginate production by *A. vinelandii* at controlled pH and/ or DO are rare.

In aerobic submerged exocellular microbial polysaccharide fermentation such as xanthan, dextran and alginate, oxygen supply to the media and mass transfer of oxygen to growing microbial cells is still a major technical problem affecting microbial productivity, since the solution becomes highly viscous and non-Newtonian during fermentation owing to the present of polysaccharide in the culture medium. The relationship between introduced mixing energy and the oxygen transfer rate in solutions with Pseudoplastic behavior is still difficult to establish (Dussap and Gros, 1982). In case of alginate production by *A. vinelandii*, the low oxygen solubility in fermention media coupled with high oxygen consumption rate make the task of

oxygen supply (aeration) difficult. On the other hand, increasing the mechanical stirring to enhance the aeration, may cause strong turbulence in local zone where damaging of the sensitive cell membrane occurs (Toma et al., 1991).

2.3.7 Organic acid

An organic acid is an organic compound with acidic properties. The most common organic acids are the carboxylic acids whose acidity is associated with their carboxyl group (-COOH). Organic acids were used supplement in medium.

Chan (1986) and Vargas-Garcia et al., (2001) demonstrated that in media supplemented with 4-hydroxybenzoic acid promoted growth cell of *A. vinelandii* and gave the highest. However, there are no references about EPS produced by *A. vinelandii* when media are supplemented with this acid as the sole carbon source.

2.4 Immobilization technology

2.4.1 Enzyme immobilization

For intracellular enzyme systems, there is obviously a need to choose between using the extracted enzyme or the cell-bound enzyme. In general, the latter is favored for systems requiring regeneration of a cofactor or where a sequence of several enzymes (a multi-step reaction) is operating. In these cases it is often easier to use the whole cell. The cell would also be favored for enzymes that are difficult to stabilize when they are released from the cell. In every other case the use of a cell-free enzyme would be favored. The specific activity of immobilized enzymes are usually higher

than for whole cell systems and the possibility of unwanted side-reactions is minimized (it can be avoided altogether if a purified enzyme is used).

The extent to which an enzyme is purified before immobilization will depend on the specific activity required and this, in turn, will depend upon the activity required in the reactor. It will also be related to the stability required (i.e. number of reuses) if the stability of an enzyme is reduced on purification a compromise may have to be reached where some activity improvement is traded off for a better operational stability. For large-scale industrial processes, it is important to minimize the number of purification steps required in order to improve biocatalyst yield and minimize overall costs. Thus, it is obviously advantageous to increase the productivity of the enzyme source to maximize the specific activity of the enzyme released from the cells and minimize the extent of further purification. There are many reviews that have dealt with enzyme immobilization (Goldstein, 1976; Chibata, 1978; Zaborsky, 1973; Broun, 1976; Bryjak, 2003). A very useful step-by-step guide to the subject is that of Rosevear et al., (1987). A highly readable introduction to enzyme immobilization is given by Trevan (1980).

2.4.2 β-amylase

Saccharide is widely applied in chemical process and food industries, and the production of oligo by starch hydrolysis has become the main stream of saccharide engineering in recent years. Its main industrial process is to form glucose by starch hydrolysis (Nigam and Singh, 1995; Wang et al., 1996). Acid splitting and thermal decomposition of starch are used in the production of traditional glucose syrup. Their products are rather complicated, requiring high purification cost, and are not suitable for industrial mass production. The price of using starch-hydrolyzed enzymes in

decomposing starch is lower and its procedure is much simple, which has become the main method of starch hydrolysis.

β-amylase is an exoenzyme that releases successive maltose units from the nonreducing end of a polysaccharide chain by hydrolysis of α-1,4-glucan linkages. The shortest normal saccharide attacked is maltotetraose (Myrback and Neumuller, 1950). Since it is unable to bypass branch linkages in branched polysaccharides such as glycogen or amylopectin, the hydrolysis is incomplete and a macromolecular limit dextrin remains. It can found primarily in the seeds of higher plants and sweet potatoes. It yields a single product: maltose. The enzyme is useful in structural studies of starch and glycogen.

2.4.3 Choices of support and principle method

In solution, soluble enzyme molecules behave as any other solute in that they are readily dispersed in the solution and have complete freedom of movement (Bickerstaff, 1995). There are five principal methods for immobilization of enzyme; covalent binding, adsorption, entrapment, cross-linking and encapsulation (Fig 2.7a-e). The relative merits of each are discussed briefly below;

2.4.3.1 Covalent binding

This method of immobilization (Figure 2.7a) involves the formation of a covalent bond between the enzyme/ cell and a support material (Woodward, 1985; Porath and Axen, 1976; Cabral and Kenendy, 1991). The bond is normally formed between functional groups present on the surface of the support and functional groups belonging to amino acid residues on the surface of the enzyme. A number of amino

acid functional groups are suitable for participation in covalent bond formation. Those that are most often involved are the amino group (NH_2) of lysine.

Many varied support materials are available for covalent binding, and the extensive range of supports available reflects the fact that no ideal support exists. Therefore, the advantages and disadvantages of a support must be taken into account when considering possible procedures for a given enzyme immobilization (White and Kennedy, 1980; Zaborsky, 1973). Many factors may influence the selection of a particular support and researcher work has shown that hydrophilicity is the most important factor for maintaining enzyme activity in a support environment (Gemeiner, 1992). Consequently, polysaccharide polymers, which are very hydrophilic, are popular support material for enzyme immobilization. For example, cellulose, dextran, starch and agarose are used for enzyme immobilization. The sugar residue in these polymers contains hydroxyl groups, which are ideal functional groups for chemical activation to provide covalent bond formation. In addition, hydroxyl groups form hydrogen bonds with water.

2.4.3.2 Adsorption

Immobilization by adsorption (Figure 2.7b) is the simplest method and involves reversible surface interface interactions between enzyme/cell and support material (Messing, 1976 and Woodward, 1985). The forces involved are mostly electrostatic, such as van der Waals forces, ionic and hydrogen bonding interactions, although hydrophobic bonding can be significant. These forces are very weak, but sufficiently large in number to enable reasonable binding. Existing surface chemistry between the enzyme and support is utilized so on chemical activation/ modification is required and

little damage is normally done to enzymes in this method for immobilization. The procedure consists of mixing together the biological component (s) and a support with adsorption properties, under suitable conditions of pH, ionic strength, and so on, for a period of incubation, followed by collection of the immobilized material and extensive washing to remove non-bound biological components.

2.4.3.3 Entrapment

Immobilization by entrapment (Figure 2.7c) differs from adsorption and covalent binding in that enzyme molecules are free in solution, but restricted in movement by the lattice structure of a gel (Bickerstaff, 1995; O' Driscoll, 1976). The porosity of the gel lattice is controlled to ensure that the structure is tight enough to prevent leakage of enzyme or cells, yet at the same time allow free movement of substrate and product. Inevitably, the support will act as a barrier to mass transfer, and although this can have serious implications for reaction kinetics, it can have useful advantages since harmful cells, proteins and enzymes are prevented from interaction with the immobilized biocatalyst (Brodelius, 1985).

Entrapment of enzyme in calcium alginate is the most widely used immobilization technique in the biocatalyst production of chemicals (Smidsrod and Skjak-Braek, 1990) and has been implemented in biocatalystic processed utilizing whole cells (Maritz et al., 2003), as well as free enzyme (Tanriseven and Dogan, 2002). In this study, entrapment method was selected in β-amylase immobilization with alginate because of cereal β-amylases are important in brewing. As a major contributor to the "diastatic power" (i.e. the combined alpha-amylase, beta-amylase, debranching enzyme and α-glucosidase activity) of malt (i.e., artificially-germinated

cereal seeds), their activity is essential for the generation of maltose and other easily fermentable sugars from cereal grain starch in the mashing process to fuel the production of alcohol by yeast. The saccharifying activity of cereal seed β-amylase is also exploited in bread making and in the use of malt as an additive in other foodstuffs and even as a "digestive" (Doehlert et al., 1982). Cereal β-amylase also finds application in the production of maltose and maltose-rich syrups, sweeteners and vaccines (Nehete et al., 1992). Cereal β-amylase are more acid-stable, less heat-stable than α-amylase and high cost. In immobilization, are extend to reusability of enzyme, easy control reaction solution.

2.4.3.4 Cross-linking

Cross-linking of enzymes with bifuctional reagents shows Figure 2.7d. Among the most popular cross-linkers are glutaraldehyde, dimethyl adipimidate, di-methyl suberimidate and aliphatic diamines. The first three directly cross-link enzymes through their amino groups. Diamines (for instance, hexamethylene diamine) cross-link enzymes through carboxyl groups following activation of these groups with carbodiimides. Cross-linking may be both inter-molecular (forming water-insoluble aggregates) and intramolecular. In the former case, enzyme molecules can be cross-linked either with themselves or with other proteins present in solution (Bickerstaff, 1995).

2.4.3.5 Encapsulation

Encapsulation of enzymes shows in Figure 2.7e. In this approach, pioneered by Chang (1972), enzymes are enveloped with in various forms of membranes that are impermeable for enzymes and other macromolecules but permeable for low molecular weight substrates and products. Typical examples include entrapment of

enzymes in microcapsules (produced by interfacial polymerization, liquid drying, or phase separation), in liposomes and in hollow fibers.
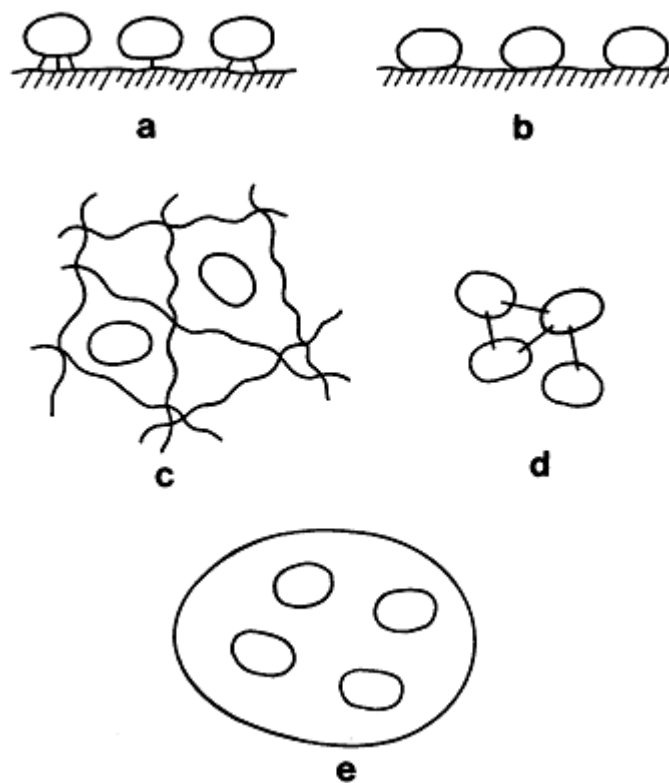
Figure 2.7 Methods of enzyme immobilization: (a) covalent attachment to solid support, (b) adsorption on solid supports, (c) entrapment in polymeric gel, (d) intermolecular cross-linking, and (e) encapsulation (Bickerstaff, 1995).

CHAPER III

MATERIALS AND METHODS

3.1 Bacterial cultivation

Azotobacter sp. was cultured in LG media contained per liter of distilled water which containing of 10 g of sucrose, 0.15 g of KH_2PO_4, 0.2 g of yeast extract, 0.05 g of K_2HPO_4, 0.02 g of $MgSO_4 \bullet 7H_2O$, 0.002 g of $CaCl_2 \bullet 2H_2O$, 0.002 g of $FeCl_3 \bullet 6H_2O$ and 0.002 g of $NaMoO_4 \bullet 2H_2O$ and adjusted the pH to 6.8 with 1M NaOH. The bacterial culture was cultivated on a rotary shaker at 200 rpm and 30^oC for 72 h.

3.2 Analytical methods

3.2.1 Apparent viscosity measurement

The apparent viscosity of culture broth was analyzed with viscometer (Well-Brookfield LVT, series 82198) and rheometer (series AR-G2, TA instrument, America). All of the determinations were carried out at room temperature (25^oC), shear rate of 12 s^{-1} and speed of 12.90 rpm using cone C-21 (Pena et al., 1997).

Shear stress (τ) was calculated as follow:

Shear stress (τ) = Apparent viscosity (μ) x Shear rate (γ)　　　(1)

where:　　Shear stress　　　=　　the shear force per unit area of plate, $cP \cdot s^{-1}$.

Shear rate　　　=　　a gradient of velocity in a flowing material, s^{-1}.

Apparent viscosity　=　the resistance of a fluid which is being deformed by either shear stress, cP.

3.2.2 Growth measurement

The bacterial cell growth was measured by viable plate count method on LG medium that incubated at $30^{o}C$ for 48 h.

Specific growth rate (μ, h^{-1}) was calculated as follow:

$$\ln x = \ln x_0 + \mu t$$

where:　　x　=　the viable cell concentration at end of time

x_0　=　the viable cell concentration at time zero

t　=　time

μ　=　specific growth rate (t^{-1})　　　(2)

According to Eq. (2), a plot of ln x versus time gives a straight line with slope μ. Because the relationship of Eq. (2) is strictly valid only if μ is unchanging, a plot of ln x versus time is often used to assess whether the specific growth rate is constant.

