IMMOBILIZATION OF PGPR TO INCREASE EFFICIENCY OF PLANT GROWTH PROMOTION IN HYDROPONIC SYSTEM

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การตรึงเซลล์แบคทีเรียกลุ่ม PGPR เพื่อเพิ่มประสิทธิภาพในการส่งเสริมการเจริญเติบโตของพืชในระบบไฮโดรโพนิกส์

นางสาวนารีรัตน์ มาอ้น

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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IMMOBILIZATION OF PGPR TO INCREASE EFFICIENCY OF PLANT GROWTH PROMOTION IN HYDROPONIC SYSTEM

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นารีรัตน์ มาอ้น : การตรึงเซลล์แบคทีเรียกลุ่ม PGPR เพื่อเพิ่มประสิทธิภาพในการส่งเสริมการเจริญเติบโตของพืชในระบบไฮโดรโพนิกส์ (IMMOBILIZATION OF PGPR TO INCREASE EFFICIENCY OF PLANT GROWTH PROMOTION IN HYDROPONIC SYSTEM) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.โชคชัย วนภู, 111 หน้า.

Plant Growth Promoting Rhizobacteria หรือ PGPR เป็นกลุ่มของแบคทีเรียหลากหลาย species ที่สามารถช่วยกระตุ้นการเจริญเติบโตในพืชได้ แต่แบคทีเรียกลุ่ม PGPR นั้นมีความสามารถในการอยู่รอด และเพิ่มจำนวนในดินต่ำเมื่อเทียบกับจุลินทรีย์ดั้งเดิมที่อยู่ในพื้นที่นั้นๆ เนื่องจากการตรึงเซลล์สามารถเพิ่มอัตราการอยู่รอดของจุลินทรีย์ได้ และไม่เป็นพิษต่อเซลล์ เป็นวิธีการที่ง่าย ค่าใช้จ่ายในการทำน้อย วัตถุประสงค์ของการศึกษาครั้งนี้เพื่อประยุกต์ใช้เซลล์ PGPR ที่ถูกตรึงในแคลเซียม-อัลจิเนท เพื่อศึกษาการส่งเสริมการเจริญเติบโตของพืชในระบบไฮโดรโปนิกส์ การดำเนินการครั้งนี้เลือกใช้แบคทีเรีย *Azotobacter* sp. เป็นตัวแทนของ PGPR เซลล์ *Azotobacter* sp. ถูกตรึงในแคลเซียม-อัลจิเนท แล้วนำไปทดสอบการอยู่รอดของ *Azotobacter* sp. ที่ถูกตรึง พบว่า *Azotobacter* sp. สามารถอยู่รอดได้นานกว่า 3 สัปดาห์ ในอาหารเลี้ยงเชื้อสูตร LG ที่ pH เริ่มต้น 4-7 แต่ที่ pH เริ่มต้น 3.5 พบเพียงหนึ่งสัปดาห์ ซึ่ง *Azotobacter* sp. อิสระไม่สามารถอยู่รอดได้ และทำการศึกษาการผลิตฮอร์โมนออกซิน (Auxin) หรือ Indole-3-acetic acid (IAA) ในอาหารเลี้ยงเชื้อสูตร LG ที่เติม L-tryptophan พบว่า เซลล์ *Azotobacter* sp. ถูกตรึงสามารถผลิต IAA ได้ และผลิตได้มากขึ้น เมื่อเติมกลูโคส และแหล่งไนโตรเจนในอาหารเลี้ยงเชื้อสูตร LG สำหรับอุณหภูมิที่เหมาะสมในการผลิต IAA โดย *Azotobacter* sp. ที่ถูกตรึง คือ 20 องศาเซลเซียส และ pH เริ่มต้นของอาหารเลี้ยงเชื้อสูตร LG เท่ากับ 6.5-7.5

การศึกษาครั้งนี้ได้ทำการศึกษาผลของ *Azotobacter* sp. ที่ตรึงในแคลเซียม-อัลจิเนทต่อการเจริญเติบโตของพืชในระบบไฮโดรโปนิกส์ 2 รอบการทดลอง โดยใช้ผักกวางตุ้งเป็นต้นแบบ โดยแต่ละรอบการทดลองแบ่งเป็น 2 วิธีคือ 1) *Azotobacter* sp. ที่ตรึงในแคลเซียม-อัลจิเนทถูกนำไปแทนที่วัสดุปลูกในถ้วยปลูก 2.5-12.5 กรัมเจลต่อต้น และ 2) สารละลาย IAA ความเข้มข้น 10-100 μM ถูกเติมให้พืช 1 มิลลิลิตร/ต้น/สัปดาห์ และทำการสำรวจปัจจัยชี้วัดการเจริญเติบโตของพืช ซึ่งประกอบด้วย น้ำหนักสดต้น, น้ำหนักสดราก, น้ำหนักแห้งต้น, น้ำหนักแห้งราก, ความยาวต้น, ความยาวราก และวิเคราะห์ความเข้มข้นของธาตุอาหารในต้นพืช (ไนโตรเจน, ฟอสฟอรัส, โพแทสเซียม และแคลเซียม) จากผลการศึกษาในครั้งที่ 1 พบว่าทั้ง *Azotobacter* sp. ที่ตรึงในแคลเซียม-อัลจิเนท และสารละลาย IAA สามารถส่งเสริมการเจริญเติบโตของพืชได้ดีกว่าในต้นควบคุม ($P \leq 0.05$) การนำ *Azotobacter* sp. ที่ตรึงในแคลเซียม-อัลจิเนท 2.5 กรัมเจลต่อต้น ใส่ในถ้วยปลูกสามารถส่งเสริมการเจริญเติบโตของพืชได้ดีที่สุด สำหรับการเติมสารละลาย IAA 100 μM

1 มิลลิลิตรต่อต้นต่อสัปดาห์ ทำให้พืชมีการเจริญเติบโตดีที่สุด และการศึกษาผลของ *Azotobacter* sp. ที่ถูกตรึงต่อการเจริญเติบโตของพืชในระบบไฮโดรโปนิกส์ครั้งที่ 2 พบว่า การนำ *Azotobacter* sp. ที่ตรึงในแคลเซียม-อัลจิเนท 5 กรัมเจลต่อต้น ใส่ในถ้วยปลูกสามารถส่งเสริมการเจริญเติบโตของพืชได้ดีที่สุด และสำหรับการเติมสารละลาย IAA ที่ความเข้มข้น 100 μM 1 มิลลิลิตรต่อต้นต่อสัปดาห์ พืชมีการเจริญเติบโตดีที่สุด นอกจากนี้ได้ทำการศึกษาผลของ *Azotobacter* sp. ที่ถูกตรึง ต่อการปลูกพืชในห้องแสง สำหรับสภาวะที่เหมาะสมต่อการส่งเสริมการเจริญเติบโตของพืชมากที่สุด คือ การประยุกต์ใช้ *Azotobacter* sp. ที่ถูกตรึง 5 กรัมเจลต่อต้น และการเติมสารละลาย IAA ให้พืชที่ความเข้มข้น 40-60 μM 1 มิลลิลิตรต่อต้นต่อสัปดาห์

สาขาวิชาเทคโนโลยีชีวภาพ
ปีการศึกษา 2552

ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____
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NAREERAT MA-ON : IMMOBILIZATION OF PGPR TO INCREASE EFFICIENCY OF PLANT GROWTH PROMOTION IN HYDROPONIC SYSTEM. THESIS ADVISOR : ASST. PROF. CHOKCHAI WANAPU, Ph.D., 111 PP.

IMMOBILIZATION/ INDOLE-3-ACETIC ACID/ PLANT GROWTH PROMOTION/ PLANT GROWTH PROMOTING RHIZOBACTERIA

Plant growth-promoting rhizobacteria (PGPR) are free-living bacteria that can promote plant growth, because of its weak competitive ability as compared to many other microorganisms in the soil during plant growth. Immobilization provides a gentle, simple, rapid, nontoxic, versatile and cheap method of immobilizing bacterial cell. In addition, this technique is ensuring high retention of cell viability. The objective of this experiment was to apply immobilized PGPR for promoting plant growth in hydroponic system. *Azotobacter* sp., a representative of PGPR strains, it was immobilized in calcium-alginate. The immobilized *Azotobacter* sp. was able to survive in LG medium longer than three weeks in pH 4-7, and a week in pH 3.5. The immobilized gel could protect toxicity from high acidity while the free cells were killed. The IAA production by immobilized *Azotobacter* sp. was tested in LG medium containing L-tryptophan, glucose and nitrogen, pH 6.8. The optimal temperature for IAA production by immobilized *Azotobacter* sp. was 20 ^{o}C at initial pH 6.5-7.5.

This study observed the effect of immobilized *Azotobacter* sp. on plant growth in hydroponic system for two batches. To estimate plant growth parameters, two factors were tested: i) immobilized *Azotobacter* sp. in the series of 2.5-12.5 g bead/ plant, and ii) IAA solution of 10-100 μM concentration. Choy sum (*Brassica*

chinensis var *parachinensis*) was used as a model to study the effect of immobilized *Azotobacter* sp. on plant growth. The parameter of plant growth, i.e. stem wet weight, root wet weight, stem dry weight, root dry weight, stem length, root length and element analysis (N, P, K and Ca) was investigated. In the first batch, results showed that all treatments were able to promote plant growth better than the control group but there were no significant differences ($P \leq 0.05$). However, the optimum condition for growth of Choy sum in hydroponic system showed that 2.5 g of immobilized *Azotobacter* sp. bead per plant and 100 μM IAA solution (1 ml/plant/week), manifested the best condition for stimulating plant growth. In the second batch, the optimum condition for growth of Choy sum in hydroponic system showed in 5 g of immobilized *Azotobacter* sp. bead per plant and 100 μM IAA solution (1 ml/plant/week). Furthermore, the effect of immobilized *Azotobacter* sp. was studied in a light controlled room, and the result revealed that 5 g of immobilized *Azotobacter* sp. bead per plant and 40-60 μM IAA solution (1 ml/plant/week) yielded the highest growth.

School of Biotechnology

Academic Year 2009

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LIST OF ABBREVIATIONS

°C	degree Celsius
µM	micromolar
A.	*Azotobacter*
Ag.	*Agrobacterium*
Ar.	*Arabidopsis*
Az.	*Azospirillum*
Ca	calcium
E.	*Enterobacter*
et al.	Et alia (and other)
g	gram
IAA	indole-3-acetic acid
K	potassium
l	litre
mg	milligram
ml	milliliter
N	nitrogen
P	phosphorus
P.	*Psuedomonas*
Pa.	*Pantoea*
SEM	scanning electron microscope

CHAPTER I

INTRODUCTION

1.1 Significance of the study

Intensive farming practices, that warrant high yield and quality, require extensive use of chemical fertilizers, which are costly and create environmental problems. Therefore, more recently there has been a resurgence of interest in environmental friendly, sustainable and organic agricultural practices (Esitken et al., 2005). However, yield reduction is an important problem in organic production system (Lind et al., 2003). Use of biofertilizers containing beneficial microorganisms instead of synthetic chemical are known to improve plant growth through supply of plant nutrients and may help to sustain environmental health and soil productivity. So far, considerable number of bacterial species mostly associated with the plant rhizosphere, have been tested and found to be beneficial for plant growth, yield and crop quality. They have been called "plant growth promoting rhizobacteria (PGPR)" (O'Connell, 1992).

PGPR is a heterogeneous group of bacteria that can be found in the rhizosphere, at root surfaces and in association with roots, which can improve the extent or quality of plant growth directly and or indirectly. In last few decades a large group of bacteria including species of *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligens, Arthobacter, Burkholderia, Bacillus* and *Serratia* have reported to enhance plant growth (Kloepper et al., 1989; Okon and

Labandera-Gonzalez, 1994; Glick, 1995). The direct promotion by PGPR entails either providing the plant with a plant growth promoting substances that is synthesized by the bacterium or facilitating the uptake of certain plant nutrients from the environment. The indirect promotion of plant growth occurs when PGPR lessen or prevent the deleterious effect of one or more phytopathogenic microorganisms.

Production of plant growth regulators by inoculation with PGPR has also been suggested as one of the most plausible mechanism of action affecting plant growth. Numerous studies have shown the improvement in plant growth and development in response to seed or root inoculation with various microbial inoculants capable of producing plant growth regulators (Zahir et al., 2004).

However, it has been shown that the organism performs by colonization of the plant roots, the longest demonstrated in artificial and sterilized subtrates (Batinic et al., 1998, Grosch et al., 1999). This is often regarded as a consequence of its weak competive ability as compared to many other microorganisms in the soil during plant growth (Grosch et al., 1999).

One of the main problems of microbial introduction in practice is that the applied microorganisms sometimes do not survive, or do not execute their specific function (Jagnow, 1987; van Elsas and Heijnen, 1990; Jagnow et al., 1991; van Veen et al., 1997). Nor does successful introduction of a PGPR strain to one soil-plant environment guarantee its survival in another (van Elsas and Heijnen, 1990; Jagnow et al., 1991; Kravchenko and Makarova, 1993; Pillay and Nowak, 1997). Similar problems are also reported for hydroponic or soilless systems of plant growth (Paulitz, 1997; Paulitz and Belanger, 2001).

Immobilization

Immobilized cell technology has a large potential for using in biotechnological processes and immobilization of bacteria has been shown to be a valuable tool for increasing the performance of microbial bioreactors (Groboillot et al., 1994). Different types of immobilization methods have been suggested. A popular method for immobilization of bacteria is entrapment in gel materials especially in seaweed gel-materials like carrageenan or alginate (Willaert and Baron, 1996). This provides a gentle, simple, rapid, nontoxic, versatile and cheap method of immobilizing bacteria (Nilsson, 1987; Palmieri et al., 1994; Park and Khang, 1995). In addition, this technique is ensuring high retention of cell viability. Immobilized bacteria in gels like alginate are surrounded by a gel network, which strongly limits their movement.

1.2 Research objectives

The purposes of this study are as follows:

1. To apply immobilized PGPR for promoting plant growth in hydroponic system
2. To determine the efficiency of IAA production by immobilized PGPR
3. To analyze the structural properties of immobilized PGPR in alginate beads

1.3 Research hypothesis

The hypothesis of this study is immobilization technique could enhance the viability of PGPR in hydroponic system. Moreover, the immobilization technology could increase the efficiency of plant growth by PGPR. And the immobilized PGPR could be applied to promote plant growth in hydroponic system.

1.4 Scope and limitation of the study

Azotobacter sp. was immobilized in form of Ca-Alginate bead. The immobilized PGPR were analyzed including of optimum pH for indole acetic acid (IAA) production, pH stability, optimum temperature for IAA production, viability of PGPR in bead and density of cell in the bead. Morphology of immobilized cell bead was considered by electron microscope. The immobilized *Azotobacter* sp. was applied in hydroponic system and efficiency of plant growth promotion was determined.

1.5 Expected Results

- Immobilized PGPR can produce IAA to promote plant growth in hydroponic system.

- The immobilization can increase availability of PGPR in hydroponic system.

CHAPTER II

LITERATURE REVIEW

2.1 Plant Growth Promoting Rhizobacteria

Plant growth-promoting rhizobacteria (PGPR) are free-living, soil-borne bacteria, isolated from the rhizosphere, when applied to seeds or crops, enhance the growth of the plant or reduce the damage from soil-borne plant pathogens (Kloepper, 1980). It has been estimated that more than 100 million tonnes of nitrogen, potash and phosphate-chemical fertilizers have been used annually in order to increase plant yield (1999). The potential negative effect of chemical fertilizers on the global environment and the cost associated with production has led to research with the objective of replacing chemical fertilizers with bacterial inoculants.

Bacterial inoculants which help in plant growth are generally considered to be of two types a) symbiotic and b) free-living (Frommel, 1991; Kloepper and Frauley, 1988). Beneficial free-living bacteria referred to as PGPR, were found in the rhizosphere of the roots of many different plants (Kloepper, 1989). Breakthrough research in the field of PGPR occurred in the mid 1970s with studies demonstrating the ability of *Pseudomonas* strains capable of controlling soil-borne pathogens to indirectly enhance plant growth and increase the yield of potato and radish plants (Burr, 1978; Howie, 1983; Kloepper, 1980; Kloepper, 1981). The effect of PGPR on agricultural crops has been investigated and published by various authors in the last two decades with recent applications on trees (Bashan, 1998; Enebak, 1998). During

1983 and 1984 more than 4,000 bacterial strains were isolated from the rhizosphere of plants grown in the Canadian High Arctic and screened for the ability to fix nitrogen. Some strains demonstrated the ability to reduce acetylene and colonize roots of canola when grown at low temperatures (Lifshitz, 1986). Strains which exhibited the potential to be PGPRs were identified as *Pseudomonas putida*, *P. putida* biovar B, *P. fluorescens*, *Arthobacter citreus* and *Serratia liquefaciens* (Kloepper and Frauley, 1988; Lifshitz, 1986). The ability of these strains to be used as bacterial inoculants in agriculture was tested in greenhouse and field trials with different formulations and they increased the yield of canola in both types of trial. Salamone (2000) reported the growth-promoting effect of *P. fluorescens* strain G20-18 on wheat and radish plants by production of cytokinin phytohormones. As the effect of PGPR on plants was demonstrated, the concept of PGPR began to gain importance and a large number of bacterial strains have been isolated, screened (Bertrand, 2001; Cattelan, 1999; Chanway, 1993) and evaluated for plant growth promotion (Abbas, 1993; Bashan, 1998; Bent, 2001; Chanway, 1989; Glick, 1997; Lifshtiz, 1987; Mayak, 1999; Salamone, 2000; Zhang, 1997).