3.2.3 Total sugar content analysis

The total sugar content in medium was quantified by High Performance Liquid Chromatographic (HPLC). The Thermo Separation Product (tsp) HPLC system composed of autosampler AS 3000 and RI-1530 detector (Jasco, Japan) was used. The Phenomenex® Rozex RPM-Monosaccharide column (300 x 7.8 mm) was operated at $75^{o}C$. The deionized water was used as mobile phase at flow rate of 0.6 mL/min

Sugar utilization rate, (g/L/h) was calculated as follow:

$$S = S_0 \text{ x } e^{\text{Sugar utilization rate x t}}$$

where: S   =   sugar concentration at time (g/L)

S_0 =   initial sugar concentration (g/L)

t   =   time (h)

3.2.4 Organic acid analysis

Determination of some organic acid was analyzed by High Performance Liquid Chromatographic (HPLC). The tsp HPLC system composed of autosampler AS 3000 and UV 6000 LP detector was set at 210 nm. The Phenomenex® Rozex ROA organic acid column (300 x 7.8 mm) was operated at $55^{o}C$. The 0.005 N of H_2SO_4 was used as mobile phase at flow rate of 0.5 mL/min.

3.2.5 The alginate concentration measurement

Alginate concentration was determined as followed. A 10 mL sample of culture broth was mixed with 1 mL of EDTA (0.1 M) and 1 mL of NaCl (1.0 M) and then centrifuged at 12,000 rpm for 20 min. The supernatant was added to 30 mL of isopropanol and the mixture was shaken vigorously. After 10 min, the resultant precipitate was filtered through a Whatman filter paper (Qualitative No. 1), dried at $70^{o}C$ for 24 h and weighted (Pena et $al.$, 1997).

Yield of alginate (Yp/s, g/g) was calculated as follow:

$$Yp/s = P / S$$

where: P   =   product was produced, (g)

S   =   substrate was utilized, (g)

3.2.6 The molecular weight determination

The alginate molecular weight distribution was estimated by gel permeation chromatography (GPC) with an Ultrahydrogel 500 column (Water, America), using a HPLC system. The tsp HPLC system composed of autosampler AS 3000 and RI - 1530 detector (Jasco, Japan) was used. The Water® Ultrahydrogel 500 column (300 x 7.8 mm) was operated at $40^{o}C$. The 0.1 M $NaNO_3$ was used as mobile phase at flow rate of 0.9 mL/min. Calibration method using pullulans of *Aureobasidium pullulans* as molecular mass standards (Shodex, Japan; Pena et al., 1997).

3.3 Optimization of alginate production conditions in shake flask

3.3.1 Source and concentration of carbon

The LG media was used for determined the optimum source and concentration of carbon was described in section 3.1 (except section 3.2.5), the additions of different of source and concentration were different. Eleven sources of carbon; glucose, arabinose, maltose, rhamnose, lactose, mannose, fructose, trehalose, xylose, sorbital and sucrose were replaced carbon source in LG medium and the concentrations of each carbon source were varied from 0 to 40% (w/v), differing by 5% (w/v) from one flask to the others. All of determinations were analyzed follow as section 3.2 (except section 3.2.6). The optimum source and concentration of carbon were selected for further study.

3.3.2 Temperature

The LG medium was used to determine for optimum alginate production that incubated at different temperature between 20 to 37°C according to Clementi et al., (2002). All of determinations were analyzed follow as section 3.2 (except section 3.2.6). The optimum temperature was selected for further study.

3.3.3 Initial pH

The LG medium and analytical methodologies were followed in section 3.1 and 3.2 (except section 3.2.6), respectively. The pH was adjusted in the range between of 5 to 9 with 1N HCl or 1N NaOH. The optimum initial pH was selected for further study.

3.3.4 Nitrogen source

The LG medium was used for determining the optimum nitrogen source. Five

sources of nitrogen; ammonium chloride (NH$_4$Cl), di-ammonium phosphate (DAP) and ammonium dihydrogen phosphate (NH$_4$H$_2$PO$_4$) was substituted as nitrogen in LG medium and various the concentration of each nitrogen source in LG medium was tested from 0 to 20 g/L, differing by 5 g/L from one flask to the others. All of determinations were analyzed follow as section 3.2 (except section 3.2.6). The optimum nitrogen source was selected for further study.

3.4   Optimization of alginate production conditions in batch fermentation

3.4.1 Oxygen concentration

The optimum air flow rate for increasing alginate productivity was determined in batch culture. Air flow rate was varied at 0 to 5 vvm (volume of air/volume of liquid/min) in 2L fermenter (micro DCU-300, B. Braun Biotech international, Germany) with working volume 1.5L and used optimum conditions (carbon source, temperature, pH and nitrogen source) were gave the highest alginate production from shake experiment. During the whole fermentation process, samples were kept every 4 h within 24 h and kept every 6 h after 24 h. The analytical methodology was followed as section 3.2 (except section 3.2.6). The optimum nitrogen source was selected for further study.

3.4.2 Agitation

For determine optimum speed stirrer for increasing alginate productivity at the initial operation of batch culture. Speed stirrer was varied at 100 to 600 rpm in 2L fermenter with working volume 1.5L. The suitable carbon source, temperature, pH

and nitrogen source were used for alginate production in future study. The analytical methodology was followed as section 3.2 (except section 3.2.6).

3.5 Increasing properties of alginate production

3.5.1 Addition organic acids in shake flask

The organic acids were added for increasing properties of alginate production or cell mass such as alginate productivity, apparent viscosity and bacterial cell growth. All of the optimum conditions obtained from shake flask were used in this experiment. Ten of organic acids; succinic acid, fumaric acid, propionic acid, phytic acid, malic acid, adipic acid, 4-aminobenzoic acid, lactic acid, tartaric acid and 4-hydroxybenzoic acid at 0.1% w/v were added into LG medium. The organic acid that gave the highest of alginate productivity, bacterial cell growth and apparent viscosity were selected for determining the optimum organic acid concentration. The concentration of the selected organic acids was 0.05, 0.1, 0.15 and 0.2% (w/v). The analytical methodology was followed as section 3.2 (except section 3.2.6). The optimum of organic acid was used again for increasing efficiency alginate productivity, bacterial cell growth and apparent viscosity in 2L fermenter.

3.5.2 The organic acid on alginate production in 2L fermenter

All of the optimum conditions obtained from shake flask (source and concentration of carbon, temperature, initial pH and nitrogen source) and 2L fermenter (stirrer speed and air flow rate) were used for increasing quality of alginate in 2L fermenter with working volume 1.5L fermenter. During the whole fermentation

process, sample were kept every 4 h within 24 h and kept every 6 h after 24 h. The

kept samples were analyzed follow as section 3.2.

3.6 Alginate production in 5L fermenter

The suitable carbon source, temperature, initial pH, nitrogen source, stirrer

speed, oxygen concentration and organic acid were used for alginate production in

fermenter, the pH maintained by addition of 0.1 N H_2SO_4 or 1 N NaOH. Batch

experiments were performed in a 5L fermenter (micro DCU-300, B. Braun Biotech

international, Germany) with a working volume of 3L for 72 h. During the whole

fermentation process, sample were kept every 4 h within 24 h and kept every 6 h after

24 h. The kept samples were analyzed follow as section 3.2, except section 3.2.6.

3.7 Application of alginate for β-amylase immobilization

3.7.1 Enzyme immobilization

Entrapment of the enzyme in alginate was carried out by extrude through a

Pasteur pipette (1mm diameter) a mixture of alginate (2.5 % w/v for alginate-

produced by *Azotobacter* sp. and 2% w/v of algae alginate and 2 mL of 200 mg/L β-

amylase (Sigma, Singapore) into gently stirred 5% (w/v) $CaCl_2$ solution for 2 h to

give bead size of 3 mm. The capsules formed were recovered by filtration using a

Buchner funnel and were thoroughly washed 0.02 M of acetate buffer (pH 4.8) in

order to remove excess of $CaCl_2$ and non-entrapped amount of enzyme. The capsules

were dried firstly between two sheets of filter paper and then in the open air for 2 h.

Beads were stored in 0.02 M of acetate buffer at 4°C (Konsoula *et al*., 2006; Chang and Juang, 2005).

3.7.2   Determination of enzyme activity

The activity of free β-amylase was analyzed as follows (Bergmeyer, 1983). A starch solution (0.5% v/v) was prepared by dissolving 0.25 g of soluble starch from potato (Sigma, Singapore) in 12.5 mL of 0.02 M of acetate buffer (pH 4.8) through gently heating. After rapid cooling, 37.5 mL of the sample acetate buffer and 0.073 g of NaCl were immediately added in the solution. An enzyme solution was also prepared by adding an aliquot of free β-amylase in DI water containing Lugol reagent (0.01 M I$_2$ and 0.08 M KI in 0.02 M HCl) and 0.02 M acetate buffer (pH 4.8). Then, 0.1 mL of enzyme solution and 0.5 mL of the starch solution were taken and mixed with 0.9 mL of DI water. After 10 min incubation at 37°C, the reaction was forced to stop by adding 2 mL of 0.02 HCl. The mixture was diluted by adding 0.5 mL of Lugol reagent and 1 mL DI water and the absorbance at 601 nm was read with an UV/ Visible spectrophotometer (Ultraspec 2000 UV/Visible Spectophotometer, Pharmacia Bitech, England). One activity unit (U) of enzyme is defined to be the amount of enzyme required hydrolysis 10 mg of starch within 10 min.

The activity of immobilized enzyme was analyzed following the procedure for corresponding free enzyme with minor modification. In this case, 0.05 g of enzyme-immobilized beads and 0.5 mL of the starch solution were mixed with 1 mL of DI water for 15 min incubation. (Change and Juang, 1983).

3.7.3   Reuseability of enzyme

The immobilized enzyme was stored in 0.02 M of acetate buffer (pH 4.8) at 4°C for 7 days before used. The immobilized enzyme was reused 8 times in every 3 day

and the enzyme activity was measured following in section 3.7.2. After each assay, immobilized preparation was washed with 0.02 M of acetate buffer and then stored at 4°C.

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Conditions for alginate-production by *Azotobacter* sp. in shake flask

4.1.1 Carbon source and concentration

Cell growth and EPS production usually depends on carbon sources that affect quality, sugar component and/or molecular weight of EPS (Wachenheim and Patterson, 1992). Glucose, arabinose, maltose, rhamnose, lactose, mannose, fructose, trehalose, xylose, sorbital and sucrose were used for study optimal carbon source. The effect of carbon source on alginate production, apparent viscosity and growth of bacterial are presented in Fig 4.1. The data for effects of carbon sources (1%) on alginate production from *Azotobacter* sp. showed that trehalose and sucrose (sugar cane) gave similar alginate yield (156.67 and 206.67 g/mol, respectively) that had apparent110 and 295 cP, respectively and had growth 1.25×10^8 and 9.1×10^7 cfu/mL in 72 h and at 30^oC. However, this experiment sucrose was used because of its lower cost than trehalose and highest alginate.

Figure 4.1 Effect of carbon sources on alginate production, apparent viscosity and bacterial cell growth by *Azotobacter* sp. in LG medium and 1% inoculum size, pH 6.8 at 30°C for 72 h on rotary shaker of 200 rpm.

The optimal sucrose concentration for alginate production from *Azotobacter* sp. was 1% w/v and beyond this concentration the production was decreased. Fig 4.2 showed the growth of bacterial and its maximum alginate concentration. The alginate production and bacterial cell growth were increased with an increase of glucose concentration for 1% (w/v). A reduction of alginate production and the growth of *Azotobacter* sp. were decreased when sucrose concentration was greater than 1% (v/v). It is apparent viscosity that sucrose concentration could be efficiently converted to alginate. The results also indicated to a certain extent that the inhibitory effects of high sucrose concentration on the alginate production was occurred. Sucrose

inhibition is related principally to osmotic effects. As pointed out by Prasertan et al., (2008) a high concentration of sucrose in the medium inhibits the growth of *Azotobacter* sp. and the production of alginate, and the inhibitory effect is attributed to high osmotic pressure.
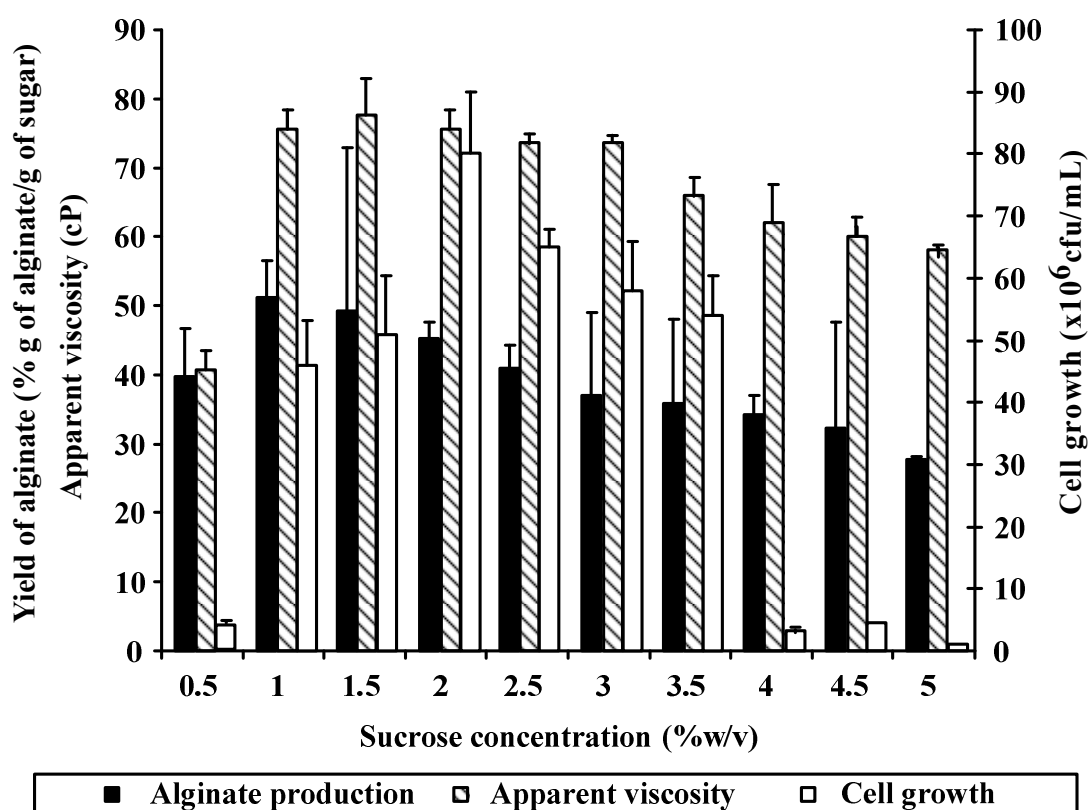
Figure 4.2 Effect of various different sucrose concentration on alginate production, apparent viscosity and bacterial cell growth by *Azotobacter* sp. in LG medium at1% inoculum size, adjusted initial pH of medium of 6.8, incubated at 30°C for 72 h and incubated on rotary shaker of 200 rpm.

4.1.2 Temperature

Incubation temperature is often a critical factor in polysaccharide biosynthesis. All commercial polysaccharide-producing microorganisms are mesophiles (Lawson and Sutherland, 1978). The optimum temperature for polysaccharide production depends on the type of microorganism. The optimum temperature for growth was 25-30°C while that for alginate production was found to be 30°C (Fig 4.3) which was similar to those bacterial strains such as 28-30°C for Plant-Pathogenic *Pseudomonads* (Fett et al., 1986).
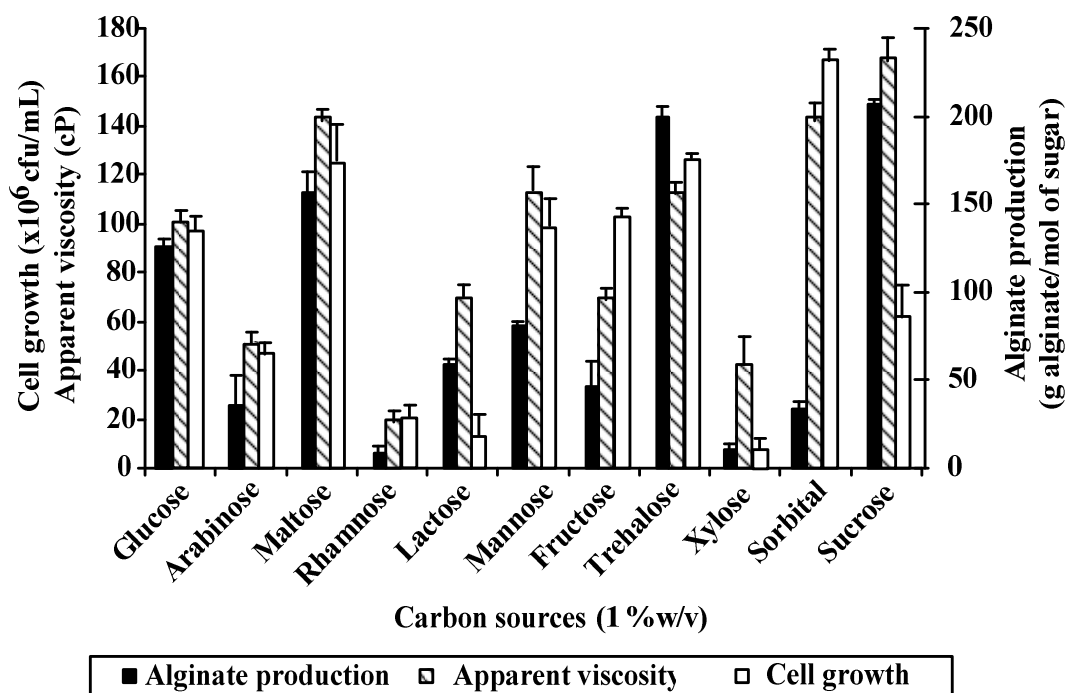

Figure 4.3 Effect of incubation temperature on alginate production, apparent viscosity and bacterial cell growth by *Azotobacter* sp. in LG medium containing 1% w/v sucrose, 1% inoculum size for 72 h on rotary shaker of 200 rpm.

4.1.3 Initial pH

Results obtained from the previous study showed that high alginate production was obtained in LG medium containing 1% w/v sucrose as a carbon source. The initial pH of 6.5-7 was found to be optimum for both bacterial cell growth and alginate production by *Azotobacter* sp. (Fig 4.4). It was reported that pH had more influence on alginate production than on cell growth (Pace, 1981) and the specific pH affected directly the synthesis of those enzymes responsible for alginate production (Lawson and Sutherland, 1978).
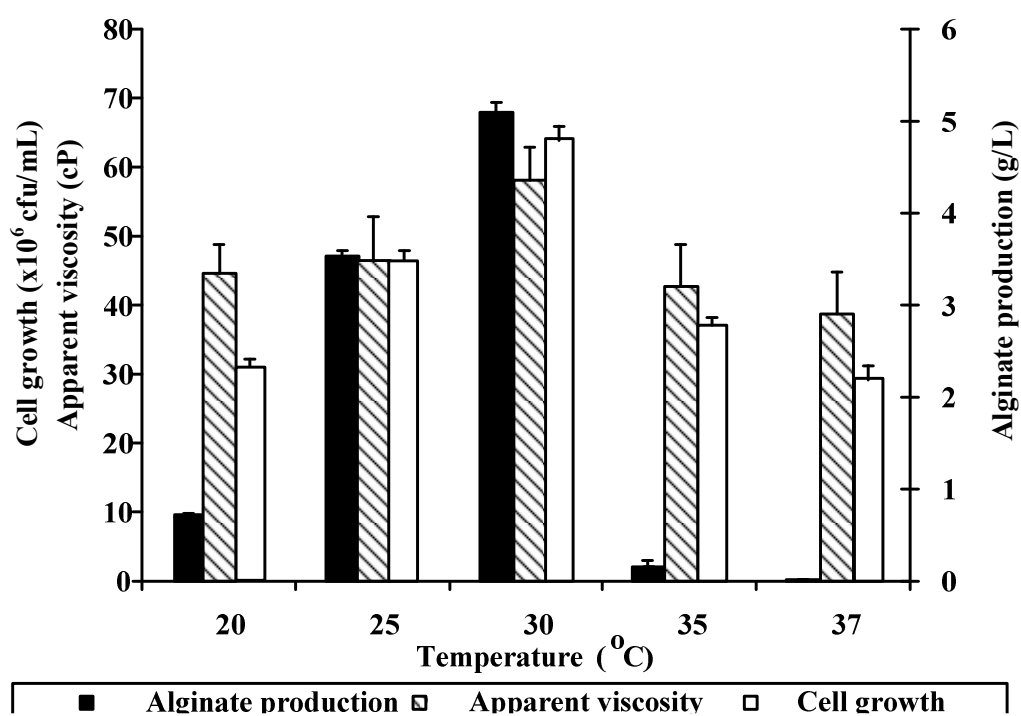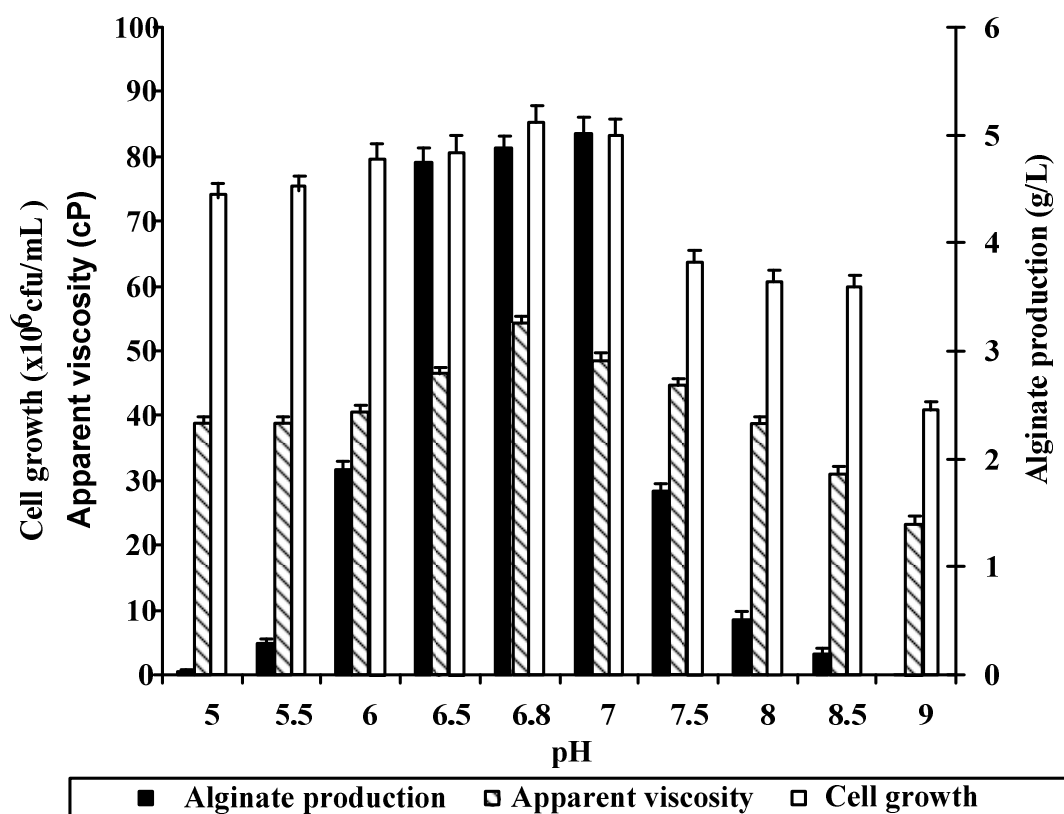

Figure 4.4   Effect of initial pH of medium on alginate production, apparent viscosity and bacterial cell growth by *Azotobacter* sp. in LG medium containing 1% w/v sucrose and 1% inoculum size, incubated temperature 30°C for 72 h on rotary shaker of 200 rpm.

4.1.4 Sources and concentrations of nitrogen

The effect of various inorganic nitrogen sources (DAP, $NH_4H_2PO_4$ and NH_4Cl) and their concentrations were examined. Fig 4.5 demonstrated that the incorporation of nitrogenous compounds into medium did not increase alginate yield and apparent viscosity. The results showed that alginate production decreased as the nitrogen concentration increased when compared LG medium which nitrogen source was not added.

Addition of inorganic nitrogen was found to effect growth of *Azotobacter* sp. with the highest value was found in the presence of 0.5-2% (w/v) of DAP ($2.15x10^7$, $2.31x10^8$, $5.3x10^9$ and $9.6x10^9$ cfu/mL, respectively), and 0.5% (w/v) $NH_4H_2PO_4$ ($8.15x10^9$) when compared LG medium (without addition of nitrogen). Adding NH_4Cl has effect on alginate production and viscosity. Conflicting reports have appeared in the literature concerning the inhibition of nitrogenase activity by added NH_4^+ in whole cells of *Azotobacter* sp. (Hardy et al., 1968). Nitrogen was also effect on nitrogen fixation which of conflict to alginate production . Page et al., 1997 reported that nitrogen fixing cells have very low alginate content and the addition of inorganic nitrogen spares the need for nitrogen fixation and the respiratory protection of the oxygen labile nitrogenase complex and thus allows the reducing power and Acetyl Co-A derived from active sugar metabolism to be used for alginate production. Similar result was obtained recently by Pal et al. (1998) using an *A. chroococcum* strain.

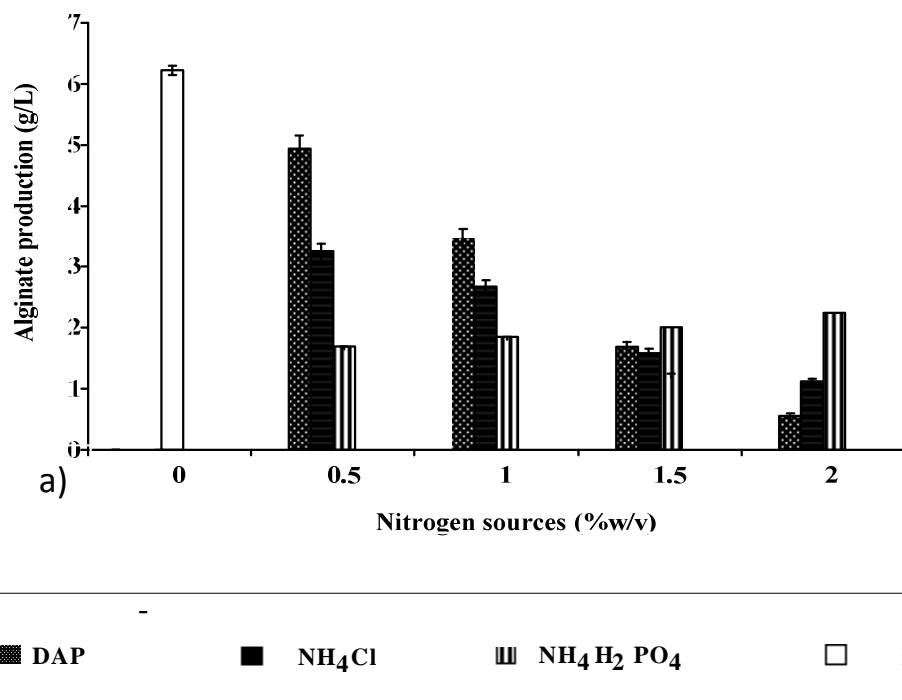**Figure 4.5**   Effect of various nitrogenous inorganic compounds on a) alginate production b) apparent viscosity and c) cell of by *Azotobacter* sp. in LG medium containing 1% w/v sucrose as carbon source and 1% that adjusted pH was 6.8, at 1% inoculum size, at 30 ^{O}C for 72 h on rotary shaker of 200 rpm.