Previous research has shown the practicality of introducing PGPR into commercial peat-based substrates for vegetable production in order to increase plant vigor, control root diseases and increase yields (Gagne´ et al., 1993; Kloepper et al., 2004; Kokalis-Burelle, 2003; Kokalis-Burelle et al., 2002a; Kokalis-Burelle et al., 2003; Kokalis-Burelle et al., 2002b; Nemec et al., 1996). In Florida various PGPR increased the growth of tomato (*Lycopersicon esculentum*) and pepper (*Capsicum annuum*) under greenhouse production significantly (Kokalis-Burelle et al., 2002b). As a result of increased growth, the time required to produce a standard sized

transplant was reduced for greenhouse applications. Also, transplant vigor and survival in the field were improved by PGPR treatments in both tomato and pepper. Trials conducted on muskmelon (*Cucumis melo*) and watermelon (*Citrullus lanatus*) resulted in reduction of root knot nematode disease severity with several PGPR formulations (Kokalis-Burelle et al., 2003). PGPR amended strawberry (*Fragaria ananassa*) plug transplants consistently had higher overall yields compared to nonamended plugs and bare root transplants, with bare-root plants having the lowest yields in 3 years of field studies. Additionally, under stress or phytotoxic conditions the PGPR amended plugs performed better than non amended plugs and bare root transplants (Kokalis-Burelle, 2003). Because typical disease control levels observed with PGPR are less than those achieved with chemicals, it is feasible to utilize PGPR as components in integrated management systems that include reduced rates of chemicals and cultural control practices. This approach is becoming increasingly more important as many agricultural chemicals undergo intense analysis with regards to their human toxicity and environmental impact.

PGPR can also stimulate growth and increase yield in apple, citrus, high bush blueberry, mulberry and apricot (De Silva et al., 2000; Esitken et al., 2002; Esitken et al., 2003; Kloepper, 1994; Sudhakar et al., 2000a). The symbiotic fixation of N_2 through inoculation of legume crops with effective rhizobia is well known (Dobereiner, 1997; Vance, 1997). Asymbiotic N_2-fixing bacteria, which live in the rhizosphere (Dobereiner, 1997; Schilling et al., 1998) and/or endophytically (Hecht-Buchholz, 1998), often increase yields of crops. Many bacterial species have N_2-fixing properties, including *Bacillus* spp., *Azotobacter* spp., *Azospirillum* spp., *Beijerinckia* spp., *Pseudomonas* spp., etc. (Dobereiner, 1997; Reis et al., 1994; Vance,

1997). Nitrogen-fixing bacteria have been used as foliar applications in mulberry (Sudhakar et al., 2000b) and apricot (Esitken et al., 2002; 2003).

Rhizosphere bacteria promote plant growth and yield either directly or indirectly (Glick, 1995; Kloepper, 1989). The direct mechanisms of plant growth promotion may involve the synthesis of substances by the bacterium or facilitation of the uptake of nutrients from the environment (Glick, 1999). The indirect promotion of plant growth occurs when PGPR lessen or prevent the deleterious effects of plant pathogens on plants by production of inhibitory substances or by increasing the natural resistance of the host (Cartieaux, 2003; Handelsman, 1996; Nehl, 1996).

The direct growth promoting mechanisms are as follows; i) nitrogen fixation, ii) solubilization of phosphorus, iii) sequestering of iron by production of siderophores, iv) production of phytohormones such as auxins, cytokinins, gibberellins, and v) lowering of ethylene concentration (Glick, 1995; Glick, 1999; Kloepper, 1989). For example, *P. putida* GR12-2, isolated from the rhizosphere of plants growing in the Canadian High Arctic, was found to promote growth of *Canola cv. Tobin* by fixing nitrogen and enhancing the uptake of phosphate under gnotobitoic conditions (Lifshitz, 1986; Lifshtiz, 1987), by synthesizing siderophores that can solubilize and sequester iron from the soil and supply it to the plants (Glick) (1995), by production of the phytohormone IAA (Xie, 1996) and by lowering of ethylene concentration via production of the enzyme ACC deaminase (Glick, 1994b).

The indirect mechanisms of plant growth promotion by PGPR include i) antibiotic production, ii) depletion of iron from the rhizosphere iii) synthesis of antifungal metabolites, iv) production of fungal cell wall lysing enzymes v) competition for sites on roots, and vi) induced systemic resistance (Dunne, 1993;

Glick, 1999; Kloepper and Frauley, 1988; Liu, 1995; Weller, 1986).

One of the direct mechanisms by which PGPR promote plant growth is by production of plant growth regulators or phytohormones (Glick, 1995). Frankenberger (1995) have discussed in detail the role of auxins, cytokinins, gibberellins, ethylene and absicisic acids (ABA) which, when applied to plants, help in increasing plant yield and growth. Microbial production of individual phytohormones such as auxins and cytokinins has been reviewed by various authors over the last 20 years (Barbieri, 1993; Fallik, 1989; Hartmann, 1983; Patten, 1996; Patten, 2002; Pilet, 1979).

As this thesis is focussed on production of phytohormones to promote plant growth especially auxin are reviewed in detail in the following sections.

2.2 Auxins

Among the plant hormones know today, auxin (from the Greek term, *auxein*, to increase) was the first phytohormone recognized and chemically detected in the 19th century. A number of indole compounds and phenylacetic derivatives have been reported with auxin activity. Among these, indole-3-acetic acid (IAA) is considered the most physiologically active auxin in plants. Auxins are involved in a variety of diverse plant growth and developmental responses; however, their mechanisms of action are not fully understood. Today, several synthetic auxins are used in commercial applications. For instance, indole-3-butyric acid (IBA) and naphthaleneacetic acid (NAA) are used commercially for initiation of rooting, especially in certain difficult-to-root cuttings.

The production of an active substance by the fungi *Rhizopus suinus* and *Absidia ramosa* was identified to be auxin by Thimann (1935), following induction of

curvature in *Avena* as demonstrated by Nielsen (Frankenberger, 1995). Various authors have identified the production of indole-3-acetic acid by microorganisms in the presence of the precursor tryptophan or peptone.

Some of the plant responses to auxin are as follows: a) cell enlargement b) cell division c) root initiation d) root growth inhibition e) increased growth rate f) phototropism g) geotropism and h) apical dominance (Frankenberger, 1995).

Eighty percent of microorganisms isolated from the rhizosphere of various crops have the ability to produce auxins as secondary metabolites (Kampert, 1975; Loper and Schroth, 1986). Bacteria belonging to the genera *Azospirillum*, *Pseudomonas*, *Xanthomonas*, and *Rhizobium* as well as *Alcaligenes faecalis*, *Enterobacter cloacae*, *Acetobacter diazotrophicus* and *Bradyrhizobium japonicum* have been shown to produce auxins which help in stimulating plant growth (Patten, 1996). Various metabolic pathways such as a) indole-3-acetamide pathway b) indole-3-pyruvic acid pathway c) tryptophan side chain pathway d) tryptamine pathway and e) indole-3-acetonitrile pathway are involved in the production of IAA.

Phytopathogens such as *Agrobacterium tumefaciens*, *Ag. rhizogenes* and *P. syringae pv. savastanoi* synthesize IAA via the indole-3-acetamide pathway (Liu, 1982; Offringa, 1986). Koga *et al*. (1991 b) suggested that *E. cloacae*, isolated from the rhizosphere of cucumber, synthesized IAA via the indolepyruvic acid pathway and promoted growth of various agricultural plants. *P. fluorescens* demonstrated the ability to convert L-tryptophan directly into indole-3-acetaldehyde (Narumiya, 1979). Strains such as *B. cereus* and *Az. brasilense* produced IAA by the tryptamine pathway (Hartmann, 1983; Perley, 1966a).

Bacterial production of IAA suggests that the pathways involved in IAA

production may play an important role in defining the effect of the bacterium on the plant (Patten, 1996). Glick et al. (1999) reported that the most of pathogenic strains of bacteria synthesize IAA via the indoleacetamide pathway while plants use the indolepyruvic acid pathway. This helps the bacteria to evade plant regulatory signals and thus the IAA produced induces uncontrolled growth in plant tissues. In contrast the benefical bacteria such as PGPR synthesize IAA via the indolepyruvic acid pathway and the IAA secreted is thought to be strictly regulated by the plant regulatory signals.

Differences in the production of IAA among bacterial strains can be attributed to the various biosynthetic pathways, location of the genes involved, regulatory sequences, and the presence of enzymes to convert active free IAA into conjugated forms. It is also dependent on environmental conditions (Patten, 1996).

Tien et al., (1979) demonstrated that production of IAA and indole lactic acid by *Az. brasilense* Sp13t SR2 increased with increasing concentrations of tyrptophan (1 - 100 µg/ml). In contrast the production of IAA and IBA by cultures of *Az. brasilense* in the absence of tryptophan was identified using gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) (Fallik, 1989). Synthesis of IAA by *Rhizobium* spp. in the presence and absence of tryptophan has also been demonstrated (Kittel, 1989). PGPR strain G20-18 and two mutants produced IAA in pure culture (Salamone, 2000). Bent et al., (2001) reported that the production of indole compounds by three different strains, *Paenibacillus polymyxa* L6, *P. poly*myxa Pw-2, and *P. fluorescens* M20 increased in concentration with increasing concentrations of tryptophan (0-200 mg/ml) at different times. Reports by Asghar et al., (2002) showed that PGPR strains produced 24.6 µg/ml of auxins in the presence

of the precursor L-tryptophan in the medium, which was 184-fold more than that without L-tryptophan.

Hartmann et al. (1983) showed that *Az. brasiliense* resistant to 5-flurotryptophan excreted 30 times higher levels of auxins compared to the wild type. Mutants grown under nitrogen fixing conditions excreted 1 μg/ml of IAA, 10 times more than the wild type. There was no difference in levels of auxin production between the mutant and wild type strain of *Az. lipoferum,* indicating differences in the tryptophan and IAA biosynthetic pathway between the two species (Hartmann, 1983). Recent studies by Patten and Glick (2002) showed IAA production by the wild-type and an IAA-deficient mutant of *P. putida* GR12-2 in the presence of varying concentrations of tryptophan. The levels of IAA secreted by the wild type ranged from 0.5 – 32.7 μg/ml, while for the mutant it ranged from 0.5 – 2 μg/ml. Similar results were reported by Asghar et al. (2002) who found that PGPR produced 24.6 μg/ml of auxins in the presence of the precursor L-tryptophan, which was 184-fold more than that without L-tryptophan.

2.2.1 IAA biosynthesis pathways in bacteria

With the analysis of additional bacterial species, different bacterial pathways to synthesize IAA have been identified. A high degree of similarity between IAA biosynthesis pathways in plants and bacteria was observed. Here an overview of bacterial IAA biosynthesis pathways is given (Fig. 1), and the current status of related genes, proteins and intermediate metabolites is discussed. Where relevant, comparisons with plant IAA biosynthesis are made.

Tryptophan has been identified as a main precursor for IAA biosynthesis pathways in bacteria. The identification of intermediates led to the identification of

five different pathways using tryptophan as a precursor for IAA.

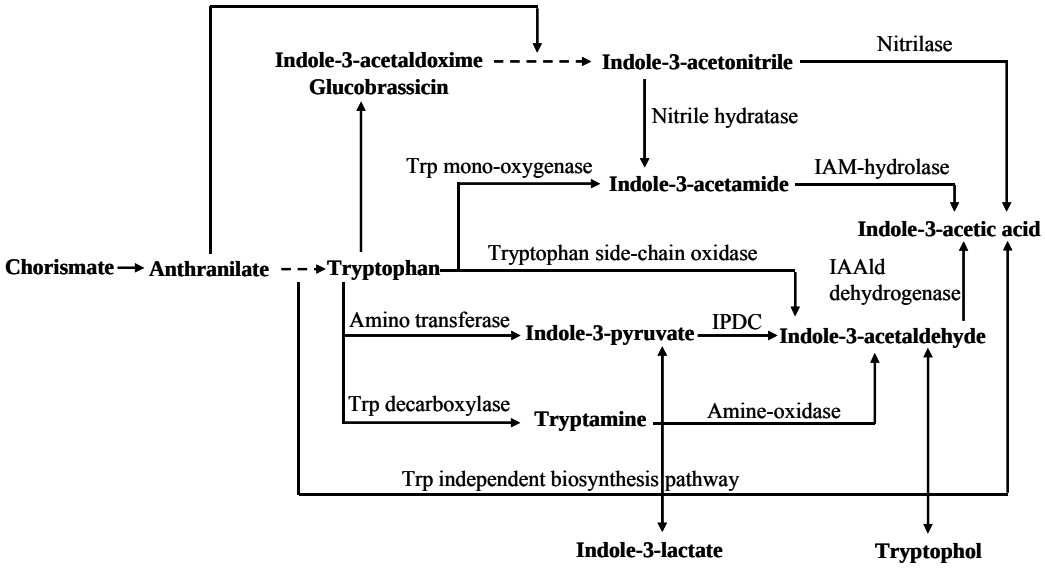

Figure 1. Overview of the different pathways to synthesize IAA in bacteria. The intermediate referring to the name of the pathway or the pathway itself is underlined with a dashed line. IAAld, indole-3-acetaldehyde; IAM, indole-3-acetamide; IPDC, indole-3-pyruvate decarboxylase; Trp, tryptophan.

2.2.1.1 Indole-3-acetamide pathway

The indole-3-acetamide (IAM) pathway is the best characterized pathway in bacteria. In this two-step pathway tryptophan is first converted to IAM by the enzyme tryptophan-2-monooxygenase (IaaM), encoded by the *iaaM* gene. In the second step IAM is converted to IAA by an IAM hydrolase (IaaH), encoded by *iaaH*. The genes *iaaM* and *iaaH* have been cloned and characterized from various bacteria, such as *Ag. tumefaciens, P. syringae, Pantoea agglomerans, Rhizobium and Bradyrhizobium* (Clark, 1993; Morris, 1995; Sekine, 1989; Theunis, 2004). The IAM-related genes

have been detected on the chromosome in different *Pseudomonas* species as well as on plasmids such as pPATH of *Pa. agglomerans* (Glickmann, 1998; Manulis, 1998).

The IAM pathway was described previously as a bacterial specific pathway, as no evidence for this pathway could be found in plants. However, with an improved, highly sensitive method for the analysis of IAM using a combination of HPLC and GC-MS/MS techniques it was proven beyond doubt that IAM is an endogenous compound of *Arabidopsis thaliana* (Pollmann, 2002). Experiments described by Piotrowski et al., (2001) and Pollmann et al., (2003) further support the operation of the IAM pathway in *Arabidopsis*.

2.2.1.2 Indole-3-pyruvate pathway

The indole-3-pyruvate (IPyA) pathway is thought to be a major pathway for IAA biosynthesis in plants. However, the key genes/enzymes have not been identified yet in plants. In bacteria, IAA production via the IPyA pathway has been described in a broad range of bacteria, such as the pythopathogenic bacterium *Pa. agglomerans*, the beneficial bacteria *Bradyrhizobium, Azospirillum, Rhizobium* and *Enterobacter cloacae*, and cyanobacteria. The first step in this pathway is the conversion of tryptophan to IPyA by an aminotransferase (transamination). In the rate-limiting step, IPyA is decarboxylated to indole-3-acetaldehyde (IAAld) by indole-3-pyruvate decarboxylase (IPDC). In the last step IAAld is oxidized in IAA (Fig. 1). The gene, encoding for the key enzyme, IPDC, has been isolated and characterized from *Az. brasilense*, *En. cloacae*, *P. putida* and *Pa. agglomerans* (Brandl, 1996; Costacurta, 1994; Koga, 1991 b; Patten, 2002). In *A. lipoferum*, the *ipdC* gene is located on the chromosome (Blaha, 2005) but in most cases the genome localization of this gene has not been determined. In these organisms, insertional inactivation of the pathway

resulted in a lower IAA production, up to 90% reduction in *Az. brasilense* (Prinsen, 1993), indicating the importance of the IPyA pathway in auxin production. However, no mutants completely abolished in IAA biosynthesis could be constructed, indicating redundancy for IAA biosynthesis pathways.

2.2.1.3 Tryptamine pathway

In bacteria, the tryptamine (TAM) pathway has been identified in *Bacillus cereus* by identification of tryptophan decarboxylase activity (Perley, 1966b) and in *Azospirillum* by detection of the conversion of exogenous tryptamine to IAA (Hartmann, 1983). In plants tryptamine was identified as an endogenous compound and genes encoding for tryptophan decarboxylases (catalyzing the decarboxylation of tryptophan to tryptamine) have been cloned and characterized from different plants, indicating an IAA biosynthetic pathway via tryptamine in plants. The rate-limiting step for this pathway in plants is probably catalyzed by a flavin monooxygenase-like protein (YUCCA) (conversion of tryptamine to N-hydroxyl-tryptamine). The presence of the intermediates, which are downstream of N-hydroxyl-tryptamine (presumably indole-3-acetaldoxime and indole-3-acetaldehyde), still needs to be confirmed (Bak, 2001; Zhao, 2001). The last step of this pathway in bacteria is different to that in plants: in bacteria TAM is directly converted to IAAld by an amine oxidase (Hartmann, 1983).

2.2.1.4 Tryptophan side-chain oxidase pathway

Tryptophan side-chain oxidase (TSO) activity has only been demonstrated in *P. fluorescens* CHA0. In this pathway tryptophan is directly converted to IAAld by passing IPyA, which can be oxidized to IAA (Oberhansli, 1991). There are no indications that this pathway exists in plants.

2.2.1.5 Indole-3-acetonitrile pathway

The biosynthesis of IAA via indole-3-acetonitrile (IAN) has been extensively studied in plants in recent years. The last step in this pathway, the conversion of IAN to IAA by a nitrilase was identified by (Bartling, 1992); the steps leading to the formation of IAN from tryptophan are still a matter of debate. Recently, two pathways were suggested for this formation: one via indolic glucosinolates and another via indole-3-acetaldoxime (Bak, 2001; Zhao, 2001). A tryptophan-independent pathway for the biosynthesis of IAN in plants has been suggested, but not further examined (Bartling, 1994; Normanly, 1993). In bacteria such as *Alcaligenes faecalis* nitrilases have been detected with specificity for indole-3-acetonitrile (Kobayashi et al., 1993; Nagasawa, 1990). In *Ag. tumefaciens* and *Rhizobium* spp., nitrile hydratase and amidase activity could be identified, indicating the conversion of IAN to IAA via IAM (Kobayashi et al., 1995).

2.2.1.6 Tryptophan-independent pathway

Analysis of knock-out mutants of *Ar. thaliana* for tryptophan biosynthesis (defective in tryptophan synthase alpha and beta) revealed increase levels of IAA conjugates, which led to the proposal of a tryptophan-independent pathway for the biosynthesis of IAA (Last et al., 1991; Normanly, 1993). This pathway branches from indole-3-glycerolphosphate or indole. However, no enzyme of this pathway has been characterized. The importance (and existence) of the tryptophan-independent pathway has been questioned (Muller and Weiler, 2000).

A bacterial tryptophan-independent pathway could be demonstrated in *A. brasilense* by feeding experiments with labeled precursors. This pathway is predominant in case no tryptophan is supplied to the medium: 90% of the IAA is

synthesized via the tryptophan-independent pathway, while 0.1% is produced via the IAM pathway (Prinsen, 1993). As no specific enzymes of this pathway have yet been identified, the existence of this pathway is currently being reexamined.

However, some bacteria possess more than one pathway i.e. *Pa. agglomerans* genes for the IAM pathway as well as for the IPyA pathway have been identified (Manulis, 1998).

2.2.2 Auxin transport

Auxin is translocated in plant tissues mainly by polar transport. However, both endogenous and exogenously applied auxins can move in the plant's vascular systems. Exogenously applied auxin moves in the phloem and xylem by mass flow through osmotic or water potential gradients. However, in polar transport, the direction of translocation is governed by the distribution of protein carriers, rather than by gross potential gradients. Polar transport is energy-dependent (Wilkins and Whyte, 1968), carrier-mediated (Goldsmith, 1977), and specific for active auxins (Hertel et al., 1969). Polar transport of IAA in shoots is in the basipetal direction, although lateral transport has also been demonstrated (Gardner et al., 1974). In roots, transport is generally in an acropetal direction (Wilkins and Scott, 1968), but this is still somewhat unclear.

Recently, Martens and Frankenberger (1992) provided the direct proof of movement of IAA from root to shoot as they detected labeled IAA-conjugates in wheat shoots grown in sterile nutrient solution with labeled IAA. Michalczuk and Chisnell (1982) demonstrated that labeled IAA and IAA-myoinositol applied to the endosperm of seeds moved toward the vegetative shoots. Similarly, there is indirect evidence that plants can respond to auxins applied to roots. Frankenberger et al.

(1990) applied various auxins (IAA, IAM, IBA, and ILA) to soil and observed a physiological response by *Raphanus sativus*. Inoculation with IAA producing strains of *Pseudomonas* caused a physiological effect on sugar beets, whereas inoculation with strains not producing IAA had no effect (Loper and Schroth, 1986). These studies indirectly indicated that auxins exposed to plant roots may play important roles in physiological responses of plants.

2.2.3 Uptake and metabolism of exogenous auxin in roots

Exogenously supplied auxins are readily taken up plant roots. However, the subsequent fate of auxins within the plant tissue is still not clearly known. The subsequent fate of exogenous auxin may include functioning as a supplement to the suboptimum levels of the endogenous auxin content, translocation to the shoot, transformation into conjugate forms (storage forms), catalytic oxidation, or combinations thereof. Exogenously supplied auxins may contribute to the active , but suboptimal, pool of the endogenous auxins. An excess of auxins may be translocated from the roots to the shoot through the xylem in the form of conjugates. Transformation into conjugate forms seems to be the immediate and major fate of exogenous auxins taken up by the roots. Several studies have revealed that most of the IAA supplied exogenously to roots is conjugated into IAA-aspartate (Bourbouloux and Bonnemain, 1974; Fang et al., 1960; Iversen et al., 1971; Kendall et al., 1971; Morris et al., 1969; Thurman and Street, 1962). These conjugated auxins may release free IAA whenever needed in the target tissue(s).

Although roots contain a nonspecific peroxidase and IAA oxidase that can destroy the IAA taken up, their significance for IAA metabolism is unknown (Scott, 1972). Aberg and Jönsson (1954) found no decarboxylation products of IAA

metabolism, although here is enough IAA oxidase in pea roots to destroy the endogenous IAA within a fraction of a second. This provides further evidence that the auxin supplied exogenously to the roots is not subject to rapid catabolism, but is regulated in plant tissues for physiological action.

2.3 *Azotobacter*

Azotobacter is gram negative, free living aerobic nitrogen fixing organism belonging to family Azotobacteriaceae. Among the several species, *Azotobacter chroococcum* happens to be the dominant inhabitant of the rhizosphere. There have been many reports on the beneficial effects of *Azotobacter chroococcum* on growth and yield of various agriculturally important crops. It benefits plants in multiple ways, which includes; a.) ability to produce ammonia, vitamins and growth substances that enhance seed germination; b.) production of indole acetic acid and other auxins such as gibberllins and cytokinins (Mratinez- Toledo et. al., 1988; Verma et. al., 2001) which enhance root growth and aid in nutrient absorption; c.) inhibition of phytopathogenic fungi through antifungal substances (Sharma and Chahal, 1987; Verma et. al.., 2001); d.) Production of Siderophores which solubilize Fe3+ and suppress plant pathogens through iron deprivation.

There is quite an extensive amount of work showing that inoculation with *Azotobacter* spp. leads to significant positive effects on plant growth and development. *A. chroococcum* was used as a seed inoculant in the USSR in the 1950s because it was assumed that its fixation of dinitrogen would provide a significant input to the nitrogen economy of plants (Cooper, 1959). Stimulation of plant growth was later attributed to the production of biologically active compounds (Mishustin,

1970; Rubenchik, 1963). It is now well established that various *Azotobacter* spp. are capable to synthesizing plant hormones. It has been reported that *A. chroococcum* (Brown and Burlingham, 1968; Brown and Walker., 1970; Martinez-Toledo et al., 1988; Müller et al., 1989), *A. vinelandii* (Azcón and Barea, 1975; Azcón and J., 1975; Gonzalez-Lopez et al., 1983; Lee et al., 1970), *A. paspali* (Barea and Brown, 1974), *A. agile* [*Azomonas agilis*] (Rasnizina, 1983), and *A. beijerinckii* (Azcón and Barea, 1975) produce auxins, cytokinins, and gibberellin like substances in chemically defined media. These bacteria are capable of producing auxins in absence of TRP (Table 1.1); however, Romanow (1965) reported that supplementation of the medium with TRP stimulates IAA production. The ability to produce auxin is so widespread in *Azotobacter* spp. that 13 of 14 (93%) strains from the roots of berseem, cotton, maize, pea, and wheat produced auxins (Apte and Shende, 1981). Indole compounds and other biologically active substances have been reported in the sterile soil inoculated with *A. chroococcum* (Elwan and El-Naggar, 1972). Sufficient indirect evidence indicates that *Azotobacter* can influence plant growth and development owing to their ability to produce phytohormones (Table 1.2).

Various environmental and cultural conditions affect IAA synthesis by *Azotobacter* spp. Auxin production by *A. vinelandii* in dialyzed soil medium was about twice that found in dialyzed soil medium supplemented with glucose. In a nitrogen free medium, auxin production was about three times that found in a medium containing ammonium nitrate (Gonzalez-Lopez et al., 1983). Lee at al. (1970) also reported that IAA production by *A. vinelandii* was reduced in the presence of combined nitrogen. However, El-Shanshoury (1979) found that the addition of NH_4Cl or $(NH_4)_2SO_4$ to a culture medium of *A. chroococcum* enhanced IAA synthesis. El-

Essaway et al.(1984) reported that NH_4Cl and $(NH_4)_2SO_4$ were favorable for IAA and gibberellic acid formation by *A. chroococcum*, respectively. Pyruvate and α-ketoglutarate also have positive influences on IAA synthesis. El-Essaway et al. (1984) found that vitamins did not affect IAA or gibberellic acid synthesis in nitrogen-supplemented media, whereas molybdate was required for IAA synthesis. Molybdate plus ferrous iron and boron were favorable for the active secretion of gibberellic acid. Sucrose addition (3%) stimulated IAA synthesis.

Relatively more IAA was produced by *A. vinelandii* in a shake culture compare with a stationary culture (Lee et al., 1970). The age of the culture was also of great importance (Brown and Walker., 1970; Lee et al., 1970; Vancura and Macura, 1960). Brown and Walker (1970) detected IAA only after the end of exponential growth of *A. chroococcum,* when cells had begun autolysis. The amount of IAA declined as the cultures aged. In aged cultures of *A. chroococcum*, IAA was converted into indole-3-carboxylic acid (ICA) (Vancura and Macura, 1960). Lee et al. (1970) reported that IAA synthesis by *A. vinelandii*o ccurred between 3 and 6 days of incubation and remained constant from 7 to 60 days.

Table 1.1 Production of Auxins by *Azotobactr* spp.

Azotobactr species	Substrates	Products	Method of detection	Ref.
Azotobactr spp.	Not specified	IAA	PC, UV, Spectrophotometry, Bioassay	Mahmoud et al.(1984)
Azotobactr spp.	peptone	Auxins	Bioassay	Rasnizina (1983)
A. beijerinckii	Not specified	Auxins	PC, Bioassay	Azcón and Barea (1975)
A. chroococcum	Not specified	IAA, indole derivatives, unknown	PC, Bioassay	Vancura and Macura (1960)
A. chroococcum	peptone	Auxin-like	Bioassay	Rasnizina (1983)
A. chroococcum	with and without L-, D-, DL-TRP	IAA	PC, UV, bioassay	Brown and Walker (1970)
A. chroococcum	Not specified	IAA	PC, Bioassay	Brown and Burlingham (1968)

Table 1.1 Production of Auxins by *Azotobactr* spp. (continued)

Azotobactr species	Substrates	Products	Method of detection	Ref.
A. chroococcum	Not specified	IAA	PA, Bioassay	Hennequin and Blanchere (1966)
A. chroococcum	Not specified	Auxins	PC	Salmeron et al. (1990)
A. chroococcum	With and without TRP	IAA	ELISA	Müller et al. (1989)
A. chroococcum	None	Indoles	PC, Bioassay	Elwan and El-Naggar. (1972)
A. chroococcum	Tryptone	IAA	Colorimetry	Apte and Shende (1981)
A. chroococcum	TRP	IAA	PC, Bioassay	El-Essaway et al. (1984)
A. paspali	Not specified	IAA	PC, Bioassay	Barea and Brown. (1974)
A. vinelandii	Not specified	Auxins	PC, Bioassay	Azcón and Barea (1975)
A. vinelandii	Not specified	IAA	TLC,PC, Bioassay	Lee et al. (1970)
A. vinelandii	Not specified	IAA	PC, UV,Bioassay, fluorescence	Gonzalez-Lopez et al. (1983)

Table 1.2 Secondary Metabolites of *Azotobacter* and Their Effects on Plant Growth

Azotobacter	PGRs[a] detected	Plant	Responses	Ref.
Azotobactr spp.	IAA, GLS	Barley	The metabolites showed stimulatory effect on plant height and dry weight.	Mahmoud et al. (1984)
Azotobactr spp.	Not determined	Linseed	Plants after 15 day of culture under sterile conditions showed an auxinlike effect with extreme shortening and simultaneous swelling of the root system.	Chalvignac (1961)
A. beijerinckii	IAA, GLS, CLS	*Medicago*	Cell-free supernatant and whole bacterial cultures, behaved as pure hormones (IAA, GA_3, kinetin) in improving dry weight and infection.	Azcón et al. (1978)
A. chroococcum	Not determined	Barley, oats	Affected root and shoot growth.	Vancura and Macura. (1960)

Table 1.2 Secondary Metabolites of *Azotobacter* and Their Effects on Plant Growth (continued)

Azotobacter	PGRs[a] detected	Plant	Responses	Ref.
A. chroococcum	IAA, GA_3	*Lycopersicon esculentum*	Exogenous application of IAA and GA_3 at an amount similar to those present in bacterial cultures produced an effect similar to that of inoculation.	Brown et al. (1968)
A. chroococcum	Not determined	*Zea mays*	Inoculation improved growth and yield significantly even in a nitrogen-rich environment, which suggested hormonal action by the inoculum.	Hussain et al. (1987)
A. paspali	IAA, GLS, CLS	*Lycopersicon esculentum*, *Paspalum notatum, Triticum vulgare, Lactuca sativa, Centrosema pubescens, Lolium perenne*	Inoculation affected plant growth and development significantly. Since there was no N_2-fixation, the pronounced effect was attributed to PGRs (IAA, GLS, CLS).	Barea and Brown. (1974)

Table 1.2 Secondary Metabolites of *Azotobacter* and Their Effects on Plant Growth (continued)

Azotobacter	PGRs[a] detected	Plant	Responses	Ref.
A. paspali	Not determined	*P. notatum*	Improved growth was attributed to PGRs, since no nitrogenase activity was detected in the rhizosphere.	Brown (1976)
A. vinelandii	IAA, GLS, CLS	*L. esculentum*	Treating roots with bacterial cultures accelerated plant growth and increased the yield of fruit. Effects were most likely caused by plant hormones (IAA, GLS, CLS)	Azcón and Barea (1975)
A. vinelandii	IAA, GLS, CLS	*Lavandula, Lycopersicon*	Cell-free supernatants and whole bacterial cultures behaved as pure hormones in improving dry weight and infection.	Azcón et al. (1978)

[a]IAA, indole-3-acetic acid; GLS, gibberellinlike substances; CLS, cytokininlike substances; GA_3, gibberellic acid.

2.4 Hydroponic system (Sheikh, 2006)

Hydroponic as a word was coined in 1930 by W.E. Gericke of University of California, meaning water and labour in other words i.e. water working or growing plants in a nutrient solution with out soil.

Hydroponic is a very young science. It has been used on a commercial basis for only 40 years. However, even in this relatively short period of time, it has been adapted to many situations, from outdoor field culture and indoor greenhouse culture to grow fresh vegetables. It is a space age science, but at the same time can be used in developing countries of the third world to provide intensive food production in a limited area. It's only restraints are sources of fresh water and nutrients. In areas, where even freshwater may not be available, hydroponic can be used through desalination of seawater. It has the potential application in providing food in areas having vast regions of non-arable land, such as deserts and dry coastal belts.

Characteristics and Attributes of Hydroponic

• The controlled environment growth leads to premium quality produce.

• High grade nutrients and precise control of the nutrient feed rations at the time of ripening.

• No sterilization of growing media required and plant nutrition is easily and completely controlled within the nutrient tanks.

• The lack of herbicides and pesticides influences on green house hydroponic crops (have no or very little risk of weeds, parasites, insects and pests etc.) is more acceptable to consumers.

• No soil borne diseases including weeds in hydroponic produces.

• Uses only up to 1/10th – 1/30th of the water which otherwise would have been used to grow equivalent amounts of field produce.

• System as a whole, allows for uniform water availability to plants.

• Hydroponic uses less fertilizer than is often used to grow equivalent amounts of field produce.

• Extended growing season i.e. crops can be grown all the year around so no regulated growing of crops needed or fallowing needed.

• Intensive production in a small space i.e. more plants per sq. foot and much greater yields at harvesting.

• No loss of fertility so no crop rotation as in soil borne crops.

• Superior taste, quality, appearance, uniformity, and extended shelf life of hydroponic vegetables.

• Closer plant spacing is possible and moveable plant channels allow greater production from equal areas for some crops.

• Certain vegetables i.e. Root zone heating, known to benefit tomatoes and cucumbers, is feasible and practical.

• Use of biological controls including beneficial insects and safe methods of insect control are possible and practical in a controlled environment system.

Growing methods in Hydroponic

In commercial hydroponic production, the two primary growing methods are drip (also known as substrate) and NFT (Nutrient Film Technique). There are a number of variations of these methods and also several others including the float system, ebb and flow system, aquaponics, aeroponics and passive hydroponic have also been practical. The biggest difference between the drip and NFT systems is the

use of a growing medium. In a drip system, the plant roots are in a growing medium such as perlite or rockwool and the nutrient solution is dripped onto the medium to keep it moist. In an NFT system, the plant roots are in a channel where a thin film of nutrient solution passes, keeping them moist but not water logged.

Drip (Substrate) system

The drip system is often used in commercial hydroponic facilities that grow long term crops like tomatoes, cucumbers and peppers. In this system, the nutrient solution is delivered to the plants through drip emitters on a timed basis. The emitters are usually scheduled to run for approximately 10 minutes of every hour depending on the stage of development of the plant and the amount of available light. The drip cycle flushes the growing medium, providing the plants with fresh nutrients, water and oxygen.

In a commercial drip system, the plant roots are most commonly grown in a medium of perlite or rockwool. The biggest variables in a drip system are in the growing medium and the container that holds that medium. Perlite is often bagged in thin, plastic sleeves. Holes are cut in the bag and plants usually, 3-4, are set in with the roots growing down into the perlite. Recently, a bucket system has been developed to contain perlite for drip systems. Each bucket holds lose perlite and one or two plants. In either of these methods, a slot or hole is cut in the container to allow excess nutrient solution to run out. A drain line below the bag or bucket collects the excess.