b)

c)

DAP   NH$_4$Cl   NH$_4$H$_2$PO$_4$ No N
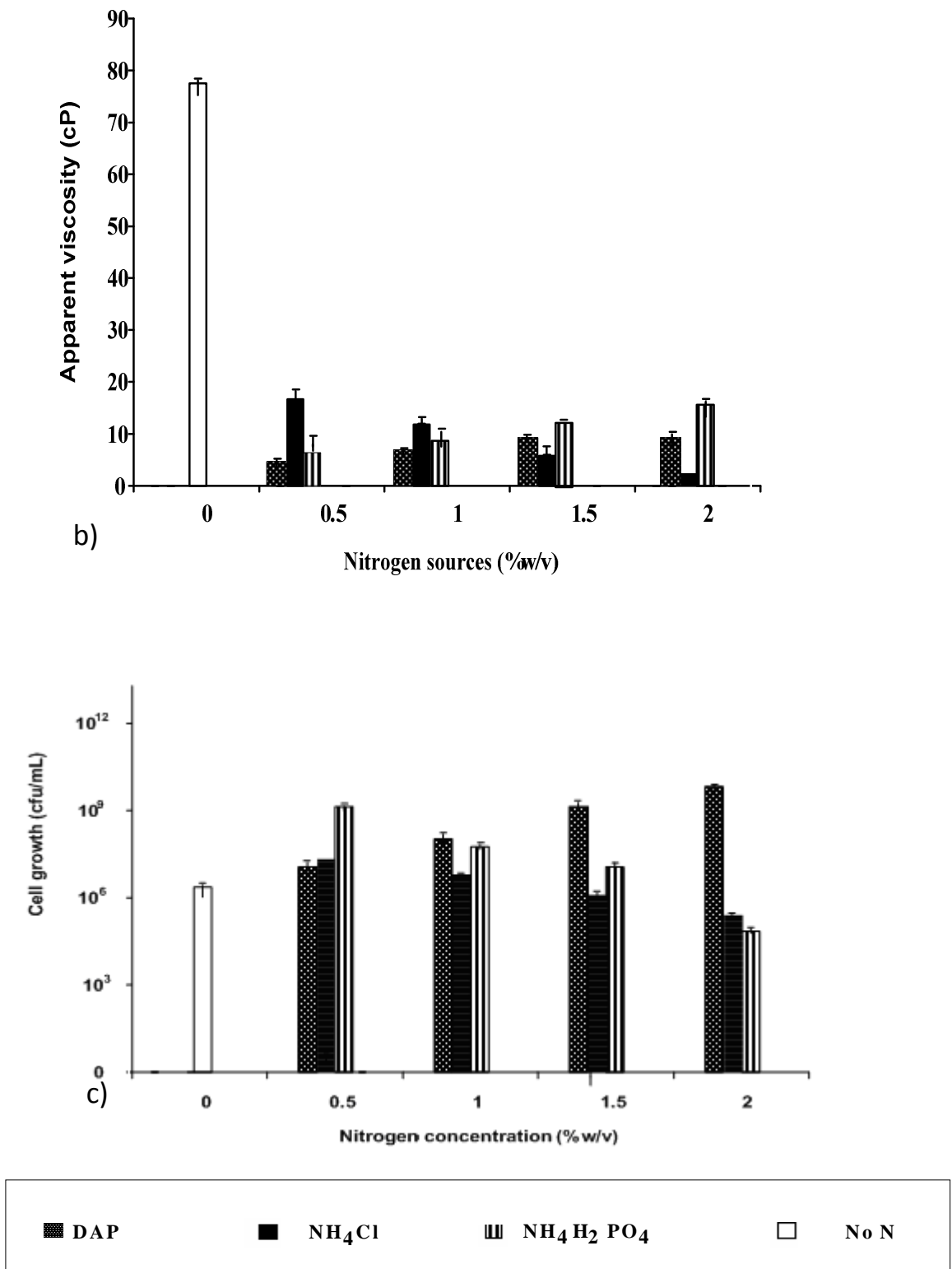
Figure 4.5 (continued).

4.2 Study the conditions for alginate production in 1.5L batch fermentation

2L of batch fermenter was run for propose of determining the alginate production by *Azotobacter* sp. The fermenter was charged with 1.5L of LG medium containing 1 % (w/v) sucrose as carbon source. The fermentations were run at 30°C and adjusted initial pH was 6.8 that both temperature and pH were controlled.

4.2.1 Stirrer speeds

The polymers synthesized in cultures carried out at different conditions were rheologically different. In figure 4.6 (a-f) showed alginate production, viscosity, residue sucrose and Cell growth of *Azotobacter* sp. from batch fermentation at different agitation speeds (100-600 rpm). Alginate formation was partially growth associated due to both alginate concentration, and growth cell sharply increased as stirrer speed increased at 100-500 rpm (Table 4.1) and slightly increased when up to 500 rpm within 24 h and slightly increased after 24 h. While apparent viscosity increased as time until end of fermentation. Kinetic parameter such as specific growth rate, maximum growth rate, sucrose utilization rate, maximum alginate yield and shear stress were showed in Table 4.1. Therefore, at stirrer speed 500 rpm was selected for further study due to save power energy and gave alginate did not different with 600 rpm. By excess turbulence, it is most likely that the decreased biomass and alginate production may be due to damaging cell membranes and limited mass transfer in localized zones (Toma et al., 1991). The higher the shear rate, the smaller the average cell surface area (and also diameter), which reached a minimum of 8.9 μm^2 at 800 rpm compared to the minimum of 33.8 μm^2 reached at 300 rpm (Sabra et al., 1999). A decrease in the cellular surface area per cell volume, which was

proposed by Post et al., (1982) as a nitrogenase protection mechanism against oxygen. Apparent viscosity viscosity of alginate was produced exhibited non-Newtonian behavior because apparent viscosity increased when shear rate increased showed in Tab 4.1.
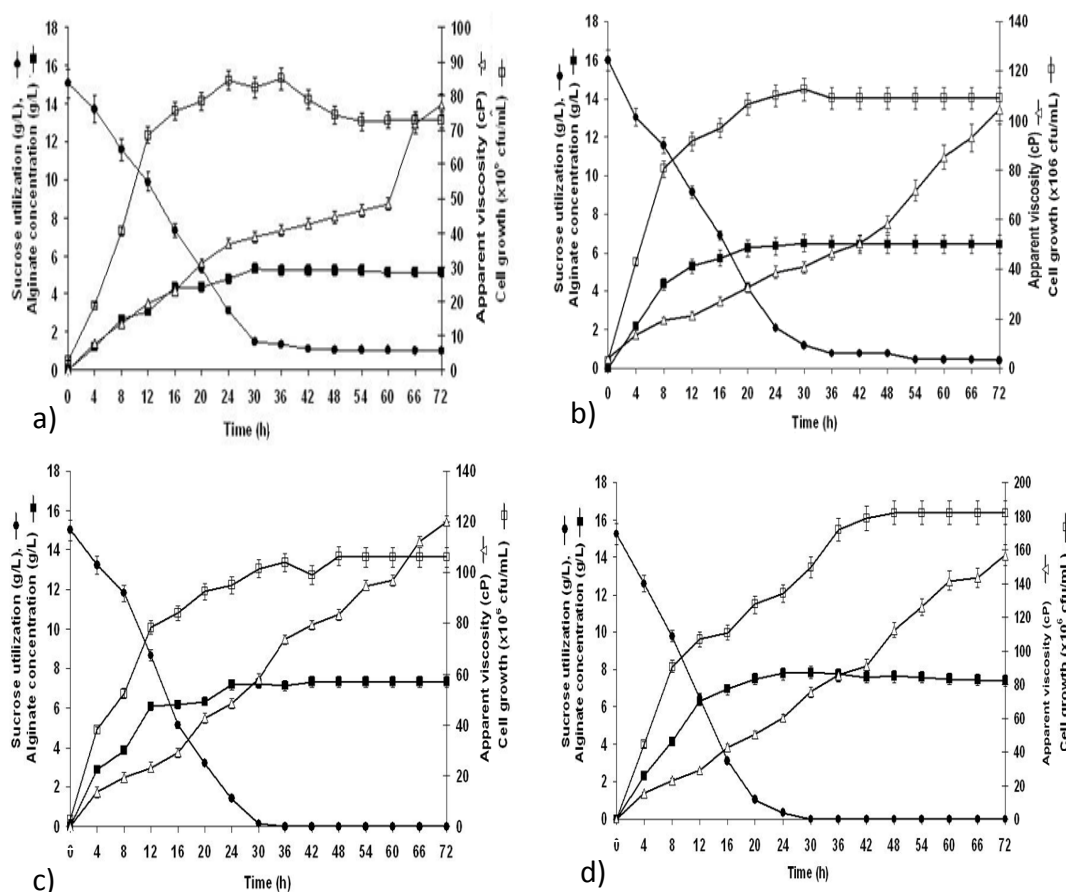
Figure 4.6 Changes in the sucrose utilization, alginate-produced concentration, apparent viscosity, residue sucrose and bacterial cell growth in batch fermentations at different agitation speeds a) 100 rpm, b) 200 rpm, c) 300 rpm, d) 400 rpm, e) 500 rpm, and f) 600 rpm with 2.5 vvm of aeration throughout fermentation, 10% inoculum size at 30°C and adjusted initial pH in the broth was 6.8.
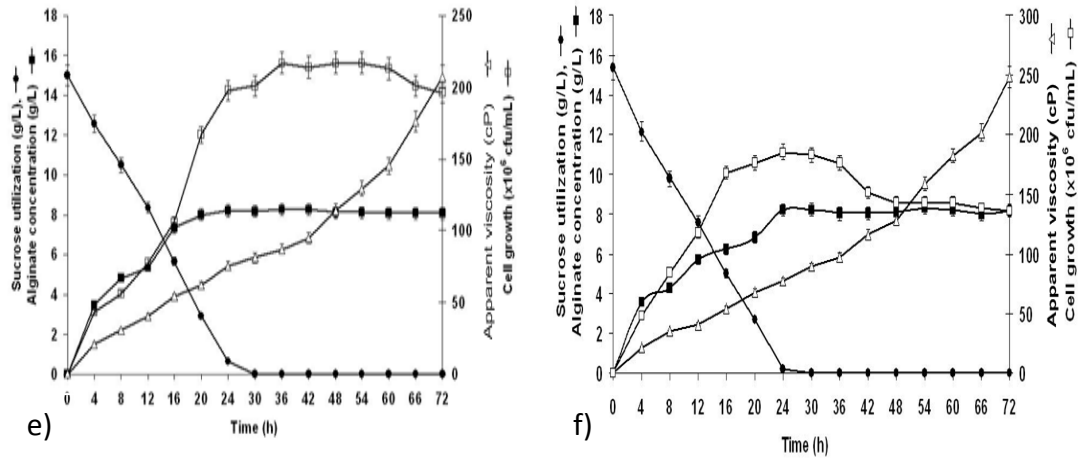
Figure 4.6 (continued)

Table 4.1 Kinetic parameters for maximum growth rate, maximum alginate yield, sugar utilization and shear stress of *Azotobacter* sp. in 2L fermenter (difference agitation speed) with 2.5 vvm of air flow rate in LG medium, pH 6.8 at 30°C

Kinetic Parameters	Agitation speed (rpm)					
	100	200	300	400	500	600
Maximum growth rate, μ_{max} (h^{-1})	0.205	0.242	0.257	0.278	0.295	0.301
Maximum alginate yield, $Y_{p/s}$ (g/g)	0.398	0.432	0.457	0.489	0.503	0.510
Sugar utilization (g/L/h)	0.361	0.428	0.470	0.533	0.582	0.602
Shear stress, τ (Pa·s^{-1})	1.29	3.48	6.01	10.47	16.59	24.81