Another method of a drip system that is becoming popular for lettuce and herb production is the perlite tray, usually about 24 inches wide and 10- 14 feet long. An aluminum tray, coated with a nontoxic material, is filled with perlite and set on a gentle slope of 1-inch to every 10 feet. The nutrient solution is continuously dripped

in, at the higher end of the tray and allows trickling through the perlite to the other end. Essentially, this system is a combination of drip and NFT techniques.

In most drip systems, injectors are used to add nutrient concentrates to water when the feed cycle starts. In this case, there is no need for a large nutrient reservoir tank or the periodic dumping of used nutrient.

Nutrient Film Technique (NFT) system

With the NFT technique, the plants are grown in channels (also called gullies) through which the nutrient solution is pumped. The plant roots are kept moist by the thin film of nutrient solution as it passes by. Ideally, the bottom of the roots are exposed to the nutrient solution while the top are kept moist but not water logged.

Most NFT channels are fed continuously at a rate of approximately 1 liter per minute. Since the plant roots are not in a growing medium, it is crucial that they are kept moist at all times. In most NFT systems, the nutrient solutions mixed in a primary reservoir, are cycled through the channels and back to the reservoir. With the development of on-demand dosing equipment, a nutrient reservoir can automatically be adjusted, and with proper aeration and pH adjustment can effortlessly be kept fresh for weeks at a time.

NFT is ideal for lettuce, leafy crops and herbs, all of which are short terms crops. Larger NFT channels are used for long term crops such as tomatoes and cucumbers in many locations around the world. One great benefit of NFT, especially for leafy crops, is that the crops are clean and no washing is necessary. Growers, grocers and consumers all appreciate this type of crop even if to be marketed as such.

NFT channels are usually set up on waist-high stands that slope slightly to allow the nutrient solution to drain to one end. Although round pipes have been used

in NFT production, most growers have found that flat bottomed channels or gullies provide greater surface area for root development and oxygen uptake, resulting in better and faster plant growth.

Float system

Float systems have the advantage of the economy and the surface for the nutrient solution. Most float systems are long, rectangular reservoirs built out of cement or wood and lined with a durable polyliner. Holes are cut in a foam board which floats on the surface of the water and plants in net pots are set in the holes. The plant roots dangle in heavily aerated nutrient solution.

In areas where raw materials are limited and manufactured hydroponic systems are not available, the float system can be an economical means of hydroponic crop production.

Ebb and Flow system

The Ebb and Flow (also known as flood and drain) method of hydroponic simply floods a growing area for 5 or 10 minutes and then the nutrient solution drains away. The nutrient solution is stored in a reservoir that can be located under the grow table. Ebb and Flow is common in hobby systems but not often found in commercial production. In an Ebb and Flow system, the plant roots are usually grown in a medium of perlite, rockwool or expanded clay pebbles.

Aquaponics system (Integrated system approach)

In hydroponic, a specific nutrient formula is mixed in solution and is fed to the plants. In aquaponics, aquaculture (fish farming) is combined with hydroponic production. The nutrient-rich waste water from the fish tank is pumped through plant beds. Although not as precise as a hydroponic fertilizer mix, the effluent from a fish

tank is high in nitrogen and many other elements and most plants do quite well in aquaponics, through this integrated system approach. The key to aquaponics is the establishment of a healthy bacteria population. Beneficial bacteria that naturally occur in the soil, air and water convert ammonia (the primary form of fish waste) to nitrite and then to nitrate, which the plants readily uptake. In consuming the nitrate and other nutrients in an aquaponic system, the plants help to purify the water. Although the combination of hydroponic and aquaculture is quite new, the interest in this technology is booming. Aquaculturists who normally have to buy expensive water purification equipment to purify the water see aquaponics as a great way to clean the water and end up with another, very marketable crop. Hydroponic growers see the value in a natural source of nutrients, already in solution. The water from a fish tank can be pumped through any hydroponic grow bed in place of a hydroponic fertilizer solution. The commercial aquaponic production system is designed to show great promise as it may include the float, NFT and ebb and flow, methods.

Aeroponics

Aeroponics is the method of growing where the plant roots are constantly misted with a nutrient solution. Designs include frame with boards on each side, plant plugs set in each side and a mister between the boards spraying the roots. A round, large diameter PVC pipe set vertically with plant plugs all the way around and a mister mounted inside is another way to set up an aeroponic system. Although aeroponics is a unique way of growing, it is not a common means of commercial production.

Passive hydroponic

Passive hydroponic systems are sometimes used by hobbyists. A passive

system does not use pumps or timers to flood the root zone. The roots usually dangle into the nutrient solution and draw what they need. A passive system is generally slower growing and not as productive as the other methods discussed, above.

Pythium root rot in hydroponic

Pythium root rot is ubiquitous and frequently destructive in almost all kinds of plants produced in hydroponic systems, including cucumber, tomato, sweet pepper, spinach, lettuce, arugula, and roses. *Pythium* root rot is also considered a potential threat to plant biomass production in manned space vehicles and at extraterrestrial installations in projected space missions (Jehkins et al., 2000; Sehuerger, 1998). Indeed *Pythium* has already been encountered on plant materials in space vehicles operated in earth orbit. In greenhouse and growth room studies, Pythium root rot became severe in hydroponic tobacco, *Arabidopsis* and antirrhinum, which are key plants employed for genetic, molecular and physiological studies (N.Ortiz, W. Liu, & J.C. Sutton, 2003 unpublished observations).

Management of Pythium root rot in the production of hydroponic crops is generally a difficult challenge. Extraordinary sanitation measures do not necessarily exclude or destroy the causal pathogens, and once initiated, epidemics are difficult to contain. Recent advances in knowledge and understanding of the etiology and epidemiology of root rot, and in methods and approaches to control the disease, are providing a framework for major improvements in root rot management and in the overall health and productivity of hydroponic crops.

Production of antibiotics by *Azotobacter* is a major factor affecting phytopathogenic organisms. Literature data are available on a single antifungal agent produced by *Azotobacter chroococcum* 92 composed of a methyl ester of a tetraenoic

aliphatic acid. The antibiotic was shown to suppress growth of such phytopathogenic fungi as *Bipolaris sorokiniana, Botrytis cinerea, Pythium debarianum, Verticillum dahliae*, and *Fusarium* sp. (Pridachina et al., 1982).

Chetverikov and Loginov (2009) reported that the *Azotobacter* strain under investigation revealed a wide spectrum of antagonistic activity against phytopathogenic fungi of the genus *Fusarium* (Table 1.3).

Table 1.3 Antifungal activity of *Azotobacter vinelandii.*

Phytopathogenic fungi	Diameter of the growth inhibition zone, mm
Fusarium culmorum	19.0 ± 2.5
Fusarium gibbosum	18.0 ± 2.2
Fusarium graminearum	14.4 ± 1.4
Fusarium nivale	9.5 ± 0.5
Fusarium semitectum	13.2 ± 1.1
Fusarium solani	8.0 ± 0.5
Fusarium avenaceum	19.0 ± 2.3
Bipolaris sorokiniana	12.0 ± 2.1
Alternaria alternate	20.0 ± 2.0

2.5 Immobilization technology

Immobilized microbial enzymes, organelles, and cells have been used in a variety of scientific and industrial applications. The economic importance of immobilization has resulted in considerable research for industrial applications. Immobilization technology has been used extensively in commercial bioreactor fermentations (Nunez and Lema, 1987). Reviews on the use of immobilized

organelles (Mattiasson, 1983a; Mattiasson, 1983b) and enzymes (Mosbach, 1987a; Mosbach, 1987b) have addressed the importance of these respective technologies. Immobilized cells have also been used in bioreactors, and production of useful compounds such as amino acids, organic acids, antibiotics, steroids and enzymes using immobilized cells have been reviewed by Brodelius and Vandamme (1987) and Tanaka and Nakajima (1990). Various whole cell techniques (Akin, 1987; Klein, 1985; Klein and Vorlop, 1985; Klein and Wagner, 1983; Mattiasson, 1983a; Mattiasson, 1983b; McLoughlin, 1994; Westmeier and Rehm, 1985) and the many applications possible have been examined (Akin, 1987; Coughlan and Kierstan, 1988; Scott, 1987; Tanaka and Nakajima, 1990). The commercial success of these processes illustrates the value of using immobilization technology under controlled conditions.

Immobilization is a general term that describes many different forms of cell attachment or entrapment. These different forms include flocculation, adsorption on surfaces, covalent bonding to carriers, cross-linking of cells, encapsulation in a polymer-gel and entrapment in a matrix (Figure 2). The advantages of various methods of cell immobilization for bioreactor systems have been addressed in several reviews (Dervakos and Webb, 1991; Klein and Vorlop, 1985; Klein and Wagner, 1983; Kolot, 1981; Kolot, 1988; Kren et al., 1987). One method that has emerged as successful in the laboratory and useful in commercial applications is the encapsulation of cells in a polymer gel-matrix. Results from bioreactor studies have demonstrated that encapsulated cells have advantages over free cells under numerous conditions. For example, increased metabolic activity and metabolite production (Gadkari, 1990; Scherer et al., 1981), protection from toxic substances (Dwyer et al., 1986; Keweloh et al., 1990; Zache and Rehm, 1989), and increased plasmid stability of encapsulated

cells compared to free cells have all been observed (Berry, 1988; Nasri et al., 1987a; Nasri et al., 1987b).

Increased awareness of the profound effects of environmental problems, such as toxic waste sites and aspects of pesticide usage, has stimulated investigations of technologies which avoid, reduce or eliminate these problems. The use of immobilized cells has been investigated as an alternate technology for environmental applications. For example, the use of immobilized cells in agriculture (Bashan, 1986; Trevors et al., 1992), biocontrol (Axtell and Guzman, 1987), pesticide application (Connick, 1982) and pollutant biodegradation in contaminated soil or groundwater (Hu et al., 1994; Lin and Wang, 1991; O'Reilly and Crawford, 1989; Zhou et al., 1993) have all been examined. However, most of the investigations were performed at the laboratory level, and applications of immobilized cells in the open environment have yet to be realized.

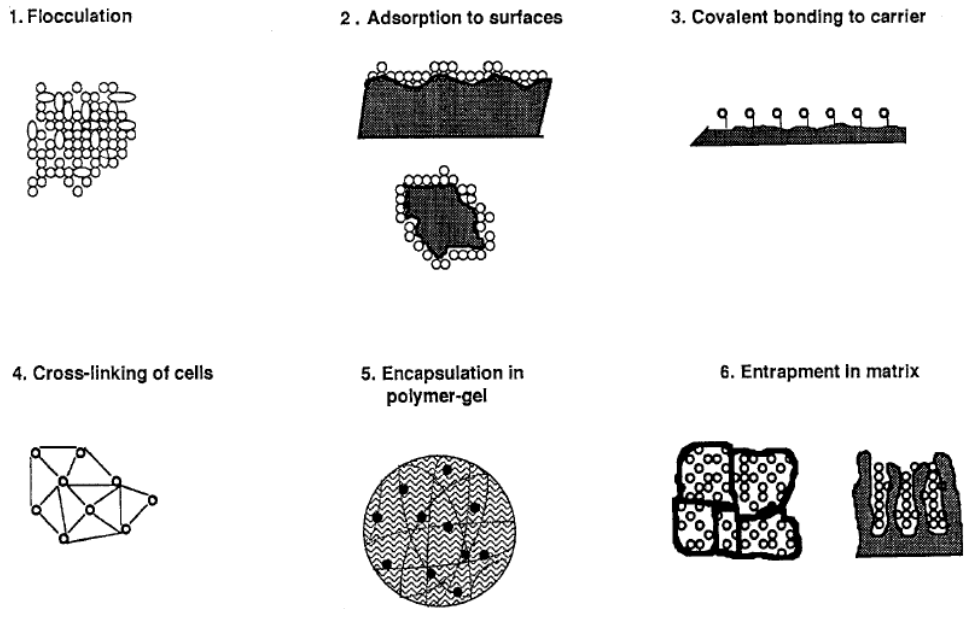

Figure 2. Methods for immobilization of viable microbial cells(Cassidy et al., 1995a).

Uses of microorganisms in soil and their application

Microbial inoculants have been investigated for soil applications such as enhancement of symbiotic or associative N_2 fixation, biological control of soil-borne plant pathogens, biological control of frost injury and biodegradation of xenobiotic compounds (Van Elsas and Heijnen, 1990). Microorganisms have been introduced into soils as liquid suspensions or adsorbed to different carriers such as peat, charcoal, vermiculite and other organic particulate matter (Sparrow Jr and Ham, 1983). Formulations can contain microorganisms in a usable form and optimize the efficacy, stability, safety and ease of application (Rhodes, 1993). Liquid cell-suspensions may be more easily contaminated, more difficult to contain in case of an accidental spill, and may have greater potential for off-site dispersal during application than dry carrier/cell formulations.

Once applied, the success of a bioremediation procedure depends on establishment of microorganisms and on expression of the necessary degradative genes [(Daubaras and Chakrabarty, 1992; Providenti et al., 1993). Various methods of microbial introduction into soil have been reviewed (Van Elsas and Heijnen, 1990). The authors cited examples of variations in survival observed with inoculation into soil from a progressive decline in bacterial numbers to improved survival of rbizobia with the addition of clay, and they suggested results have been variable because soil is a highly heterogeneous and unpredictable environment for introduced bacteria. Both abiotic and biotic factors play critical roles in determining microbial survival. Soil moisture content, temperature, pH, texture, oxygen availability, rate of oxygen diffusion and nutrient availability have been suggested as abiotic factors controlling survival of introduced bacteria in soil (Van Elsas et al., 1986). Biological factors

include predation by protozoans, a lower level of starvation resistance of the introduced bacteria (Acea et al., 1988), and lack of suitable soil niches for extended cell survival (Van Elsas and Heijnen, 1990).

The use of carriers for microorganisms has been proposed as a means with which to overcome some of the problems associated with microbial survival in soil after inoculation. The advantages and disadvantages of various solid carriers for use in soil have been reviewed (Trevors et al., 1992). The authors suggested that an effective and safe carrier for soil-applied microorganisms should be non-toxic and nonpolluting, consistent in quality, have a long shelf life, allow sufficient cell activity and cell density, and permit accurate release of bacteria into the target site. Encapsulation emerged as one of the best candidates because it provides many features which may aid in the introduction and establishment of microorganisms. McLoughlin (1994) suggested that microenvironments in the bead may initially protect cells from the soil macroenvironment. Microorganisms are released after adaptation to prevailing environmental conditions. This may enable cells to overcome the numerous changing conditions in soil and increase microbial survival. He also suggested that immobilizing cells by encapsulation in a gel-matrix offers a stable, defined, consistent, protective environment, without the immediate release of large number of cells, where cells can survive and metabolic activity can be maintained for extended periods of time.

Advantages of using encapsulated bacterial cells for soil applications

The use of encapsulated cells for soil applications provides a number of advantages over free cells or other carriers such as peat, vermiculite or charcoal. These are outlined in Table 1.3 and discussed further below. Formulations must be

carefully designed to maintain viability and activity during extended storage and distribution. Immobilization under aseptic conditions allows entrapment of only the cells of choice. Cells adsorbed onto activated carbon and kept at 4^{o}C kept their degradation capacity for up to 12 months (Mörsen and Rehm, 1987). Dried beads can be stored for up to 3 years at 4^{o}C (Cassidy et al., 1995b; Mugnier and Jung, 1985), and the bead structure provides protection against contamination during storage, transport and application (Jung et al., 1982). Alginate carrier formulations prolonged storage life and provided protection from mechanical abrasion during transport and application (Axtell and Guzman, 1987). Existing mechanical equipment can be used for the application of inocula, and since no bioaerosol formation occurs, the possibility of drift off-site is minimized. The importance of bacterial containment, detection and survival in the environment was reviewed by Jackman *et al* (Jackman et al., 1992). These areas are of prime concern, particularly when considering the use of genetically-engineered microorganisms (GEMs). Accidental spills of encapsulated cells in dried beads can likely be contained and cleaned up more easily than a liquid cell-suspension spill.

Table 1.4 Advantages and limitations of using encapsulation for soil applications (Cassidy et al., 1995a).

Advantages
1) Reduced possibility of inoculum contamination during storage, transport and application
2) Reduced possibility of off-site drift during application

Table 1.4 (Continued).

3) Beads are non toxic, biodegradable and non-polluting
4) Can be produced in large quantities, stored for extended periods as dried beads and used with existing mechanical application equipment
5) Provides protection from biotic and abiotic environmental stresses leading to increased microbial survival
6) Increased metabolic activity of encapsulated cells
7) Slow cell release with reduced cell movement through soil from water flow-induced transport
8) Increased plasmid stability
Limitations
1) Gas and solute diffusion may be restricted
2) Reduced oxygen consumption rates of encapsulated cells may occur
3) Cell morphological or metabolic alterations may have a detrimental effect
4) Effects of changes in water activity may limit effectiveness
5) Cells may not establish colonies outside of beads. Repeat applications of beads may be required

CHAPTER III

MATERIALS AND METHODS

3.1 Apparatus

Autoclave:	Hiclave HA-3000MIV, Hirayama, Japan
Balance:	Precisa 205A, Precisa Instruments, Switzerland
Compound microscope:	Olympus SZ2-LGCL, Olympus, Japan
	Olympus CX 31-RBSF, Olympus, Japan
Incubator shaker:	C24 Incubator shaker, New Brunswick Scientific, USA
Incubator:	Memmert, BE 500, WTB Binder BD115, Shel-lab 2020
	Low Temperature Incubator, Sheidon, USA
Laminar hood:	Holten laminAir HBB 2448, Denmark, BH2000
	Series ClassII BiologicalSafety Cabinates, BHA120 &
	BHA180, Clyde-Apac
Microcentrifuge:	Hereaus, Labofuge 400R Eppendorf 54154,
	Eppendorf, Germany
pH meter:	Hanna instruments 8519, Italy
Shaker:	Innova 2300 platform shaker, New Brunswick
	Scientific, UK
Spectrophotometer:	Ultraspec 2000 UV/Visible Spectrophotometer,
	Pharmacia Biotech, England
Stirrer:	Variomag Electronicrührer Poly 15, Germany Magnetic

stirrer MSH300, USA

Vortex: Vortex-Genie2 G506, Scientific industries, USA

3.2 Bacterial cultivation

Azotobacter sp. was cultured in LG media contained per liter of distilled water which containing of 10 g of glucrose, 0.15 g of KH_2PO_4, 0.2 g of yeast extract, 0.05 g of K_2HPO_4, 0.02 g of $MgSO_4 \cdot 7H_2O$, 0.002 g of $CaCl_2 \cdot 2H_2O$, 0.002 g of $FeCl_3 \cdot 6H_2O$ and 0.002 g of $NaMoO_4 \cdot 2H_2O$ and adjusted the pH to 6.8 with 1M NaOH. The bacterial culture was cultivated on a rotary shaker at 200 rpm, 30^oC for 48 h.

3.3 Cell Immobilization

Cells in late exponential phase were harvested from culture broth by centrifugation at 4000 g for 10 min at 4°C. The pellets were suspended in sterilized peptone. Resuspended cells were mixed with an equal volume of 2% (w/v) sodium alginate solution.

The mixture of alginate and cells were added drop-wise into a sterilized solution of 3% calcium chloride. In order to produce beads, the solution was added through the needle. To obtain aseptic operation the beads was made at a stirilized condition. The beads was stored at 4^oC in sterilized peptone until used.

Bead Coating with chitosan

Chitosan solutions were prepared as described by Zhou et al. (1998) to a final chitosan concentration of 0.4% and a pH adjusted to 5.6. This solution was autoclaved at 121°C for 15 min. To coat the beads, the beads were immersed in

chitosan solution and stirred. After 45 min, the beads were sieved off from the chitosan solution and washed with sterile distilled water. The beads was stored at 4^{o}C in sterilized peptone until used.

3.4 Properties of immobilized *Azotobacter* sp.

3.4.1 Viability of cell in Calcium-Alginate bead

Beads containing entrapped cells 1 g (10^{9} cfu/g bead) were inoculated into 5 ml LG medium in the range of initial pH 3-9 in a 10 ml tube, and were incubated on a rotary shaker (200 rpm) at 30°C 3 weeks. Number of viable cells was determined follow as section 3.4.1. once per week.

3.4.2 Bead dissolution and cell counting

Bead 1 g was liquefied in 9 ml of 2% sterilized sodium citrate solution (pH 6.0) and serial diluted with sterilized water. Plate counts were conducted in duplicate at each dilution on LG medium. Plates were incubated at 30^{o}C for 48 h, and result was reported as colony forming units per gram of bead (cfu/g bead).

3.4.3 Production and estimation of IAA

Azotobacter sp. was immobilized in Calcium alginate as described above. Beads containing entrapped cells (10^{9} cfu/g bead) were inoculated into 25 ml LG medium supplemented with tryptophan 0.061 g per liter pH 6.8 in a 50 ml flask, and were incubated on a rotary shaker (200 rpm) at 30°C 48 h. The concentration of IAA was determined using Salkowski's chemical assay which compared to a standard curve (Gordon and Weber, 1951). Pure indole-3-acetics acid (Sigma, USA) was used as a standard.

3.4.4 Optimum pH for IAA production and cell viability

Effect of pH value on immobilized *Azotobacter* sp. was carried out at pH range 5.5-7.5 adjusted with 1N HCl or 1N NaOH, concentration of IAA was determined follow as section 3.4.2. Number of viable cells was determined follow as section 3.4.1.

3.4.5 Optimum temperature for IAA production

For thermal stability immobilized *Azotobacter* sp. was incubated at a designated temperature (15-45 ^{o}C) for 48 h and IAA production were detected follow as section 3.4.2.

3.4.6 Effect of nitriogen on IAA production

Five percent of Potassiumnitrate (KNO_3) was added to LG medium to studied effects of IAA production by immobilized *Azotobacter* sp.

3.4.7 Effects of glucose on IAA production

Ten percent of glucose was supplemented in various nutrient including of nutrient for hydroponic system, N-free medium and LG medium (Table 3.1 and Table 3.2).

3.5 Observation of Scanning Electron Microscopy (SEM)

For observations with SEM, the material was treated with chemical reagents and then transferred to acetate plates prior to coated with gold following the technique proposed by Rowley and Nilsson (1972).

Table 3.1 Composition of hydroponic nutrient solution.

Composition	**Concentration**
Solution A	
KNO_3 (g)	800
KH_2PO_4 (g)	310
$MgSO_4 \cdot 7H_2O$ (g)	370
H_3BO_3 (g)	2.02
$MnSO_4 \cdot H_2O$ (g)	7.76
$ZnSO_4 \cdot 7H_2O$ (g)	0.55
$Na_2MoO_4 \cdot 2H_2O$ (g)	0.15
$CuSO_4 \cdot 5H_2O$ (g)	0.16
Water (L)	10
Solution B	
$Ca(NO_3)_2 \cdot 4H_2O$ (g)	1700
Fe-EDTA (g)	74.24
Water (L)	10

½ Solution A+ ½ Solution B in water to obtain EC value 1.8-2.2 mS/cm., pH adjusted to 6.5 with 65% HNO_3.

Table 3.2 Composition of N-free medium solution for plant growth.

Composition	Concentration
1.$CaCl_2$ (g)	55.50
2. $MnSO_4 \cdot 7H_2O$ (g)	49.50
3. KCl (g)	37.50
4. KH_2PO_4 (g)	13.60
5. Fe-EDTA·Na (g)	30
6. Mixed minerals	
6. 1 H_3BO_3 (g)	2.86
6.2 $MnCl_2 \cdot 4H_2O$ (g)	1.81
6.3 $ZnSO_4 \cdot 7H_2O$ (g)	0.22
6.4 $CaCl_2 \cdot 6H_2O$ (g)	0.10
6.5 $CuSO_4 \cdot 5H_2O$ (g)	0.08
6.6 MoO_3 (g)	0.02

For 2.5 liter culture medium; 100 ml of solution 1,2,3,4 + 10 ml of solution 5,6 adjusted to 2.5 liter with water.

3.6 Plant cultivation with immobilized *Azotobacter* sp. in light room.

3.6.1 Plant cultivation with immobilized *Azotobacter* sp.

Choy sum (*Brassica chinensis* var *parachinensis*) was cultured with immobilized *Azotobacter* sp. at 25^{o}C in light room, nutrient solution pH 6.5, EC= 22 mS cm^{-1}. The formulation of nutrients for plant growth was described as in section 3.4.7. The experiment was set out following a completely randomized factorial design. Two factorials were comprised of immobilized *Azotobacter* sp. (5 levels: 2.5,

5, 7.5, 10, 12.5 g beads per plant (1 g bead contained *Azobacter* sp. about 10^9 cfu/ml)) and IAA solution (6 levels: 10, 20, 40, 60, 80, 100 µM 1ml per plant per week). Seeds were surface sterilized before sown into sterilized pots containing substrate (vermiculite), sterilized nutrient solution were added after plant germination. Replicates were performed 5 pots per treatment. The plant was grown in light room with a 16/8 h light/dark cycle. The experiment was performed for 45 days, two factors were applied at 21 days after seedling.

3.6.2 Observation of plant growth

Forty-five days after seedling, plant growth parameters were investigated as followed; shoot and root length, fresh and dry weight and element analysis include of percent Nitrogen, Phosphorus, Potassium and Calcium. The methods for plant elements analysis were follow as;

- Nitrogen was analyzed by Kjeldahl method (Kjeldakl, 1883).
- Phosphorus was analyzed by Vanadomolybdate (Barton) method (Barton, 1948).
- Potassium and Calcium were determined by Flame photometer (Plant analysis handbook of reference method, 1998).

3.7 Plant cultivation with immobilized *Azotobacter* sp. in hydrponics system

3.7.1 Plant cultivation with immobilized *Azotobacter* sp.

Choy sum was cultured with immobilized *Azotobacter* sp. in two different crop cycles (winter and summer) under hydroponic system. The formulation of nutrients for plant growth was described as section 3.4.7. The experiment was set out

following a completely randomized factorial design. There were 2 factorials in this study; comprise of 1) immobilized *Azotobacter* sp. (5 levels: 2.5, 5, 7.5, 10, 12.5 g beads per plant (1 g bead contained *Azobacter* sp. about 10^9 cfu/ml)) and 2) IAA solution (6 levels: 10, 20, 40, 60, 80, 100 μM 1ml per plant per week), each treatment had 30 replications. Nutrient solution for plant growth was controlled to pH 6.5, EC= 22 mS/cm by adjusting everyday with stock solution A and B for EC, and HNO_3 for pH as described in section 3.4.7. The experiment was performed for 45 days, two factors were applied at 21 days after seedling. Ten samples per treatment were taken to determined plant growth parameters as described in section 3.6.2.

3.8 Statistical analysis

All data were subjected by analysis of variance (ANOVA) and means were separated by Duncan's multiple range tests (SPSS® solfware for WINDOW™, Version 13.0; SPSS, Chicago.IL). Significance of differences was established at $P \leq 0.05$.

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Cell immobilization

Azotobacter sp. was immobilized in alginate bead, each beads cover 2.5 mm diameter and contain *Azotobacter* sp. cells about 10^6 cfu (Fig. 4.1).

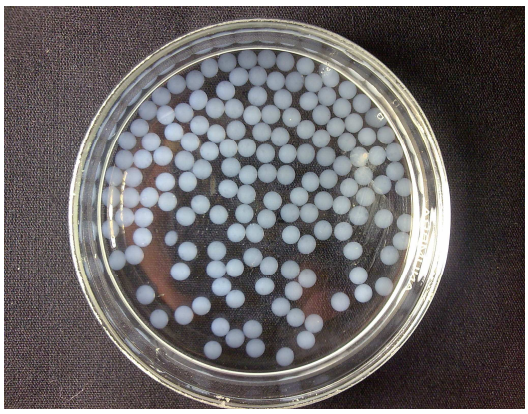

Figure 4.1 Immobilized *Azotobacter* sp. bead after dropped in 2% $CaCl_2$ solution.

4.2 Cell concentration in alginate bead encapsulated *Azotobacter* sp.

4.2.1 Free cell

For determination availability of *Azotobacter* sp. compare to availability of immobilized *Azotobacter* sp. in bead in LG medium (initial pH 3-9). The result showed that *Azotobacter* sp. can survive in LG medium in the range of pH 4 -9 for three weeks but in pH less than 3.5 bacteria can not survive. The initial pH 6-8.5 of LG medium was found to be optimum for bacterial cell growth of *Azotobacter* sp. for

three weeks (Fig. 4.2a). Several reports reviewed that growth on the alkaline side of *Azotobacter* sp. appears to stop between pH 9 and 10 (Burk et al., 1934; Yamagata and Ttana, 1923). The optimum of *A. chroococcum* and *A. vinelandii* near pH 7.5, with a considerable amplitude towards 7 and 8.

4.2.2 Ca-alginate bead encapsulated *Azotobacter* sp.

Cell concentration of *Azotobacter* sp. were found in pH 3.5 of initial pH medium for one week about 3.7×10^5 cfu ml^{-1} and can growth well in pH 4-9 for three weeks. In contrast with free cell of *Azotobacter* sp. does not survive in pH 3.5 of initial medium (Fig. 4.2b). It was reported that immobilization provides protection from biotic environmental stresses leading to increased microbial survival (Cassidy et al., 1995)

4.2.3 Ca-alginate bead encapsulated *Azotobacter* sp. coated with chitosan

Cell concentration of *Azotobacter* sp. in Ca-alginate bead coated with chitosan were found in pH 3.5 of initial pH medium about 1.3×10^6 cfu ml^{-1} higher than cell concentration of *Azotobacter* sp. in Ca-alginate bead at the same initial pH (Fig. 4.2c). This is suggested that chitosan provides a barrier to cell release because of it is a positively charged polyamine which forms a semi-permeable around a negatively charged polymer such as alginate. This membrane dose not dissolve in the presence of Ca^+ chelators or anti-gelling cations and thus enhances stability of the gel (Smidsrod and Skjak-Braek, 1990).

Viability of free *Azotobacter* sp. was higher and more stability than *Azotobacter* sp. in Ca-alginate bead and Ca-alginate bead coated with chitosan. However, this experiment performed under aseptic condition therefore environmental stresses were not happen. In fact, when applied microorganism into field, the

environmental stress both abiotic and biotic factors play critical roles in determining microbial survival. Soil moisture content, temperature, pH, texture, oxygen availability, rate of oxygen diffusion and nutrient availability have been suggested as abiotic factors controlling survival of introduced bacteria in soil (Van Elsas et al., 1986). Biological factors include predation by protozoans, a lower level of starvation resistance of the introduced bacteria, and lack of suitable soil niches for extended cell survival (Acea, 1988; Van Elsas and Heijnen, 1990). McLoughlin, (1994) suggested that microenvironments in the bead may initially protect cells from the soil macroenvironment. Microorganisms are released after adaptation to prevailing environmental conditions. This may enable cells to overcome the numerous changing conditions in soil and increase microbial survival.

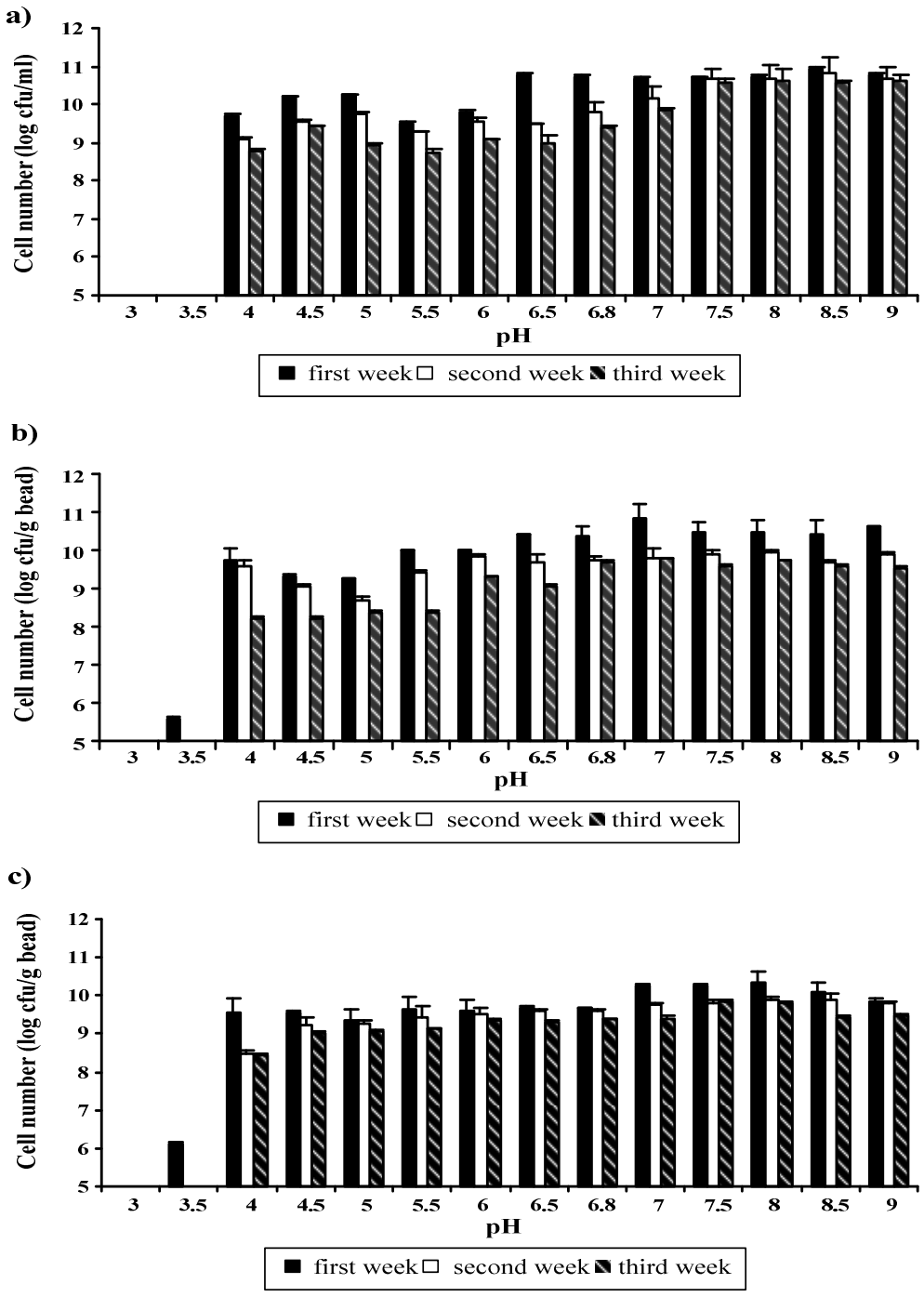

Figure 4.2 Effect of initial pH of medium on cell viability of a) *Azotobacter* sp., b) *Azotobacter* sp. in Ca-alginate bead and c) of *Azotobacter* sp. in Ca-alginate bead coated with chitosan, incubated temperature 30^{o}C for 3 weeks on rotary shaker of 200 rpm.