4.2.2 Aeration

The aeration was varied by 0, 2.5 and 5 vvm. The results of alginate-produced concentration, bacterial cell growth, apparent viscosity and residue sucrose in batch fermentation by *Azotobacter* sp. were showed in Fig 4.7 (a-c). Kinetic parameter such as specific growth rate, maximum growth rate, sucrose utilization rate, maximum alginate yield and shear stress were showed in Tab 4.2. From the results found that alginate formation was partially growth associated due to both alginate productions, cell growth increased at the same time within first 24th h and slightly increased or slightly increased until end of fermentation. At aeration 2.5 vvm gave high cell growth and alginate production while at aeration 5 vvm, all values were dropped due to antiform that added into medium for reduce air bubble and at high levels of oxygen, cell growth declined, confirming an inhibitory effect of oxygen on nitrogenase activity. So, at 2.5 vvm was selected to future study to avoid using antiform due to affects to cell wall of bacteria and apparent viscosity of alginate. (Fig 4.7c). In the biological fixation of dinitrogen depends on the activity of the highly oxygen-sensitive nitrogenase enzyme complex (Linkerhagner and Oelze, 1997). For the survival of *Azotobacter* under aerated conditions, one of the priorities of their entire metabolism is to protect the active nitrogenase from being damaged by oxygen. Protection of this enzyme from oxygen has been proposed to occur in *Azotobacters* mainly through two mechanisms: (i) high respiratory activity that removes oxygen already at the cell surface and (ii) reversible conversion of the enzyme into a protected inactivated state (Linkerhagner and Oelze., 1995; Liu et al., 1995). For *Azotobacter*, the increase of apparent viscosity of the culture broth during the course of the cultivation because of increasing biomass and alginate concentrations can reduce the

oxygen transfer rate from the gas phase to the aqueous phase and from the bulk liquid to the cell surface. To avoid a high oxygen transfer rate into the cell, an effective oxygen barrier on the cell surface can be even more important (Sabra et al., 1999).
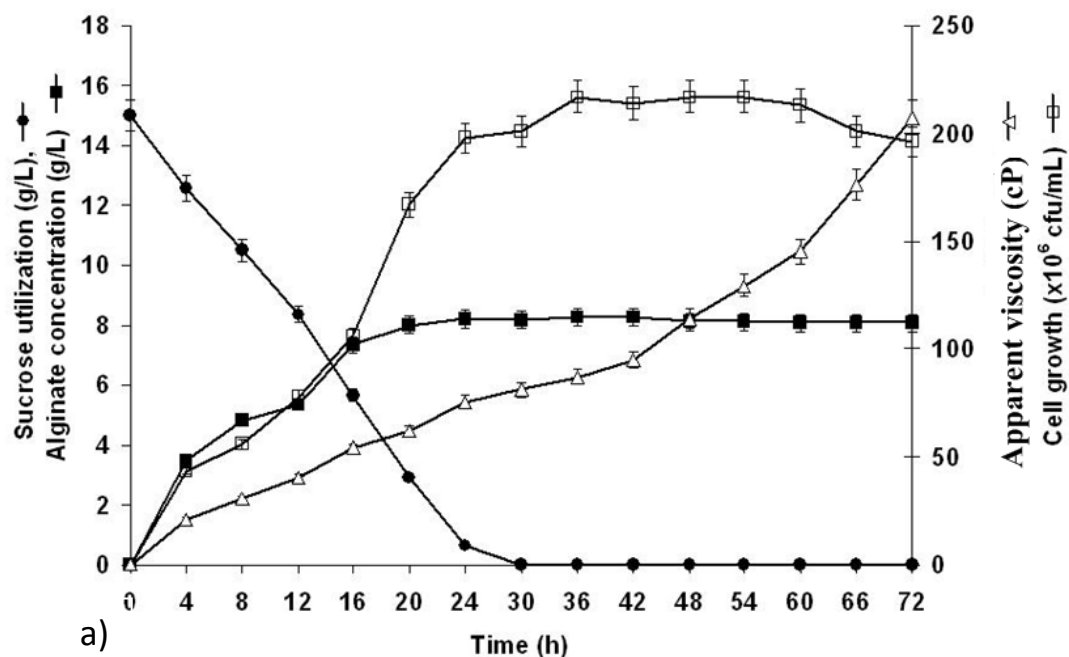
Figure 4.7 Effect of aeration rate on sucrose utilization, alginate concentrations, apparent viscosity and cell growth of *Azotobacter* sp. in LG medium in 2L fermenter, 10% inoculum size, pH 6.8 and 30°C. The aeration rate was varied at 0-5 vvm; (a) 0 vvm, (b) 2.5 vvm, and (c) 5 vvm.
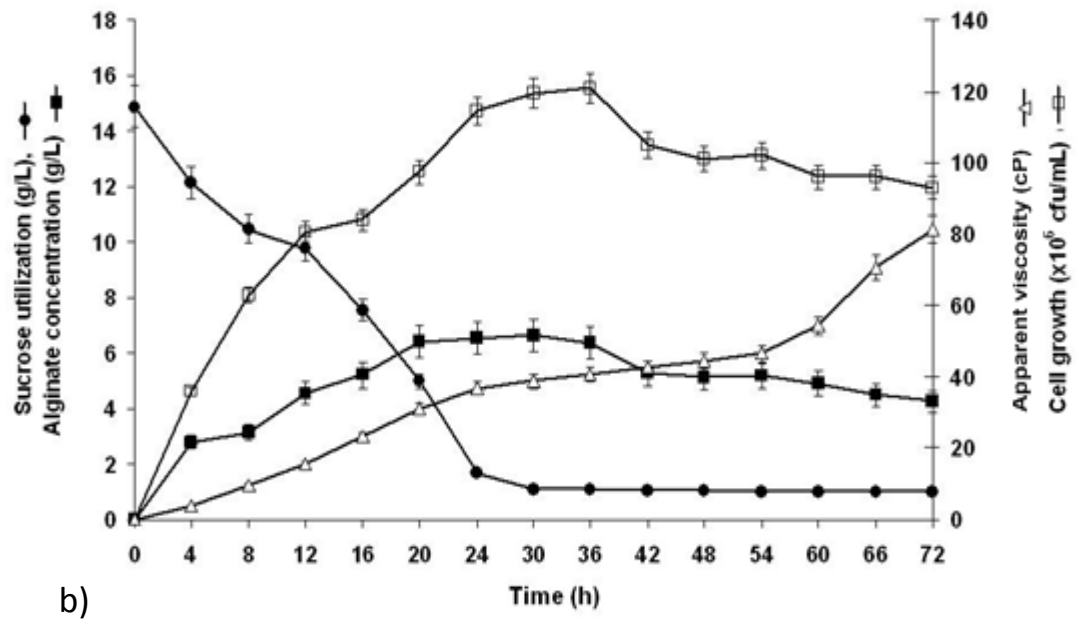
b)
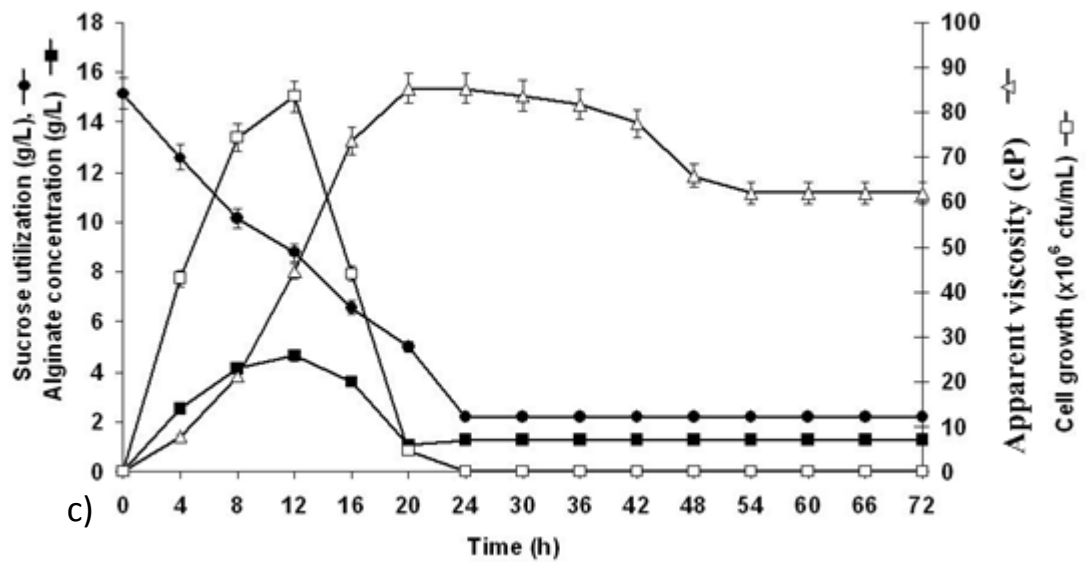
c)

Figure 4.7 (continued).

Table 4.2 Kinetic parameter for maximum growth rate, maximum alginate yield and sucrose utilization of *Azotobacter* sp. in 2L fermenter (difference aeration) at 500 rpm of agitation speed with LG medium, pH 6.8 at 30°C.

Kinetic parameters	Aeration (vvm)		
	0	2.5	5
Maximum growth rate, μ_{max} (h^{-1})	0.228	0.295	0.108
Maximum alginate yield, Yp/s (g/g)	0.375	0.503	0.215
Sucrose utilization (g/L/h)	0.287	0.583	0.242

4.3 Increasing some properties of alginate-produced by organic acids

4.3.1 Source and concentration organic acids

The results of shake flasks experiment were showed in figure 4.8 that demonstrated alginate production, apparent viscosity and cell growth. Succinic acid, fumaric acid, propionic acid, phytic acid, malic acid, adipic acid, 4-aminobenzoic acid, lactic acid and tartaric acid were used in increasing efficiency of alginate. The results showed that four of all of organic acids; succinic acid, malic acid, lactic acid and adipic acid increase apparent viscosity and alginate concentration while phytic acid promotes cell growth only.
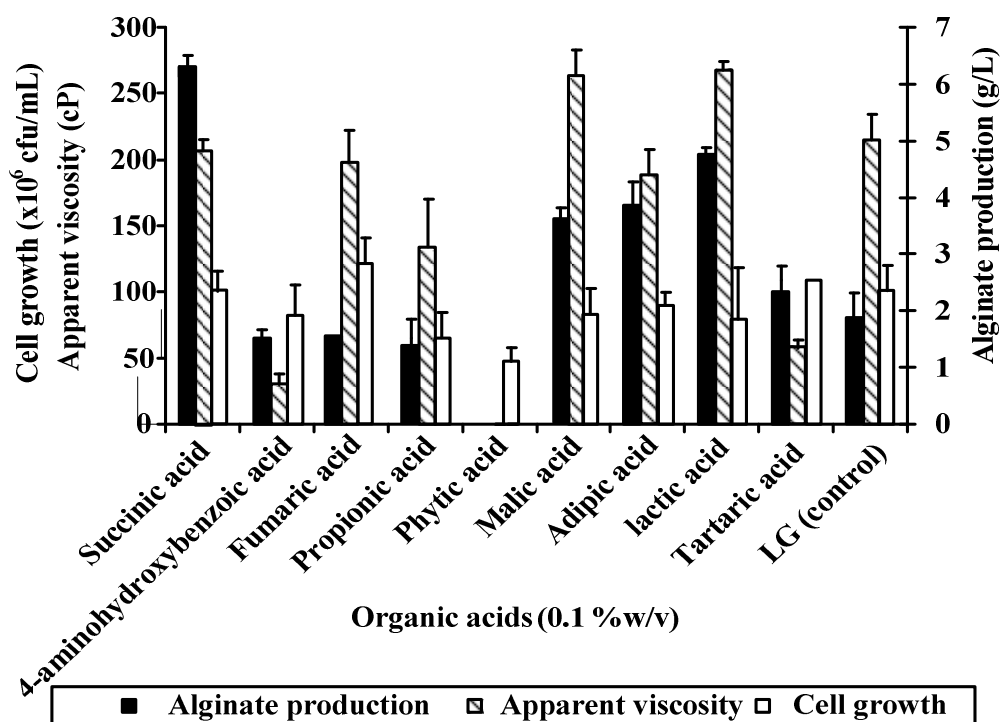
Figure 4.8   Effect of organic acids on alginate-produced, bacterial cell growth and apparent viscosity of alginate-produced in LG medium containing 1% w/v sucrose as carbon source and adjusted pH was 6.8, at 1% inoculum size, at 30°C for 72 h on rotary shaker of 200 rpm.

The concentrations of each organic acid (succinic acid, malic acid, lactic acid and adipic acid) were varied from 0-0.2 % (w/v), differing by 0.05 % (w/v) found that at 0.15% (w/v) succinic acid gave highest alginate production, cell growth and increased apparent viscosity when compared with LG medium without adding organic acid (Fig 4.9). From the result, succinic acid might be use as carbon source in TCA (tricarboxylic acid) cycle.

Vargas-Garcia et al., (2001) concluded that the EPS was produced by *Azotobacter* sp. when added 4-aminohydroxybenzoic acid as sole carbon source which leads to high apparent viscosity values, while Chan, (1986) demonstrated that the *Azotobacter* sp. can give good levels of growth when used phenolic compound as carbon source.
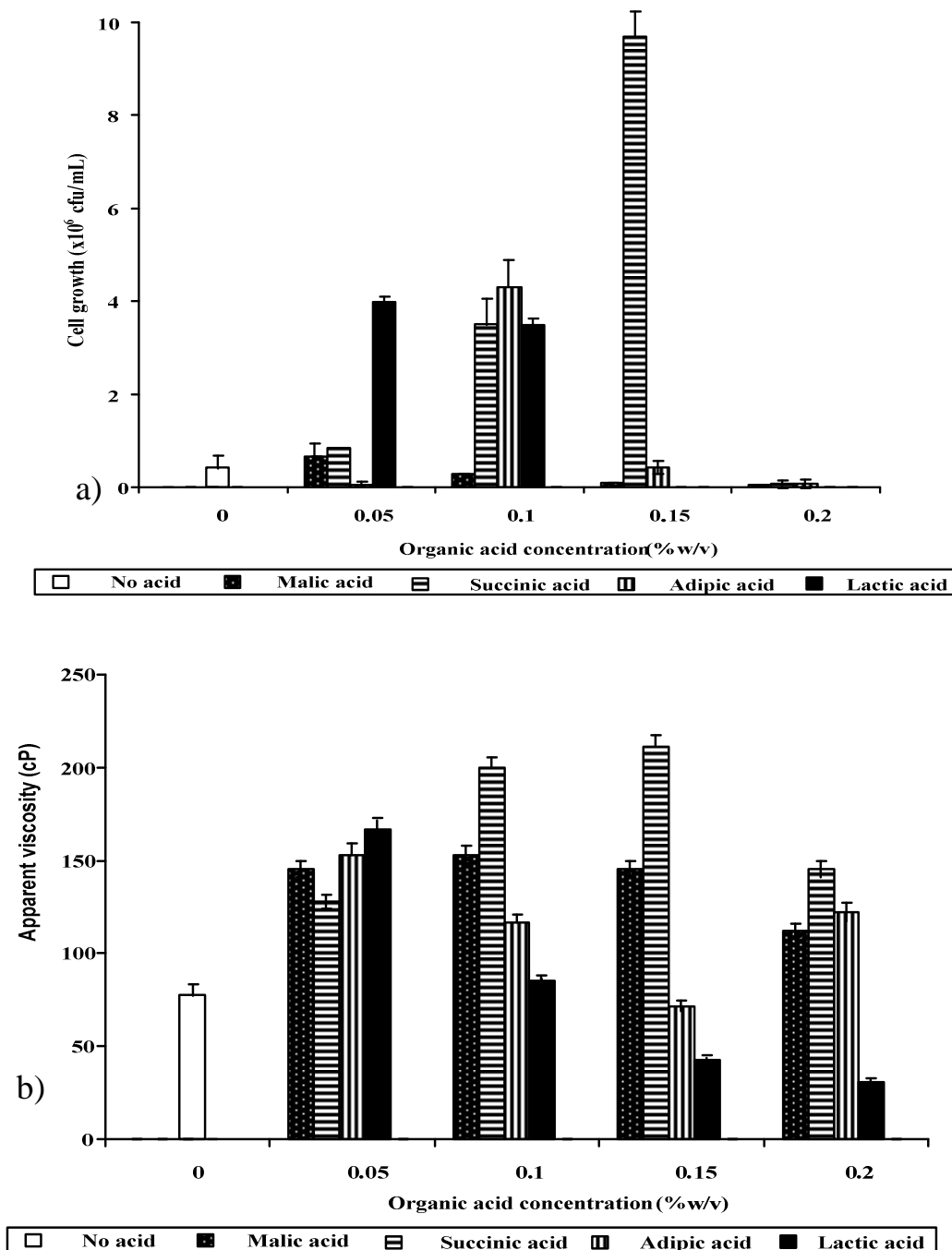
Figure 4.9 Effect of organic acids concentration on (a) bacterial cell growth (b)