4.3 Indole-3- acetic acid production in immobilized *Azotobacter* sp.

4.3.1 IAA production by immobilized *Azotobacter* sp.

Immobilized *Azotobacter* sp. can produced and secreted IAA into culture medium and the highest rate of IAA production was 5 g beads of Alginate bead per 25 ml of culture medium supplemented with L-tryptophan and decrease when increase amount of beads (Fig. 4.3). This could be attributed to the fact that, when the number of beads increases, the nutrient/bead ratio decreases, which may become limiting (Beshay, 2003). Furthermore, tryptophan as precursor of IAA synthesis in culture medium also limited. Ahmad et al., (2004) reported that increase tryptophan concentration in culture medium increased IAA production in *Azotobacter* sp. Therefore, this number of beads was used for the rest of the study.

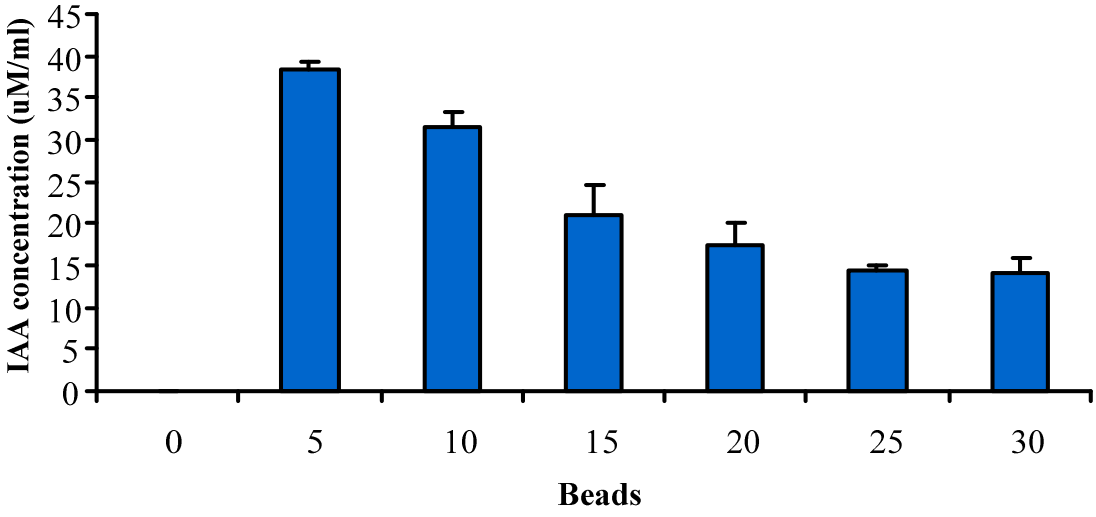

Figure 4.3 Effect of various different amount of immobilized *Azotobacter* sp. in alginate beads in the range of 5 to 30 g beads in 25 ml LG medium supplemented with L-tryptophan, adjusted pH of medium of 6.8, incubated at 30^{o}C for 48 h and incubated on rotary shaker of 200 rpm.

4.3.2 Initial pH and present of nitrogen in LG medium

The effect of nitrogen on IAA production in immobilized *Azotobacter* sp. was examined. Fig. 4.4 demonstrated that the incorporation of nitrogenous compound (KNO_3) into medium can increase IAA production in immobilized *Azotobacter* sp., and initial pH of medium has effect to IAA production. The result showed that IAA production increased when nitrogen was supplemented in LG medium compare to LG medium (without addition of nitrogen). Whereas Gonzalez-Lopez et al., (1983) found that in a nitrogen free medium, auxin production was about three times that found in a medium containing ammonium nitrate. Lee at al. (1970) also reported that IAA production by *A. vinelandii* was reduced in the presence of combined nitrogen. However, El-Shanshoury (1979) found that the addition of NH_4Cl or $(NH_4)_2SO_4$ to a culture medium of *A. chroococcum* enhanced IAA synthesis. El-Essaway et al. (1984) reported that NH_4Cl and $(NH_4)_2SO_4$ were favorable for IAA and gibberellic acid formation by *A. chroococcum*, respectively. Pyruvate and α-ketoglutarate also have positive influences on IAA synthesis. Comparable result was obtained by initial pH of 6.8-7.5 was found to be optimum for IAA production. Yamagata and Itano (1923) found an optimum pH of *A. beijerinckii* was about 6.8.

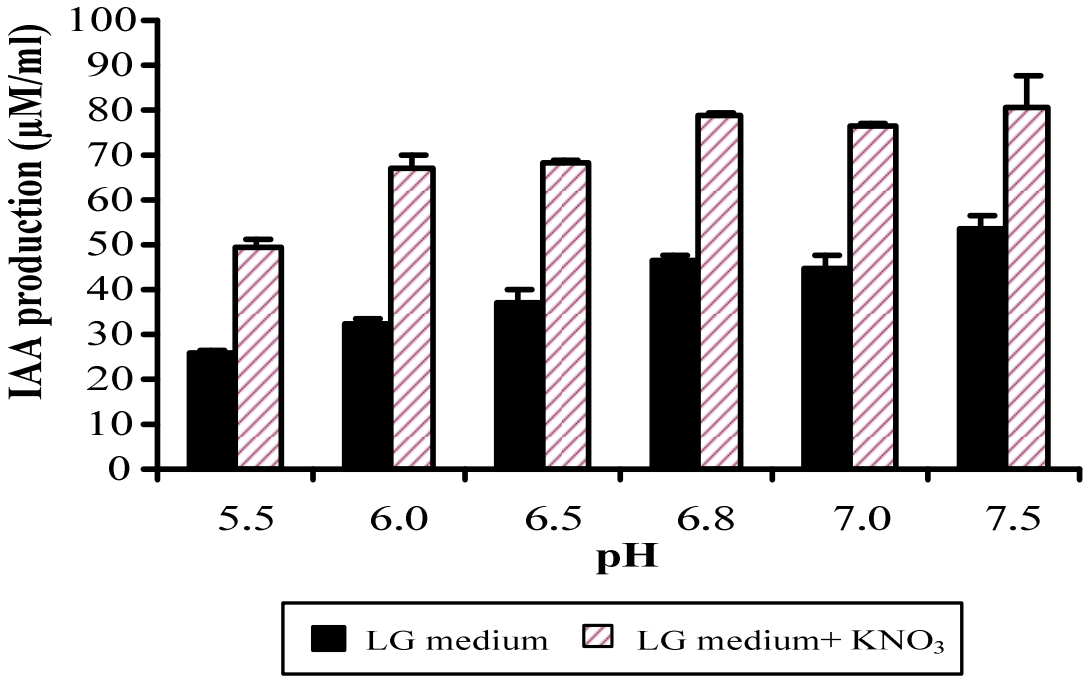

Figure 4.4 Effect of initial pH medium on IAA production by immobilized *Azotobacter* sp. 5 g beads in 25 ml. LG medium and LG medium addition with nitrogen supplemented with L-tryptophan, incubated at 30^{o}C for 48 h and incubated on rotary shaker of 200 rpm.

4.3.3 Temperature

The effect of different temperatures on IAA production by immobilized *Azotobacter* sp. was shown in Fig. 4.5. A temperature 20^{o}C appears to be the optimal for production of IAA by immobilized *Azotobacter* sp. and highest at pH 6.5. However Krzemieniewski, (1908) exposed that *Azotobacter* are typical mesophilic bacteria with minimum, optimum and maximum at approximately 10, 30 and 40-45 ^{o}C respectively. Mohammed and Belal (2007) found that optimal temperature for IAA production of *Azotobacter* sp. was 30^{o}C. Moreover, IAA was unstable to high temperature or illumination. Hiratsuka et al., (1989) reported that under light,

degradation of IAA was more rapid at 25 ^{o}C than at 4^{o} C and IAA rather stable in the dark even at 25 ^{o}C for several week, illumination treatments degraded IAA significantly.

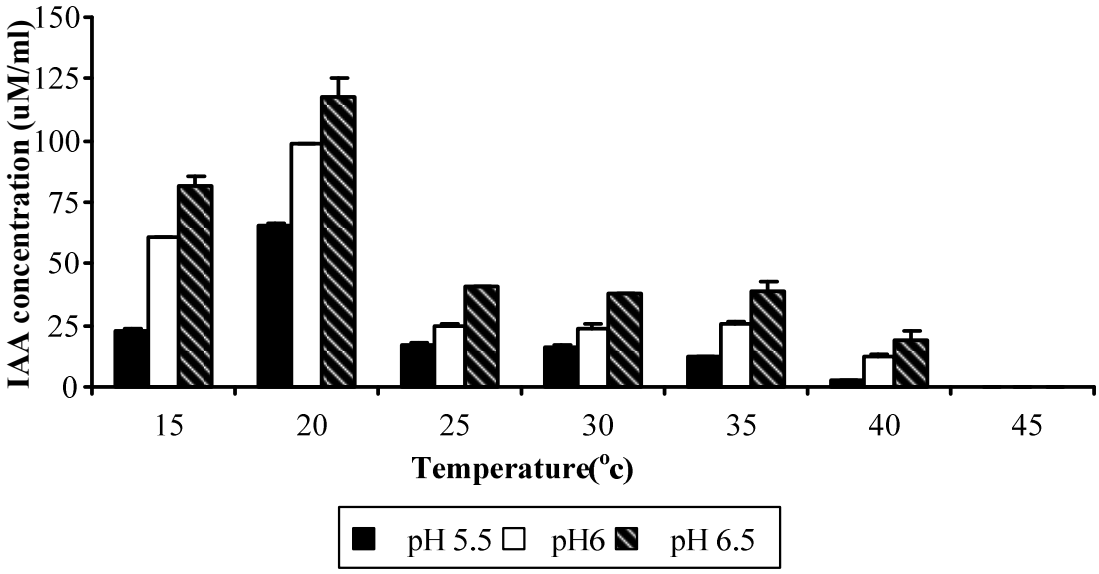

Figure 4.5 Effect of incubation temperature on IAA production by immobilized *Azotobacter* sp. 5 g beads in 25 ml. LG medium supplemented with L-tryptophan, adjusted pH of medium of 6.8, incubated at 30^{o}c for 48 h and incubated on rotary shaker of 200 rpm.

4.3.4 Glucose

To studied the effect of glucose on IAA production by immobilized *Azotobacter* sp. in various nutrient. One percent of glucose was added into LG medium containing L-tryptophan compared with nutrient not supplemented glucose shown in Fig. 4.6. The data showed that immobilized *Azotobacter* sp. can produced IAA in nutrient supplemented with glucose but not produce IAA without glucose. The highest IAA was produced in hydroponic nutrient supplemented with glucose. El-Essaway et al. (1984) reported that sucrose addition (3%) in culture

medium also stimulated IAA synthesis.

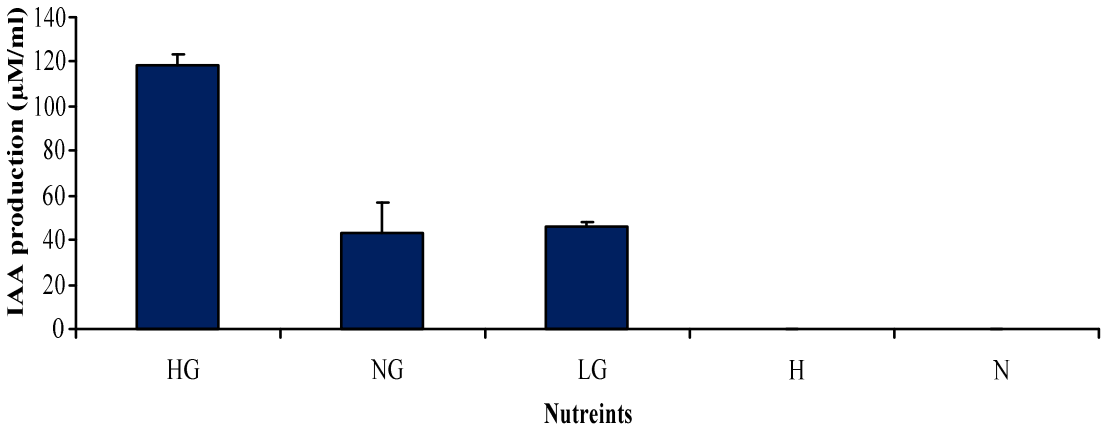

Figure 4.6 Effect of glucose and culture media (HG; nutrient for hydroponic system supplemented with 10 g l^{-1} glucose, NG; N-free medium supplemented with 10g l^{-1} glucose, LG; LG medium, H; Nutrient for hydroponic system and N; N-free medium) on IAA production by immobilized *Azotobacter* sp. 5 g beads in 25 ml. LG medium supplemented with L-tryptophan, adjusted pH of medium of 6.8, incubated at 30^{o}C for 48 h and incubated on rotary shaker of 200 rpm.

4.4 Scaning micrographs of encapsulated *Azotobacter* sp.

Scaning electron micrograph of encapsulated *Azotobacter* sp. alginate beads, alginate-chitosan beads and their surface morphologies are depicted in Fig. 4.7-4.9, for used alginate-chitosan beads were shown in Fig. 4.9. *Azotobacter* sp. alginate beads and alginate-chitosan beads were spherical in shape with a smooth out core but alginate-chitosan beads were shown more smooth skin (Fig. 4.7a, b, c and Fig. 4.8a, b, c). This can be elucidated by the positive charge of chitosan to form semi-permeable membrane around the bead. Whereas surface morphology of used alginate beads were shown numerous bulges all over the bead surface (Fig. 4.10a and b) indicated that the cross-linking of Ca-alginate bead were damaged. When the bead was cut into a thick section, the surface of section was show smooth also (Fig. 4.7 d, e, and Fig. 4.8 d, e) while a thick section of used alginate beads was shown cracked area as seen in Fig. 4.9e. Therefore, this experiment were use alginate-chitosan beads because of its higher strongly than alginate bead.

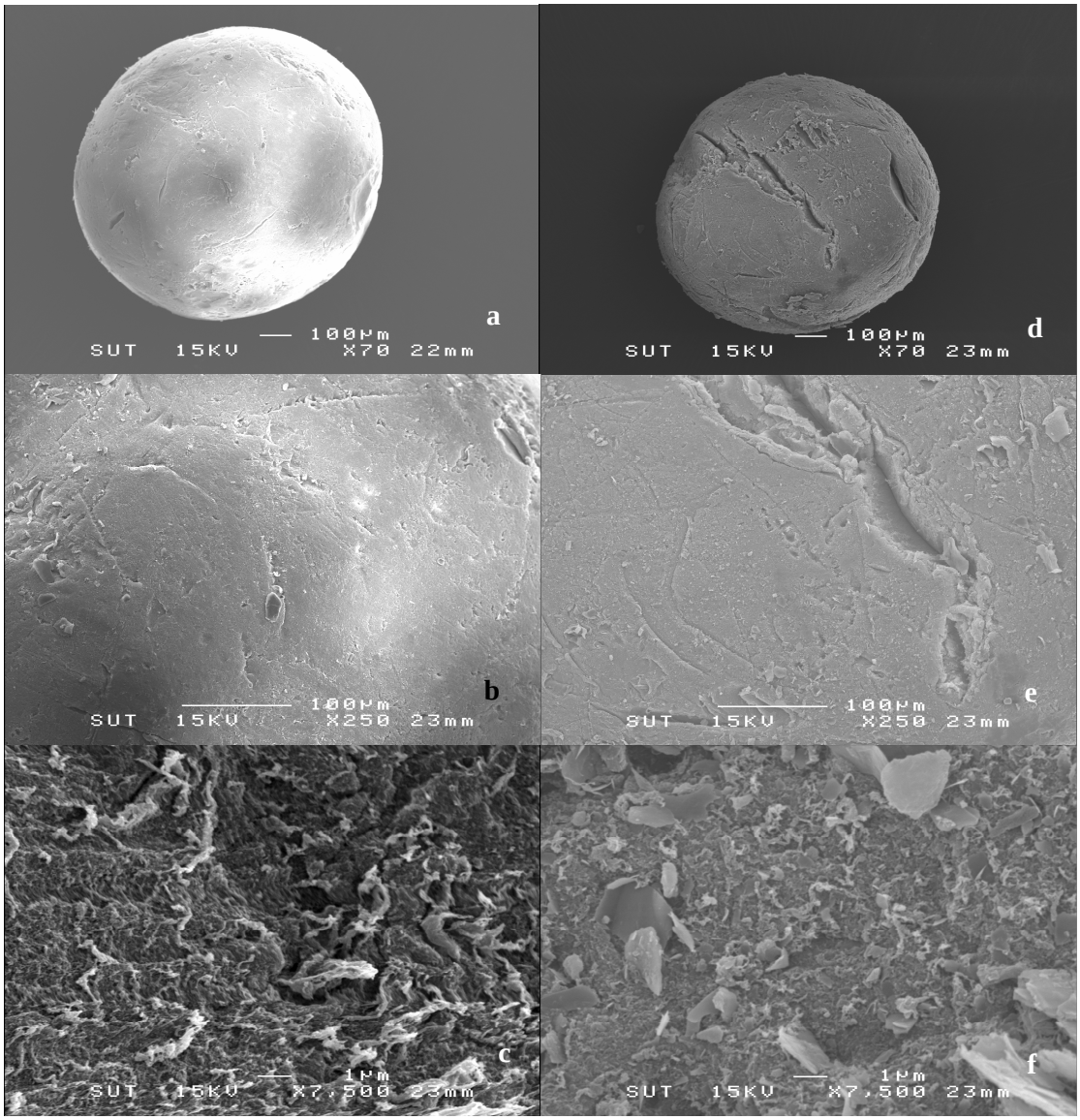

Figure 4.7 Scanning electron micrographs of immobilized *Azotobacter* sp. in alginate bead before used (a) its surface morphology, scale bar 100 µm and magnification x70 (b) its surface morphology, scale bar 100 µ and magnification x250 (c) its surface morphology, scale bar 1 µm and magnification x7500 (d) its thick-section cut, scale bar 100 µm and magnification x70 (e) its thick-section cut, scale bar 100 µm and magnification x250 (f) its thick-section cut, scale bar 1 µm and magnification x7500.

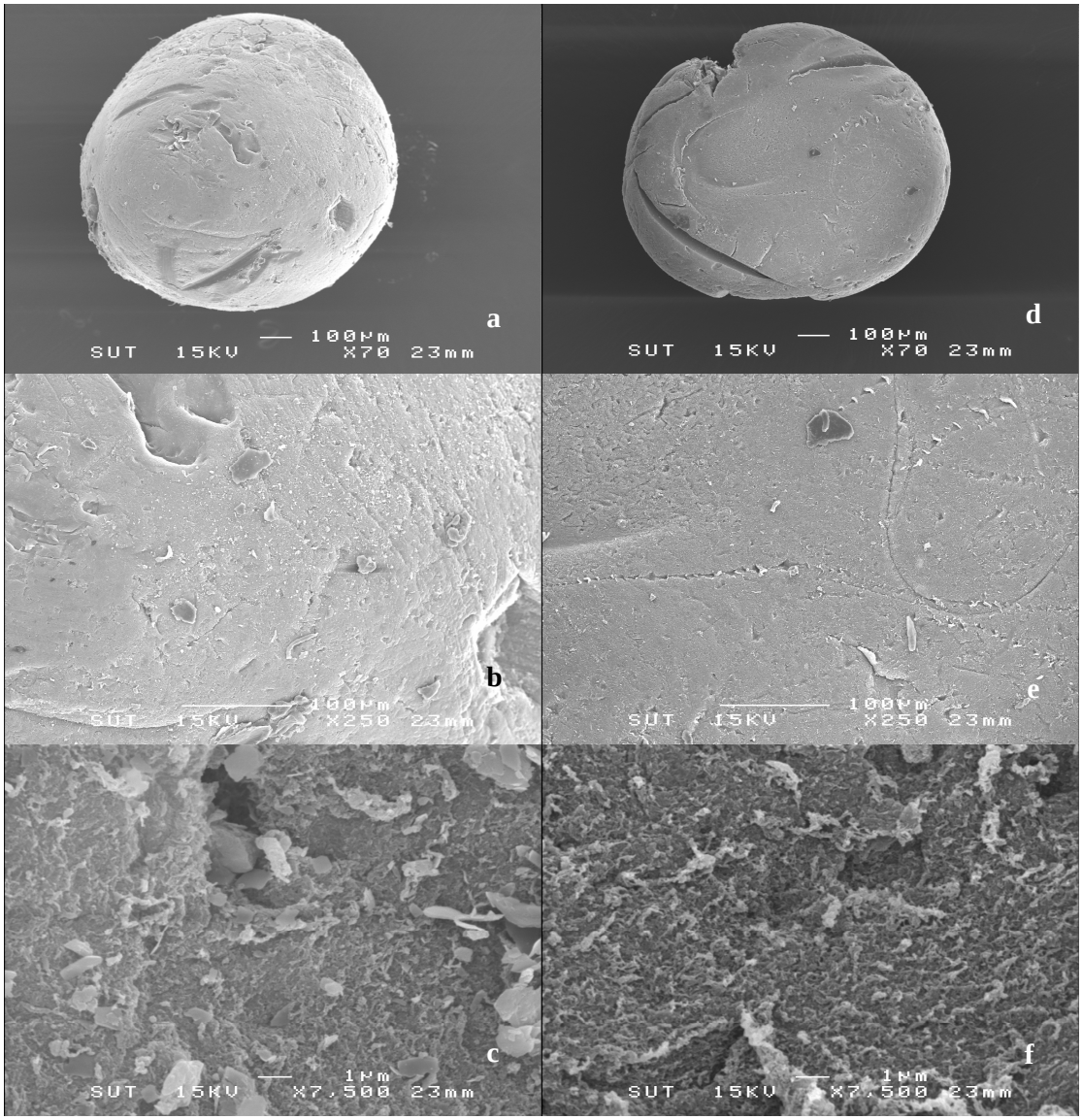

Figure 4.8 Scanning electron micrographs of immobilized *Azotobacter* sp. in alginate bead coated with chitosan before used (a) its surface morphology, scale bar 100 µm and magnification x70 (b) its surface morphology, scale bar 100 µm and magnification x250 (c) its surface morphology, scale bar 1 µm and magnification x7500 (d) its thick-section cut, scale bar 100 µm and magnification x70 (e) its thick-section cut, scale bar 100 µm and magnification x250 (f) its thick-section cut, scale bar 1 µm and magnification x7500.

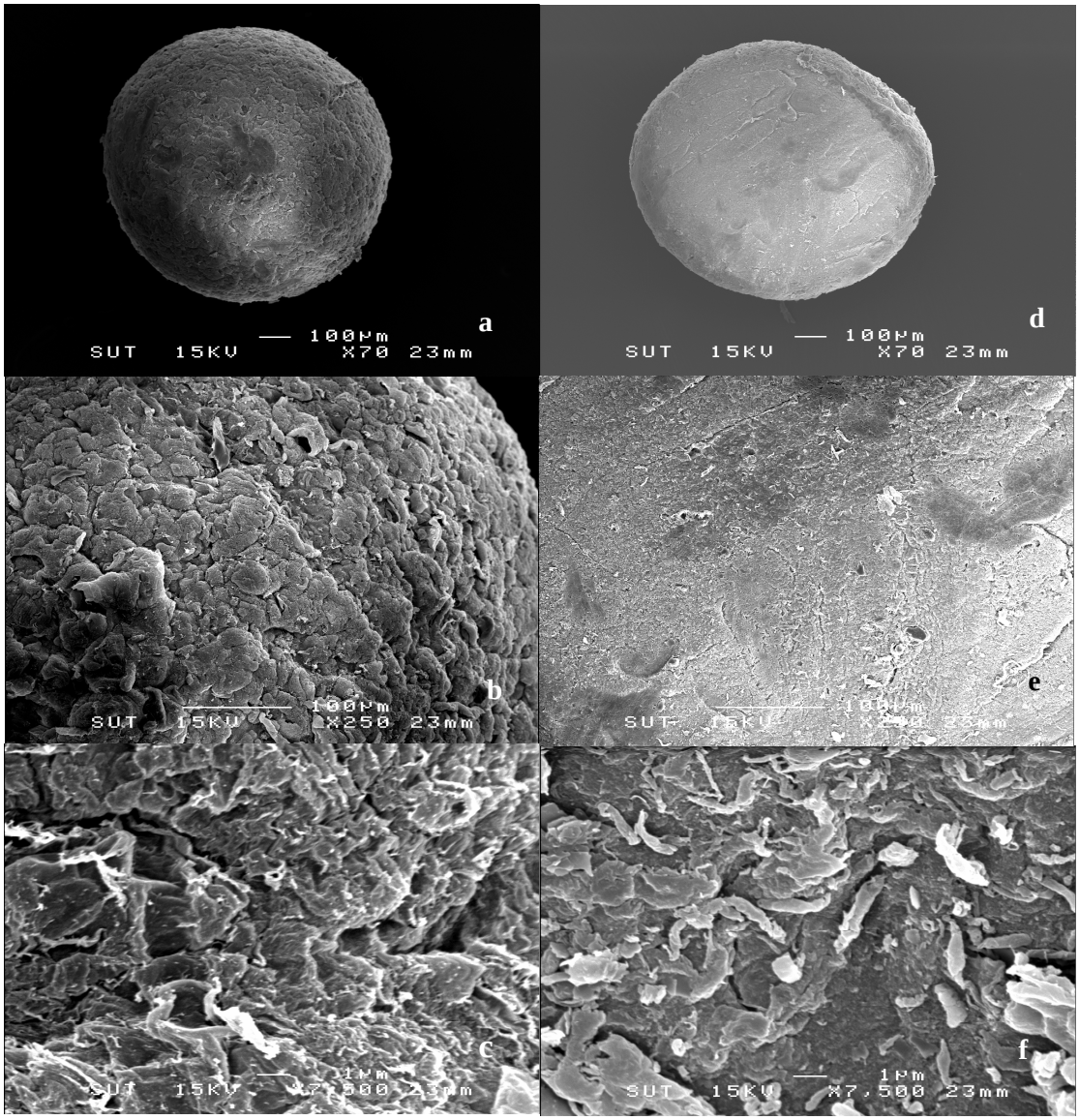

Figure 4.9 Scanning electron micrographs of immobilized *Azotobacter* sp. in alginate bead after used to determine IAA production in LG medium (a) its surface morphology, scale bar 100 µm and magnification x70 (b) its surface morphology, scale bar 100 µm and magnification x250 (c) its surface morphology, scale bar 1 µm and magnification x7500 (d) its thick-section cut, scale bar 100 µm and magnification x70 (e) its thick-section cut, scale bar 100 µm and magnification x250 (f) its thick-section cut, scale bar 1 µm and magnification x7500.

4.5 Applied immobilized *Azotobacter* sp. under hydroponic system (First batch)

The experiment were obtained for 45 days from 28th November 2008 to 11th January 2009, temperature was between 10-28°C, nutrient solution pH 6.5, EC= 22 mS cm^{-1}.

4.5.1 Applied with immobilized *Azotobacter* sp. 2.5-12.5 g bead per plant

4.5.1.1 Parameter of plant growth

Growth of Choy sum under various immobilized *Azotobacter* sp. bead concentration found that 2.5 g bead per plant showed the highest stem fresh weight, root fresh weight, stem dry weight and root dry weight. Root length was lower than control except in applied with 5 g bead per plant. However, the data showed not significant different at P value ≤ 0.05 (Fig. 4.10).

4.5.1.2 Elements analysis

Elements concentration in plant that applied with immobilized *Azotobacter* sp. 0-12.5 g bead per plant were showed in Table 4.1. Result reveal that nitrogen concentration was significant higher than control only in applied with 10-12.5 g bead per plant. Phosphorus concentration significant higher than control excepted in applied with 7.5 g bead per plant was significant lower than control. Potassium concentration also higher than control except in applied with 2.5 g bead per plant, but for calcium concentration was higher than control only in applied with 12,5 g bead per plant

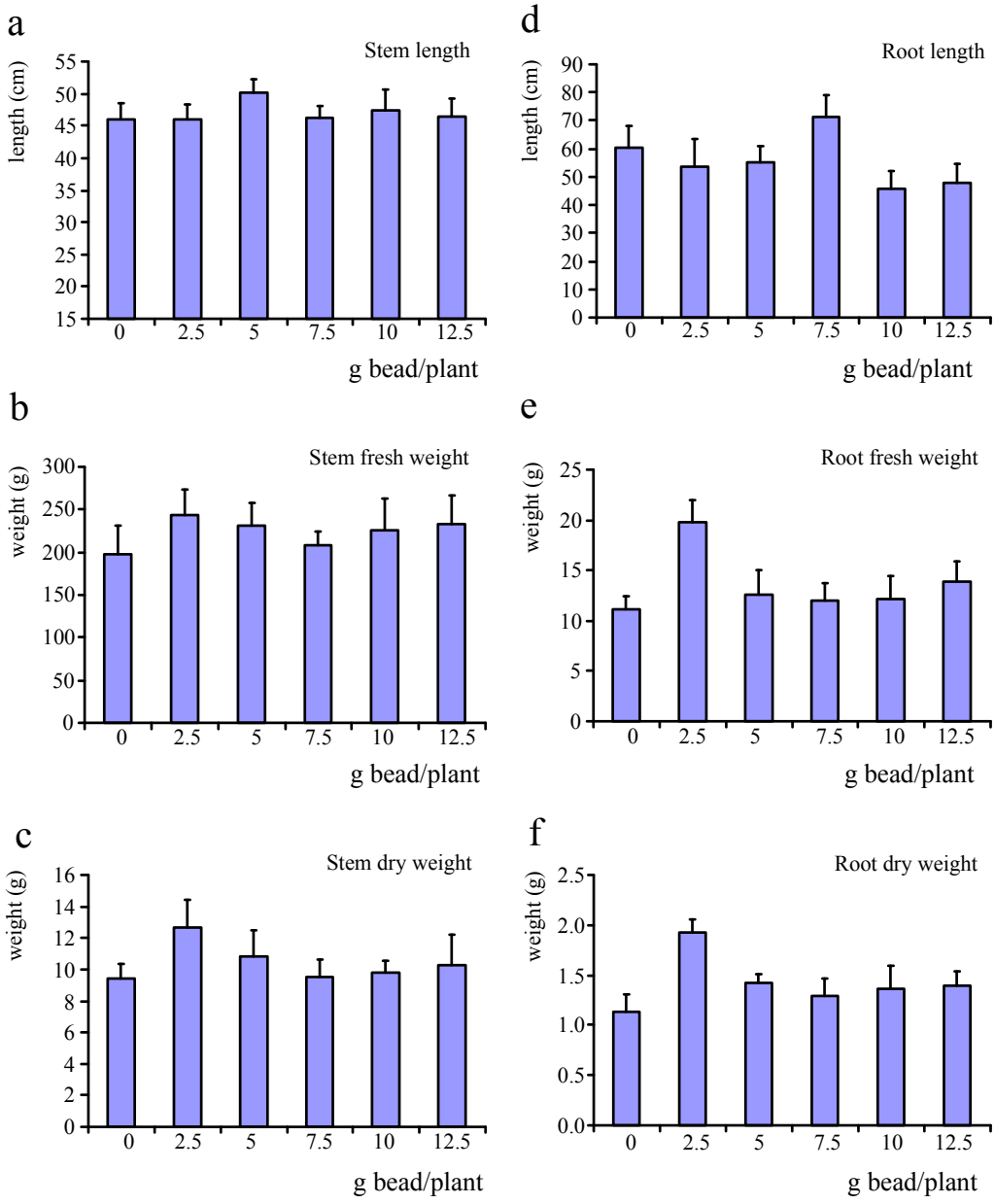

Figure 4.10 Growth parameter of Choy sum (*Brassica chinensis var parachinensis*) in hydroponic system applied with immobilized *Azotobacter* sp. 2.5-12.5 g bead/plant, temperature was 10-28^{o}C (winter season), nutrient solution pH 6.5, EC= 22 mS cm^{-1}; (a) stem length, (b) stem fresh weight, (c) stem dry weights, (d) root length, (e) root fresh weight and (f) root dry weight.

Table 4.1 Elements analysis of Choy sum (*Brassica chinensis var parachinensis*) applied with immobilized *Azotobacter* sp. 0-12.5 g bead per plant in hydroponic system, temperature 10-28 ^{o}C (winter season), nutrient solution pH 6.5, EC= 22 mS cm^{-1}.

Beads (g bead/plant)	Concentration of elements (%)			
	N	P	K	Ca
0	4.00^{c}	1.26^{d}	2.59^{d}	3.10^{bc}
2.5	3.43^{d}	1.31^{c}	2.50^{d}	3.15^{b}
5.0	4.08^{c}	1.38^{b}	2.87^{c}	3.15^{b}
7.5	4.04^{c}	0.54^{f}	3.29^{a}	2.60^{d}
10.0	4.28^{b}	1.48^{a}	3.36^{a}	3.05^{c}
12.5	5.21^{a}	1.07^{e}	3.08^{b}	3.40^{a}

Values followed by the same letter are not significantly different at $P \leq 0.05$ by DMRT

4.5.2 Applied with IAA solution 10-100 µM

4.5.2.1 Parameter of plant growth

In applied with 1 ml IAA solution 0-100 µM per plant per week, result showed stem length, stem fresh weight, root fresh weight, stem dry weight and root dry weight were higher than control. Although root length was lower than control except in applied 1 ml IAA solution 80 µM per plant per week (Fig. 4.11). All of data were not significant different at P value ≤ 0.05.

4.5.2.2 Elements analysis

Result of elements concentration of plant that applied with 1 ml IAA solution 0-100 µM per plant per week showed in Table 4.2. Nitrogen concentration of all treatments was higher than control, but significant higher only in applied with 1 ml IAA solution 20, 40 µM per plant per week. For applied with 1 ml IAA solution 60, 80 µM per plant per week were showed significant higher than control in phosphorus concentration. Potassium concentration in plant were showed significant higher than control in applied with 1 ml IAA solution 20-80 µM per plant per week. Whereas, calcium concentration in plant applied with 1 ml IAA solution 40-60 µM per plant per week were showed significant higher than control ($P \leq 0.05$).