apparent viscosity and (c) alginate production of alginate in LG medium

containing 1% w/v sucrose as carbon source and adjusted pH was 6.8, at

1% inoculum size, at 30°C for 72 h.
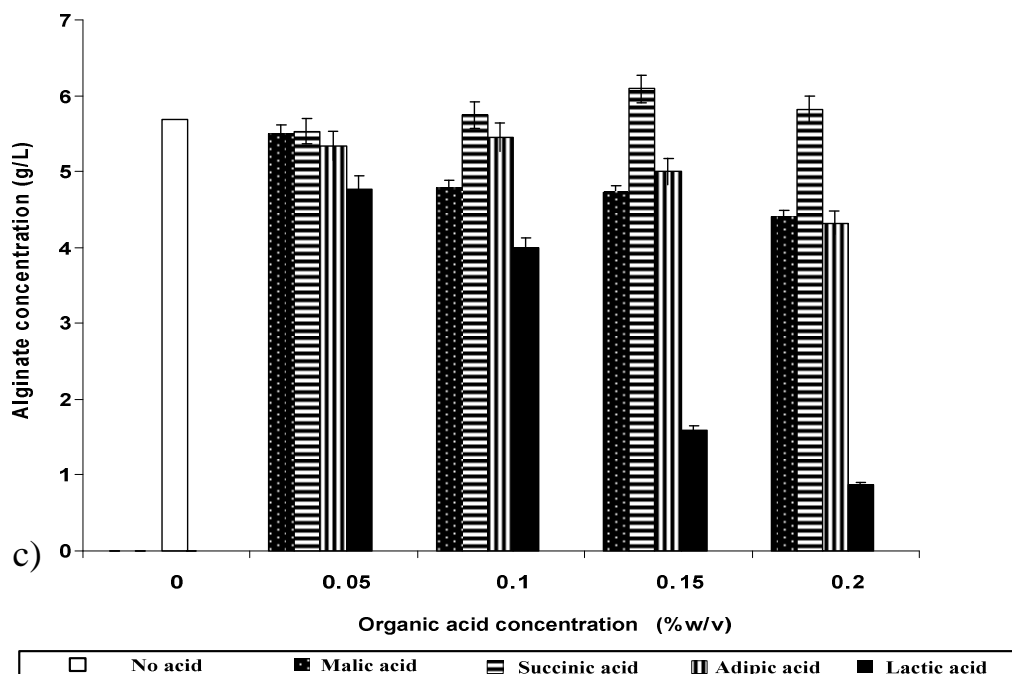
Figure 4.9 (continued).

4.3.2 Alginate production in 2L fermenter using LG medium with acid

2L of batch fermentation was run again for increase apparent viscosity and alginate production by *Azotobacter* sp. A 2L fermenter was charged with 1.5L of LG medium, at sucrose concentration of 1% (w/v) and 0.15% succinic acid as carbon source. The fermentation was run aseptically and controlled temperature at 30°C and pH 6.8.

The sucrose utilization, succinic acid utilization, alginate production, apparent viscosity and cell growth are showed in Figure 4.10. Alginate production and cell growth were increased within 24th h and constant until fermentation end while sucrose and succinic acid were exhausted within 24th h. Adding 0.15% (w/v) of succinic acid into medium LG medium was enhance alginate production, cell growth and apparent

viscosity value when compared no adding 0.15% (w/v) of succinic acid. From the result, might be confirm that succinic acid as carbon source for in alginate production by *Azotobacter* sp. The kinetic parameters were showed in Tab 4.3.
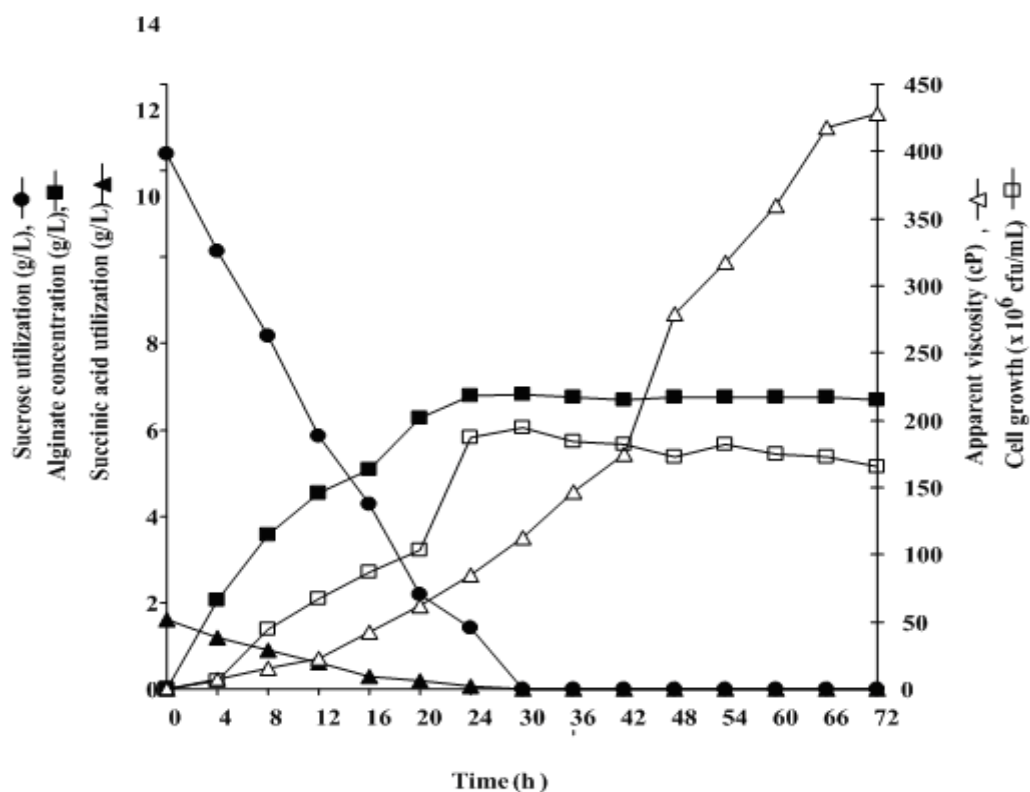
Figure 4.10 Changes of sucrose utilization, alginate concentrations, apparent

viscosity and cells growth of *Azotobacter* sp in LG medium contained

sucrose and 0.15% succinic acid as a carbon source, at 5 vvm., 10%

inoculum size, pH 6.8 and 30°C.

Table 4.3   Kinetic parameters for maximum growth rate, maximum alginate yield, sugar utilization and shear stress of *Azotobacter* sp. in 2L fermenter in LG medium containing 0.15% (w/v) succinic acid, at 500 rpm of agitation speed with 2.5 vvm, pH 6.8 at 30°C.

Kinetic Parameter	Adding 0.15% (w/v) succinic acid
Maximum growth rate, μ_{max} (h^{-1})	0.297
Maximum alginate yield, Yp/s (g/g)	0.505
Sugar utilization (g/L/h)	0.566
Succinic utilization (g/L/h)	0.327
Apparent viscosity (cP)	428.28

4.3.3 Characteristic of alginate-produced from *Azotobacter* sp.

4.3.3.1 Morphology characteristic under SEM

The cell morphology of the *Azotobacter* sp. was studied under scanning electronmicroscope. The cells morphology of *Azotobacter* sp. are rod shape, size about 1 µm (Fig 4.11) and alginate is developed (Fig 4.12-4.13). The production of alginate or slime embeds the aggregating cells to form microcolonies. Fig 4.14 showed dry form of alginate produced by *Azotobacter* sp. and alginate (commercial grade) have same crosslinked-structure but number of cross-link alginate produced by *Azotobacter* sp. less than alginate that obtained from seaweed alginate. However, the seaweed alginate is more flexible polymer and rigid than its of the *Azotobacter*.
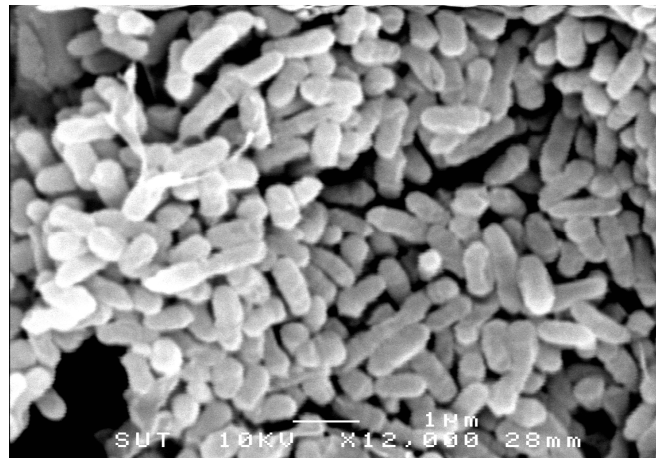
Figure 4.11 Morphological characteristic of *Azotobacter* sp. under scanning

electronmicroscope (12,000x).

Alginate →   ← *Azotobacter* sp.
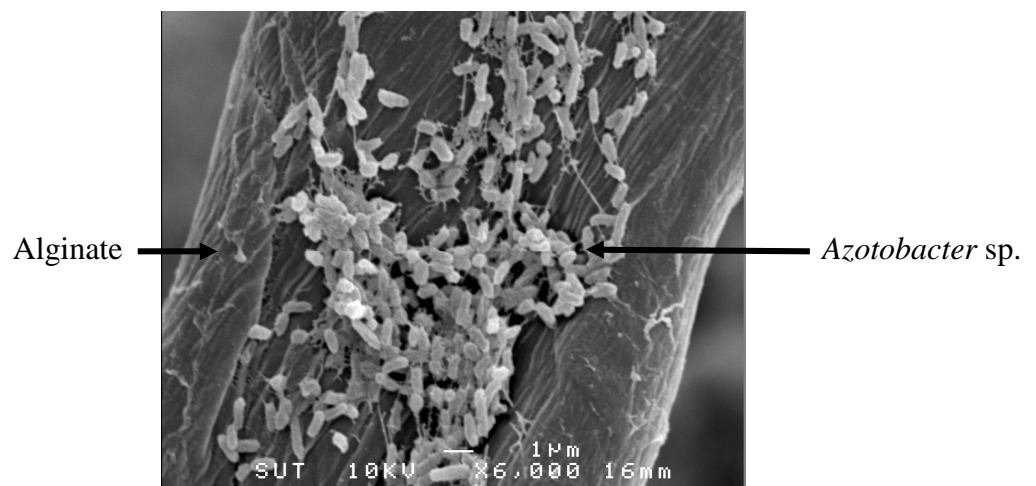
Figure 4.12 Morphological characteristics of *Azotobacter* cells and alginate in LG

medium containing 0.15% (w/v) succinic acid at 30°C for 3 days (Scale

bars = 1µm).
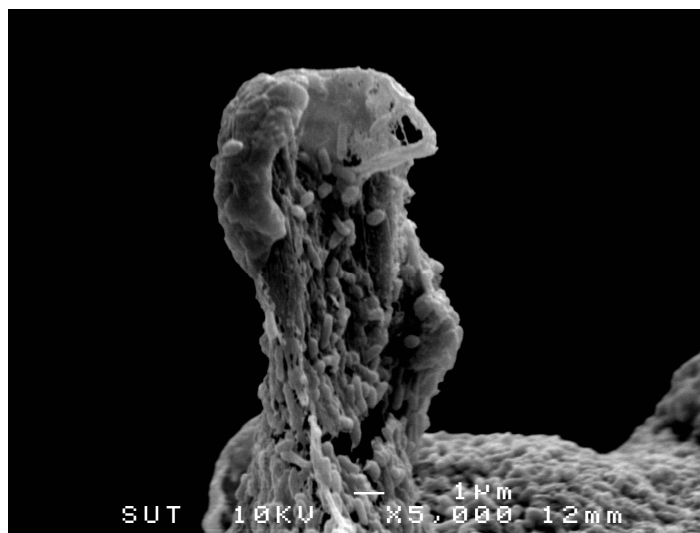
Figure 4.13 Scanning electronmicroscope of *Azotobacter* sp. alginate formation in

LG medium containing 0.15% (w/v) succinic acid incubated at 30°C for 3

days.
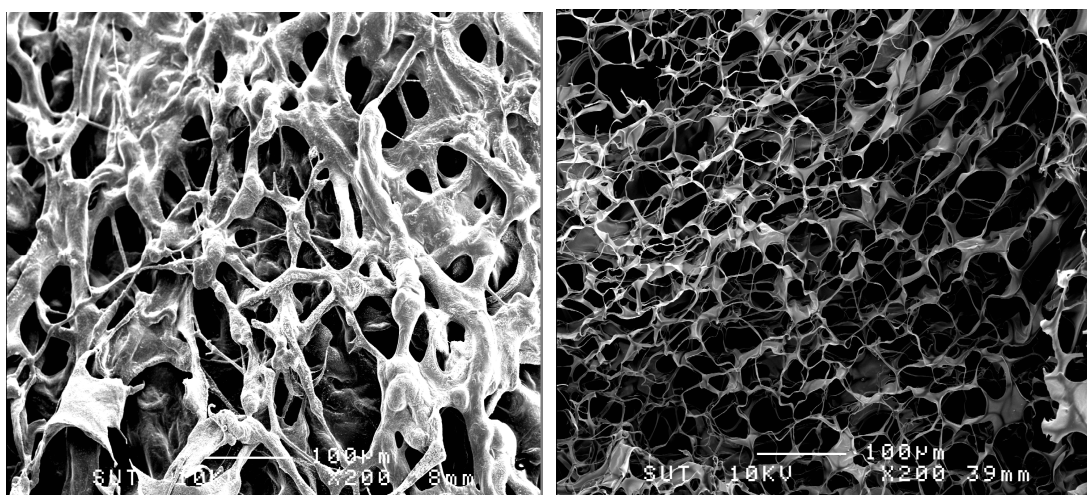
Figure 4.14 Morphological characteristic of alginate under SEM; a) dry form of

Azotobacter alginate that used LG medium containing 0.15% succinic

acid b) seaweed alginate.

4.3.3.2 Molecular weight of alginate

The molecular weight of bacteria alginate and seaweed alginate were determined when compared with pullulan kit as a standard by using HPLC technique. The peak of each of molecular weights; 2.9×10^3, 2.04×10^3, 6.75×10^3 and 0.77×10^3 kDa were found at the retention time of 6.19, 8.14, 10.143 and 10.894 minutes, respectively (Fig 4.15a). The peak of *Azotobacter* alginate was found at the retention time of 6.13, 11.29 and 11.9 minutes (Fig 4.15b) while seaweed of alginate was found only peak at the retention time of 6.14 minutes (Fig 4.15c). From the result, the molecular weight of *Azotobacter* alginate might be similar to molecular weight of seaweed alginate because occur peak at the same retention time (6.13 and 6.14 minutes) and at the retention time of 11.29 and 11.9 might be impurity compound that remain from purification processing. Molecular mass of *Azotobacter* alginate and seaweed alginate were of 2.87×10^3 and 2.88×10^3 Da, respectively.
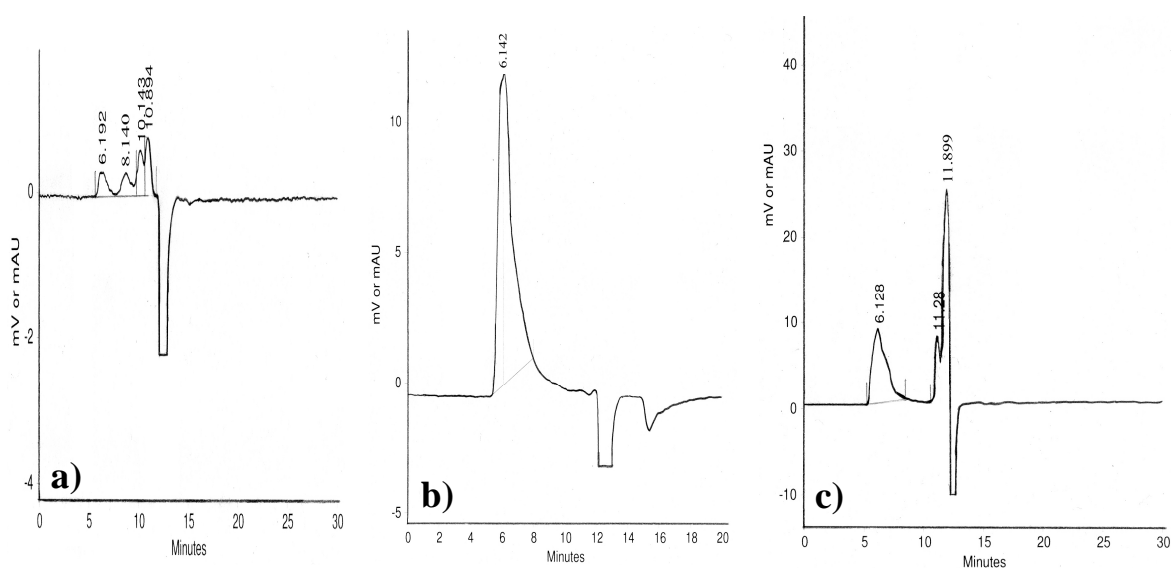
Figure 4.15 Chromatograms of molecular mass of a) pullulan strandard b) *Azotobacter* alginate and c) seaweed alginate.

4.3.3.3 Behavior fluid of alginate

The behavior fluid of seaweed alginate and *Azotobacter* alginate were determined by Rheometer which used din conical concentric cylinder as roter at 25°C and shear rate 0-300 1/s. Fig 4.16 showed fluid behavior of seaweed alginate and *Azotobacter* alginate found that both of alginate exhibited pseudoplastic behavior due to apparent viscosity decreased when shear rate increased.
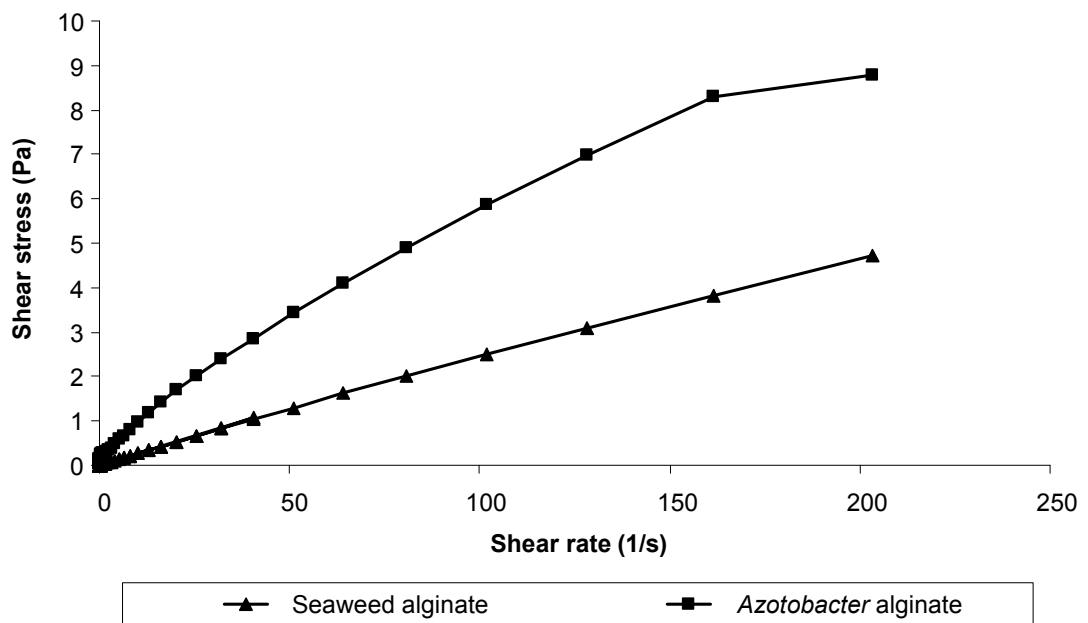
Figure 4.16 Apparent viscosity of seaweed alginate and *Azotobacter* alginate at difference shear rate.

4.4 Alginate production in 5L fermenter

Optimum condition parameters were obtained from shake flask and 2L fermenter experiments such as carbon source concentration, temperature, initial pH, stirrer speed and organic acid concentration were used for scale up of alginate production. Sucrose concentration, succinic acid utilization, alginate concentration, apparent viscosity and cell growth are also analyzed as showed in Fig 4.17. From the result found that sucrose and succinic acid were exhausted within 24th h. The cell growth and alginate production were increased within 24th h while apparent viscosity increased also. Kinetic parameters in 2L fermenter and 5L fermenter were showed in Tab 4.4. The growth rate of *Azotobacter* sp. and alginate production in 5L fermenter was lower than in 2L fermenter, this may results from many factors such as mixing, aeration, impeller design etc. due to changing the size of the fermenter.
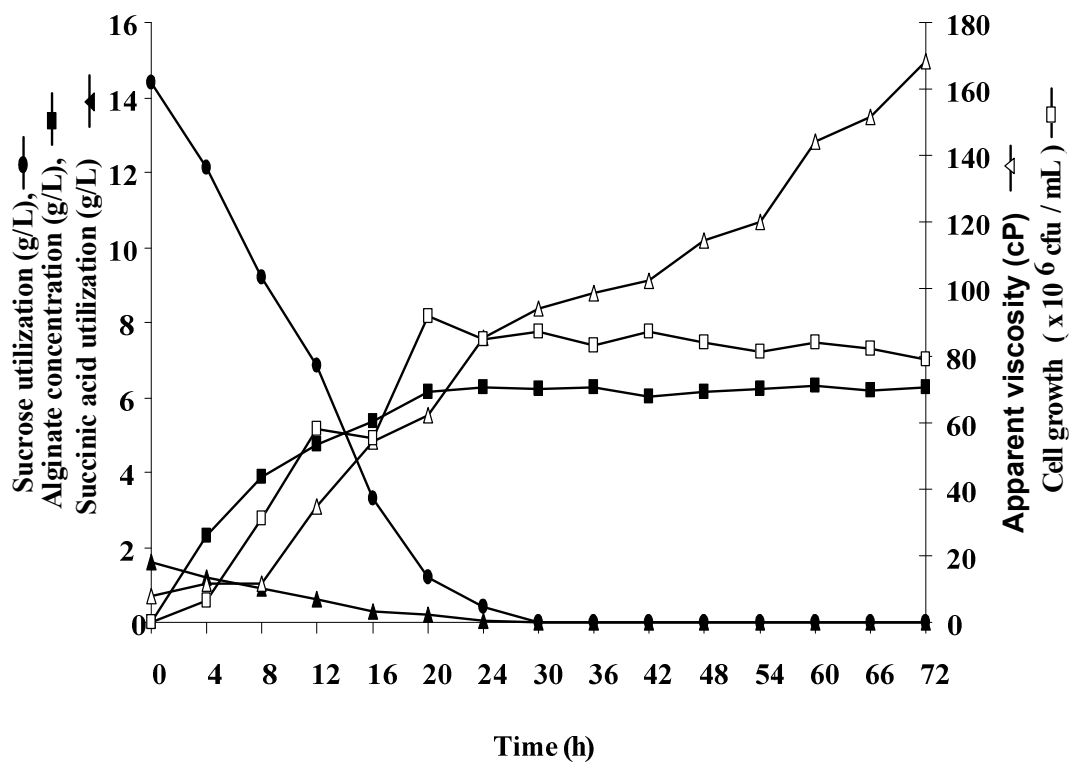
Figure 4.17 Changes of sucrose utilization, alginate production, succinic acid

utilization, apparent viscosity and cell grow of *Azotobacter* sp. in 5L

fermenter with working volume 3L in LG medium with sucrose and

succinic acid.

Table 4.4 Kinetic parameters of maximum growth rate, maximum alginate yield, sugar utilization, succinic acid utilization and apparent viscosity of *Azotobacter* sp. in 2L fermenter which which no adding 0.15% w/v succinic acid, 2L fermenter which adding 0.15% w/v succinic acid and 5L which adding 0.15% w/v succinic acid at 500 rpm of agitation speed with 2.5 vvm in LG medium, pH 6.8 at 30°C.

Kinetic Parameter	Fermentation		
	2L	2L	5L
	No adding 0.15% succinic acid	Adding 0.15% succinic acid	Adding 0.15% succinic acid
Maximum growth rate, μ_{max} (h^{-1})	0.295	0.297	0.221
Maximum alginate yield, $Y_{p/s}$ (g/g)	0.503	0.505	0.397
Sugar utilization (g/L/h)	0.582	0.566	0.514
Succinic acid utilization (g/L/h)	-	0.327	0.370
Apparent viscosity (cP)	207.52	428.28	168.78
Fermentation time (h)	72	72	72

4.5 Enzyme immobilization

4.5.1 Bead formation

Alginate produced by *Azotobacter* sp. was used in enzyme immobilization compared alginate that obtained from Sigma-Aldrich (Germany) found that at 2.5% (w/v) of alginate produced could form bead in $CaCl_2$ solution while seaweed alginate was used at 2% (w/v) due to purification and molecular weight (Figure 4.18).
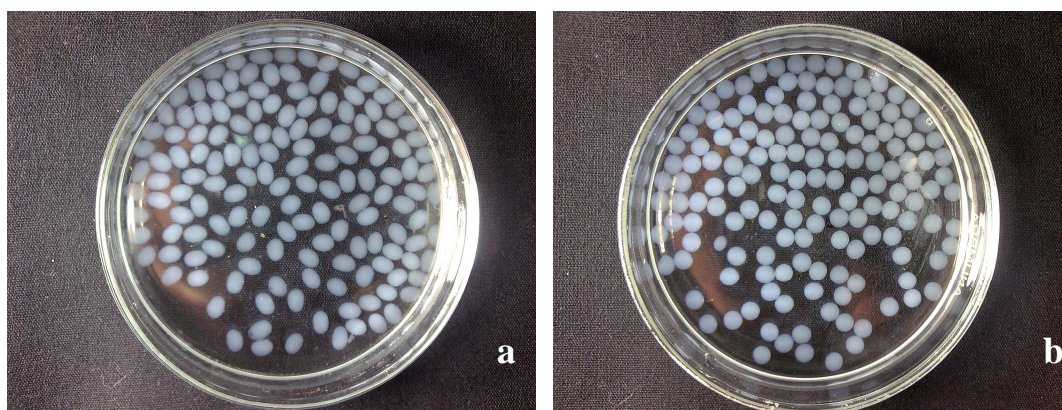
Figure 4.18 Bead immobilized enzyme after dropped in 2% $CaCl_2$ solution a) 2.5% (w/v) of alginate was produced by *Azotobacter* sp. after adding 0.15% (w/v) of succinic acid b) 2% seaweed alginate.

4.5.2 β-amylase activity

Both of *Azotobacter* alginate-coated immobilized β-enzyme and seaweed alginate-coated immobilized enzyme were kept in 0.02 M acetate buffer (pH 4.8) at 4^oC for 7 days before used for study stability of bead immobilized β-amylase enzyme. In Fig 4.19 shows the percentage of relative activity enzyme as function of reuse number for free β-amylase enzyme, *Azotobacter* alginate-coated immobilized β-amylase and seaweed alginate coated immobilized β-amylase found that efficiency in digesting solution starch decreased as number of assays. In 8 times of repeated tests,

the activity of free enzyme does not change while immobilized β-amylase drop over 50% from first time of test. Trend of β-amylase activity in immobilized bead between *Azotobacter* alginate and seaweed alginate decrease also and slightly difference about 5-6% at 8th times. However, it can be seen from the results that the reuse of the immobilized enzyme was up to 8 times due to enzyme loss out from alginate. Leakage can occur from the gel beads. This is influenced by initial alginate concentration, mechanical treatment of the beads and physical shaking produces beads (Smidsrod and Skjak-Braek, 1990). Even through stability of immobilized enzyme less than free enzyme but immobilization offers more advantages over free enzymes, such as easy of handling, relative ease of product separation, prevent of washout, reduced risk of contamination (Konsoula and Kyriakides, 2006; Laca et al., 1998), the possibility of repeated and continuous use of biocatalysts and longevity in contrast to that of free cells (Cruz et al., 2001). These factors lead to a subsequent increase in bioprocess efficiency (Tanaka and Kawamoto, 1999).
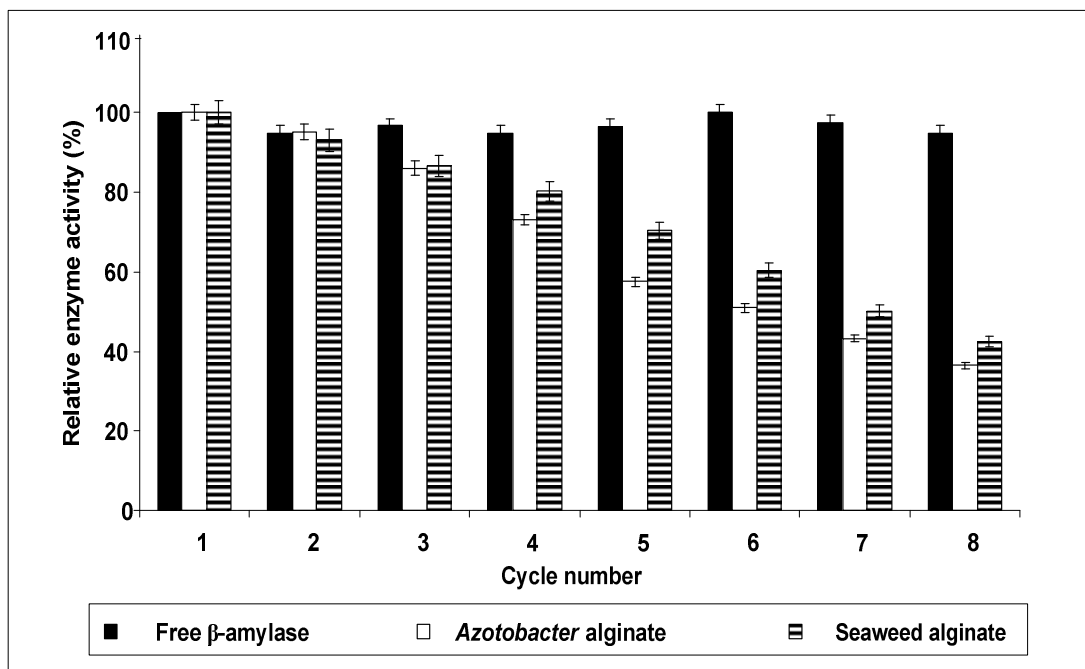
Figure 4.19 Relative β-amylase activity of reused immobilized *Azotobacter* alginate

and seaweed alginate in 8 cycles compared with free enzyme.

CHAPTER V

CONCLUSION

The optimum conditions for alginate production were preliminary studied. In shake flask experiment, to observe the carbon source, temperature, pH and nitrogen source for alginate production, LG medium was used. At 1% (w/v) sucrose or sugarcane was used as carbon source at $30^{o}C$ and initial pH of 6.8 was able to produced alginate about 4.5-5 g/L and apparent viscosity was 62.2-79.15 cP in LG medium. Adding nitrogen source did not increase the alginate yield but did increase cell growth. The optimum condition for alginate production in shake flask experiment was observed in 2L fermenter with working volume 1.5L, to observe optimum aeration and stirrer speed in alginate production. At 500 rpm and aeration 5 vvm gave the highest alginate concentration and viscosity. Alginate production was growth-associated and alginate production was increased within 24th h and stable until fermentation end. *Azotobacter* alginate exhibited non-Newtonian behaviors due to apparent viscosity increase as time (shear rate). In 2L fermenter, the *Azotobacter* alginate higher than that in shake flask due to aeration, inoculum size and stirrer speed. At 0.15% (w/v) of succinic acid was selected to increase apparent viscosity of *Azotobacter* alginate. Apparent viscosity increased was 3-fold while cell growth and alginate production did not difference from original LG medium (did not add succinic acid). All parameters that obtained from shake flask experiment and 2L fermenter, were observed again in 5L fermenter with working volume 3L. The alginate production, apparent viscosity and cell growth of *Azotobacter* sp. similar increased

within 24th h. Alginate production in 5L fermenter was lower than in 2L fermenter. Additionally, the molecular weight of *Azotobacter* alginate was investigated compared with commercial seaweed alginate by using HPLC. The molecular weight of *Azotobacter* alginate was similar to seaweed alginate. At 2.5% (w/v) of *Azotobacter* alginate was used to β-amylase immobilized bead while seaweed was used at 2% (w/v). The efficiency of bacteria alginate and seaweed alginate-coated immobilized β-amylase enzyme decreased over 50% when compared with free β-amylase enzyme. The relative β-amylase activity of immobilized enzyme decreased also.

Results obtained from this research confirmed that alginate could be produced from bacteria and its quality is similar to algae alginate. To produce bacterial alginate commercially it is important to further study the scale up production at industrial scale.

CHAPTER VI

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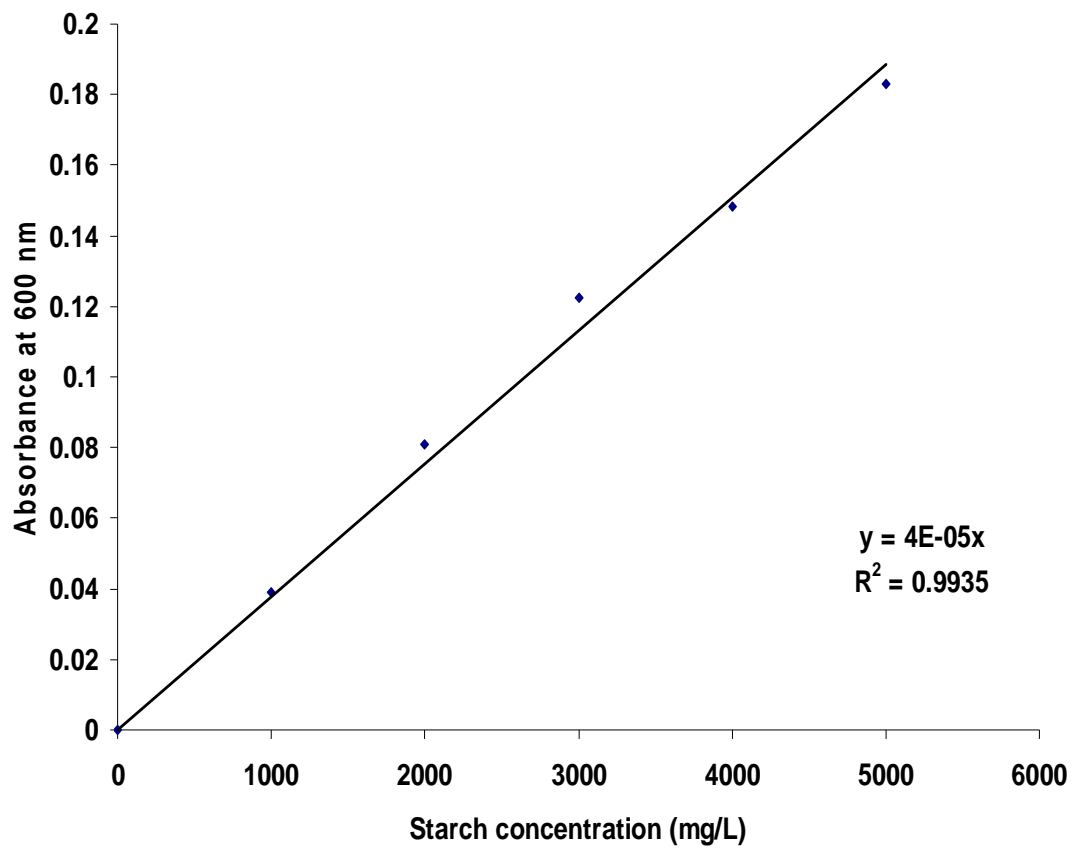
APPENDIX

RESULTS

Figure 1A Standard curve of soluble starch concentration analysis using iodine method.
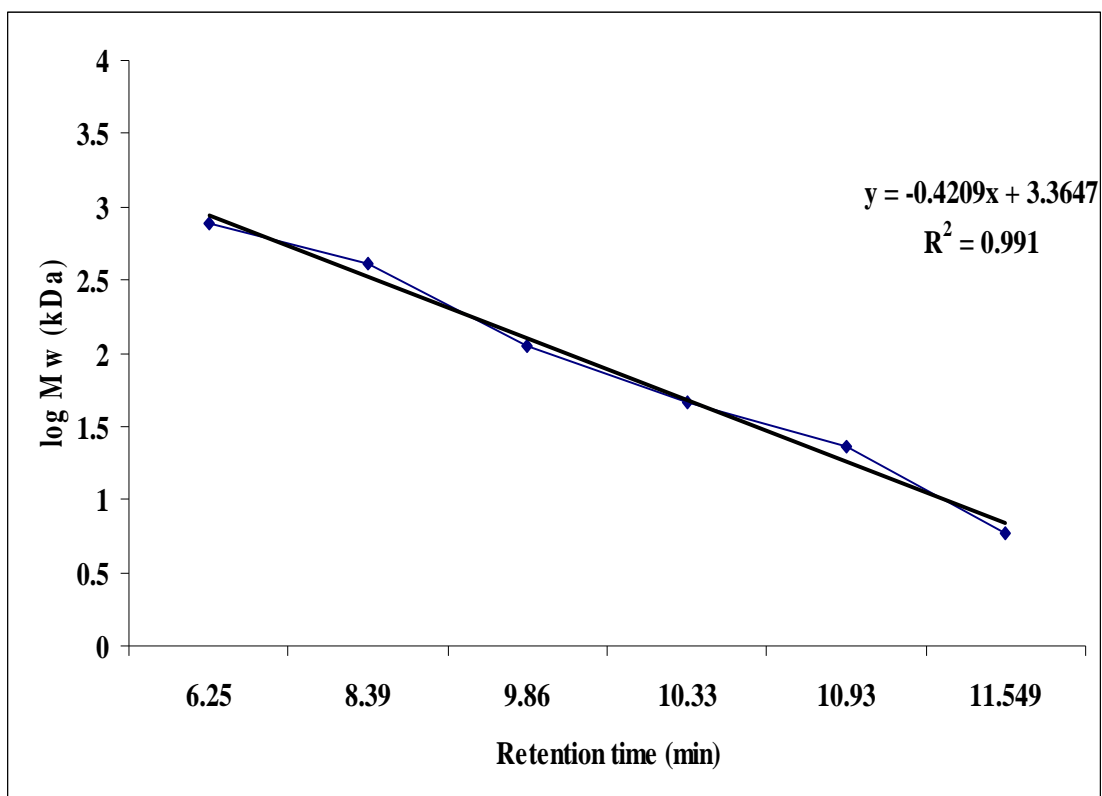
Figure 2A   Chromatogram of molecular mass standard of pullulan with DI water

as mobile phase and RI detection.

BIOGRAPHY

Anyanee Prompaphagorn was born in Nakhonratchasima, Thailand. In 2001, she studied in the Department of Microbiology, Faculty of Science, Khon Kaen University, Khon Kaen. She graduated with a Bachelor of Science in Microbiology in 2005. After graduation, in 2005, she was a Master student in School of Biotechnology at Suranaree University of Technology. She had an experience on poster presentation in the title of "Alginate production by *Azotobacter* sp." of the 34th Congress on Science and Technology of Thailand at Queen Sirikit National Convention Center, Bangkok, Thailand, October 1-4, 2008.