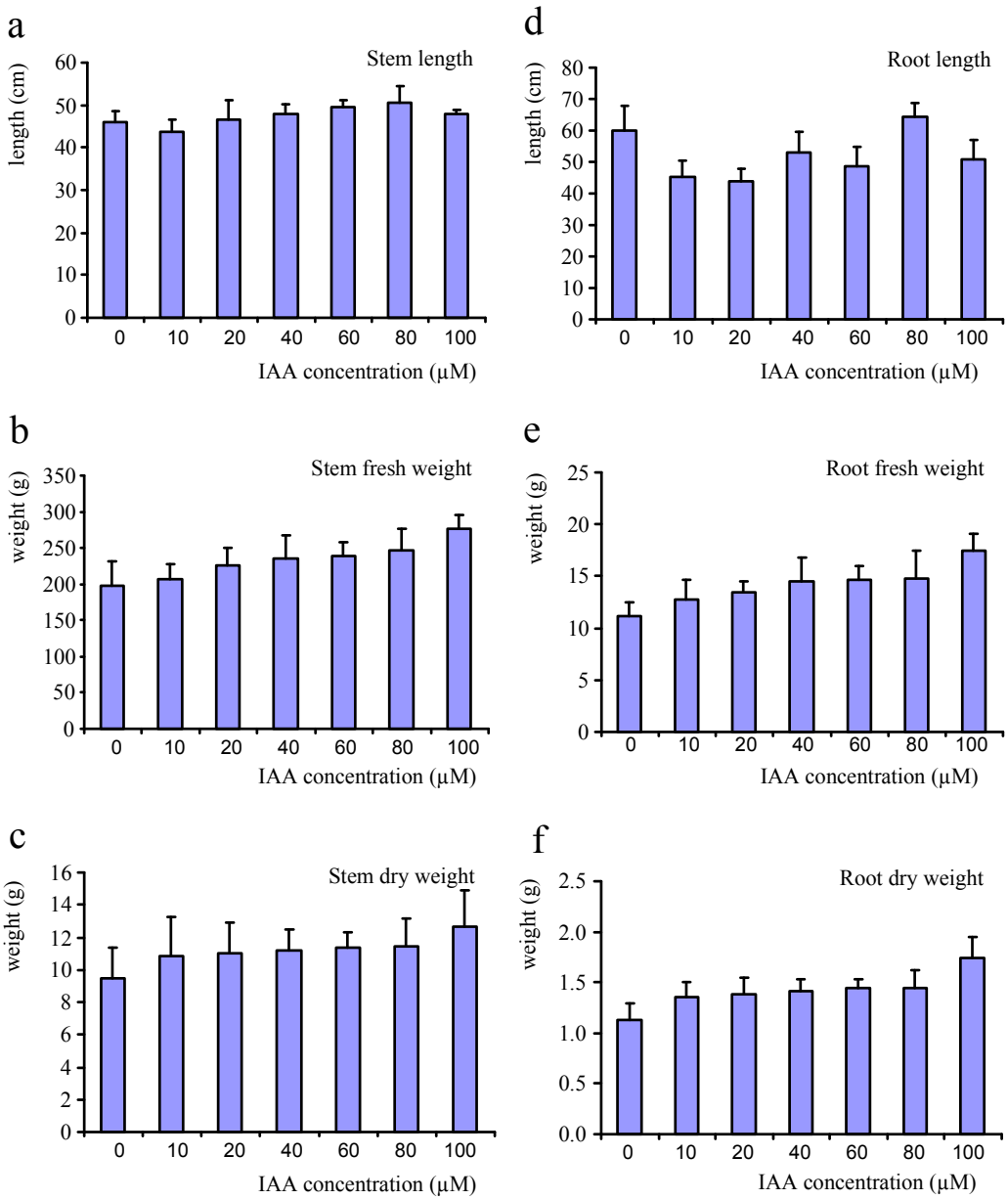

Figure 4.11 Growth parameter of Choy sum (*Brassica chinensis* var *parachinensis*) in hydroponic system applied with IAA solution in the range of 0-100 µM per week per plant, temperature was 10-28^{o}C (winter season), nutrient solution pH 6.5, EC= 22 mS cm^{-1}; (a) stem length, (b) stem fresh weight, (c) stem dry weights, (d) root length, (e) root fresh weight and (f) root dry weight.

Table 4.2 Elements analysis of Choy sum (*Brassica chinensis var parachinensis*) applied with IAA solution 10-100 µM in hydroponic system, temperature 9-28 ^{o}C (winter season), nutrient solution pH 6.5, EC= 22 mS cm^{-1}.

IAA solution (µM)	Concentration of elements (%)			
	N	P	K	Ca
0	4.00^{c}	1.26^{c}	2.59^{c}	3.1^{b}
10	4.04^{bc}	1.1^{e}	2.07^{d}	1.74^{c}
20	4.52^{a}	1.13^{d}	3.83^{a}	1.82^{c}
40	4.44^{ab}	1.02^{g}	3.69^{a}	3.33^{a}
60	4.12^{abc}	1.28^{b}	3.42^{b}	3.34^{a}
80	4.28^{abc}	1.45^{a}	3.36^{b}	3.09^{b}
100	4.04^{bc}	1.06^{f}	2.35^{c}	3.17^{b}

Values followed by the same letter are not significantly different at $P \leq 0.05$ by DMRT

4.6 Applied immobilized *Azotobacter* sp. under hydroponic system (Second batch)

The experiment were obtained from 28th February 2009 to 13th April 2009, temperature was between 27-37°C, nutrient solution pH 6.5, EC= 22 mS cm^{-1}.

4.6.1 Applied with immobilized *Azotobacter* sp. 2.5-12.5 g bead per plant

4.6.1.1 Parameters of plant growth

Effect of immobilized *Azotobacter* sp. on plant growth showed that all of growth parameters comprise of stem length, root length, stem fresh weight, root fresh weight, stem dry weight and root dry weight were higher than control, but significant higher only in stem length and stem dry weight (Fig. 4.12).

4.6.1.2 Elements analysis

The elements concentration of plant that applied with immobilized *Azotobacter* sp. 2.5-12.5 g bead per plant were showed nitrogen and phosphorus concentration significant higher than control, whereas potassium and calcium concentration were significant higher than control simply in applied with immobilized *Azotobacter* sp. 7.5-12.5 g bead per plant (Table 4.3).

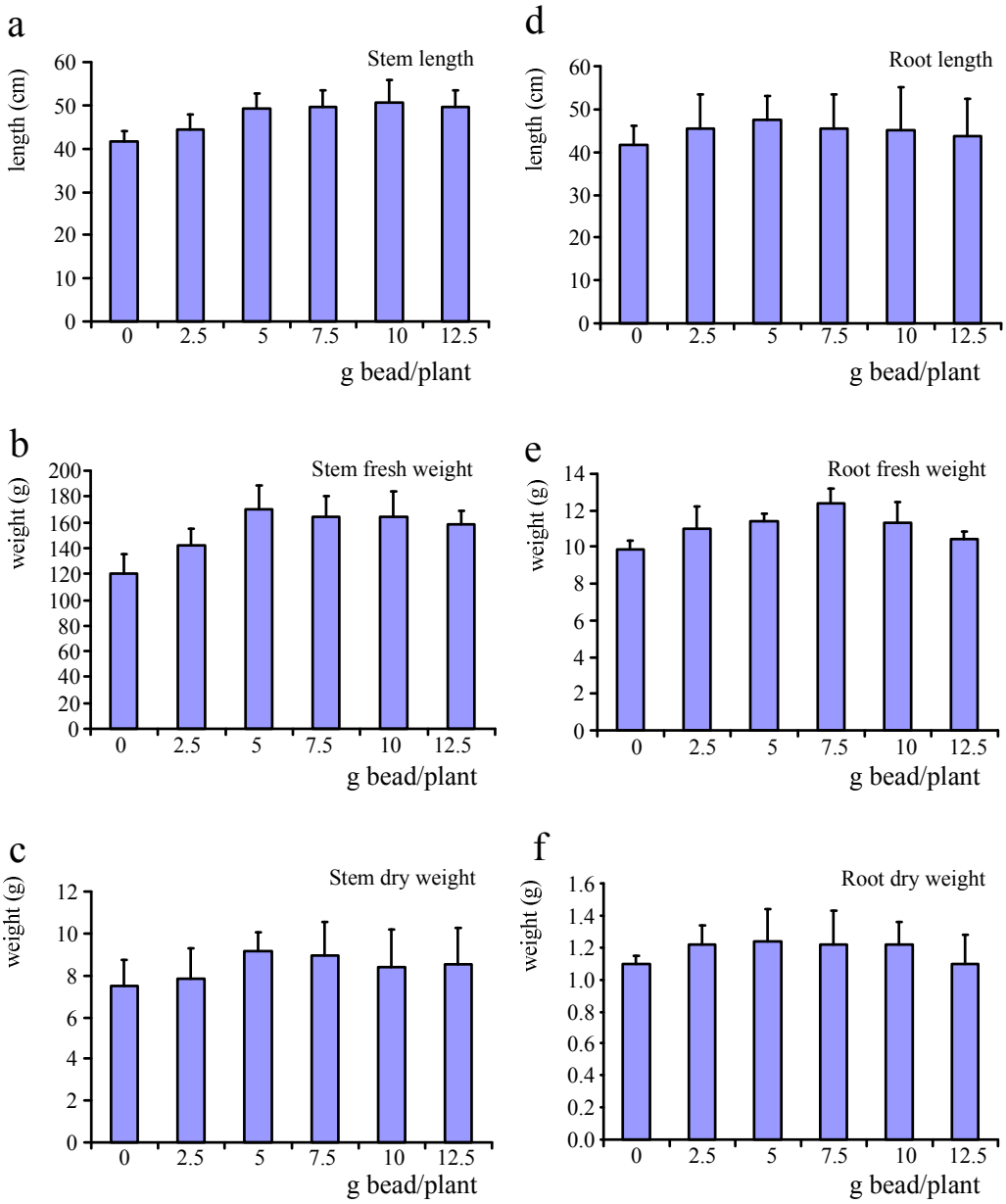

Figure 4.12 Growth parameter of Choy sum (*Brassica chinensis var parachinensis*) in hydroponic system applied with immobilized *Azotobacter* sp. 2.5-12.5 g bead/plant, temperature was 28-37^{o}C (summer season), nutrient solution pH 6.5, EC = 22 mS cm^{-1}; (a) stem length, (b) stem fresh weight, (c) stem dry weights, (d) root length, (e) root fresh weight and (f) root dry weight.

Table 4.3 Element analysis of Choy sum applied with immobilized *Azotobacter* sp. 2.5-12.5 g per plant temperature was 28-37^{o}C (summer season), nutrient solution pH 6.5, EC = 22 mS cm^{-1}**.**

Beads (g/plant)	Elements concentration (%)			
	N	P	K	Ca
0	4.33^{c}	0.4^{b}	2.6^{c}	1.73^{a}
2.5	4.91^{ab}	0.48^{a}	2.43^{cd}	1.17^{c}
5	4.83^{ab}	0.46^{a}	2.29^{d}	1.33^{b}
7.5	5.26^{a}	0.47^{a}	3.29^{b}	1.71^{a}
10	4.59^{bc}	0.46^{a}	3.92^{a}	1.72^{a}
12.5	4.88^{ab}	0.46^{a}	3.96^{a}	1.78^{a}

Values followed by the same letter are not significantly different at $P \leq 0.05$ by DMRT

4.6.2 Applied with IAA solution 10-100 µM

4.6.2.1 Parameter of plant growth

Plant were applied with 1 ml IAA solution 10-100 µM per plant per week, the result of plant growth showed in Fig. 4.13. Stem length, stem fresh weight and stem dry weight of Choy sum were slightly higher than control as a result to not significant different at P value ≤ 0.05. Moreover, Root was lower than control except in applied with 1 ml IAA solution 100 µM per plant per week, root fresh weight and root dry weight were also lower than control except in applied with 1 ml IAA solution 80-100 µM per plant per week.

4.6.2.2 Elements analysis

Plant element analysis of Choy sum applied with 1ml IAA solution 10-100 µM per plant per week were showed in Table 4.4. The result of nitrogen concentration were that significantly higher than control. Phosphorus concentration was significantly higher than control except in applied with 1ml IAA solution 10, 20 µM per plant per week was significantly lower than control. Percent potassium were similarly to control except in applied with 1ml IAA solution 20 µM per plant per week was significantly higher than control and calcium concentration was significantly higher than control.

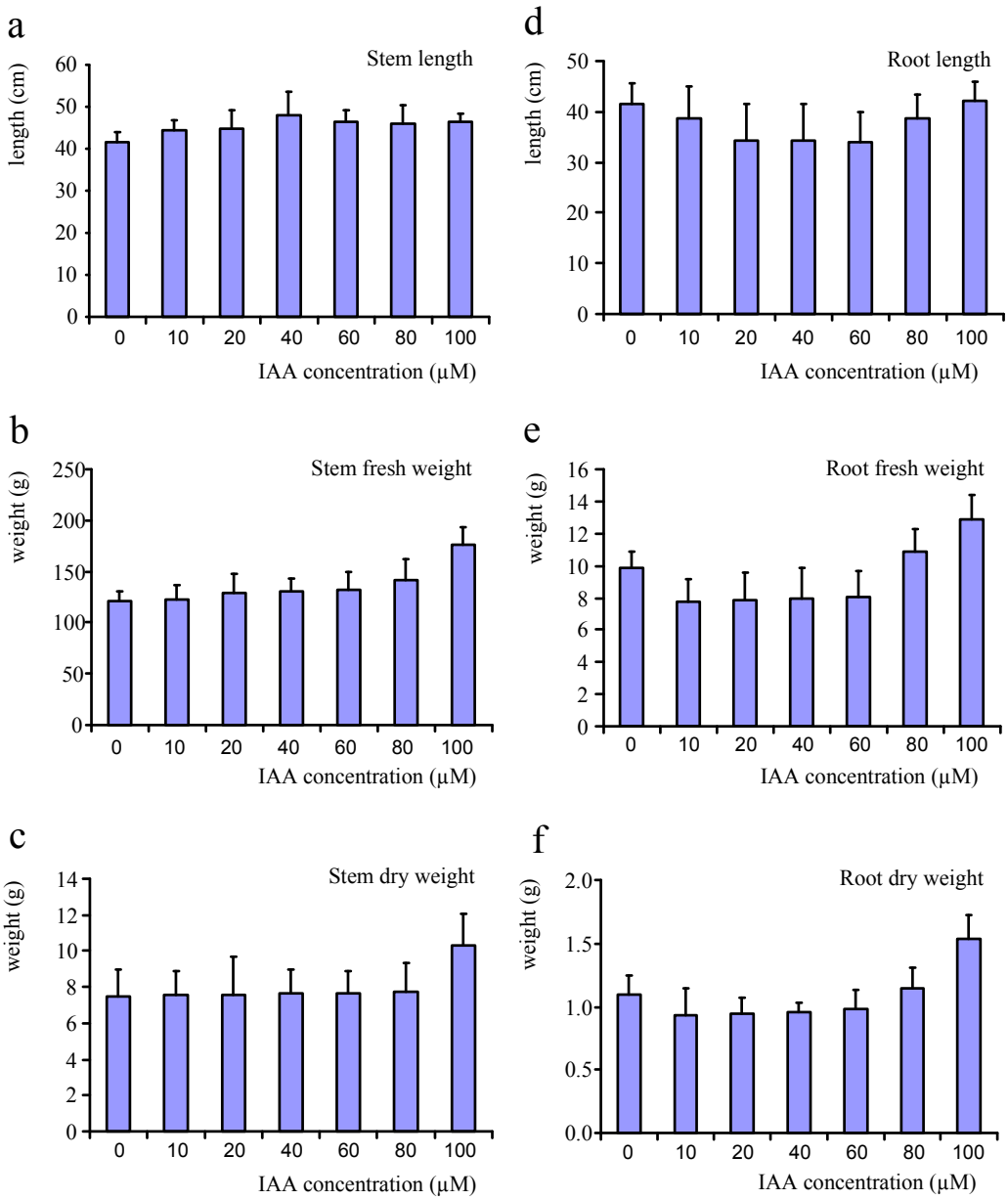

Figure 4.13 Growth parameter of Choy sum (*Brassica chinensis* var *parachinensis*) in hydroponic system applied with IAA solution in the range of 0-100 µM per week per plant; (a) stem length, (b) stem fresh weight, (c) stem dry weights, (d) root length, (e) root fresh weight and (f) root dry weight.

Table 4.4 Element analysis of Choy sum applied with IAA solution 10-100 µM.

IAA solution (µM)	Element concentration (%)			
	N	P	K	Ca
0	4.33^{d}	0.4^{c}	2.6^{bc}	1.73^{c}
10	4.97^{a}	0.41^{c}	2.72^{bc}	1.74^{c}
20	4.56^{c}	0.39^{c}	3.28^{a}	1.72^{c}
40	4.74^{bc}	0.5^{ab}	2.84^{b}	1.86^{b}
60	4.83^{ab}	0.52^{a}	2.57^{bc}	1.8^{bc}
80	4.59^{c}	0.47^{b}	2.7^{bc}	2^{a}
100	4.83^{ab}	0.46^{b}	2.37^{c}	1.2^{d}

Values followed by the same letter are not significantly different at $P \leq 0.05$ by DMRT

4.7 Applied immobilized *Azotobacter* sp. in light room

This experiment was established in light room, the same plant as section 4.5 was used, the factors were similar also (immobilized *Azotobacter* sp. 0-12.5 g bead per plant and IAA solution 0-100 µM). The experiment were obtained from 25th February 2009 to 11th April 2009, temperature was 25^{o}C, nutrient solution pH 6.5, EC= 22 mS cm^{-1}.

4.7.1 Applied with immobilized *Azotobacter* sp. 2.5-12.5 g bead per plant

4.7.1.1 Parameter of plant growth

Plant growth of Choy sum applied with immobilized *Azotobacter* sp. 2.5-12.5 g bead per plant revealed that root length, stem fresh weight, root fresh weight, stem dry weight and root dry weight were higher than control but was not significantly different. Whereas, stem length was lower than control and slightly decrease when amount of bead applied increase. Furthermore, stem dry weight and root dry weight were highest when applied with 5 g bead per plant and significant higher tan control ($P \leq 0.05$) (Fig. 4.14).

4.7.1.2 Elements analysis

Plant element analysis of Choy sum applied with immobilized *Azotobacter* sp. 2.5-12.5 g bead per plant were showed in Table 4.5. The result of nitrogen, phosphorus and potasium concentration similarly to control except in applied with immobilized *Azotobacter* sp. 2.5-12.5 g bead per plant was significant higher than control. Whereas calcium concentration in applied with immobilized *Azotobacter* sp. 2.5-5 g bead per plant were significant higher than control**.**

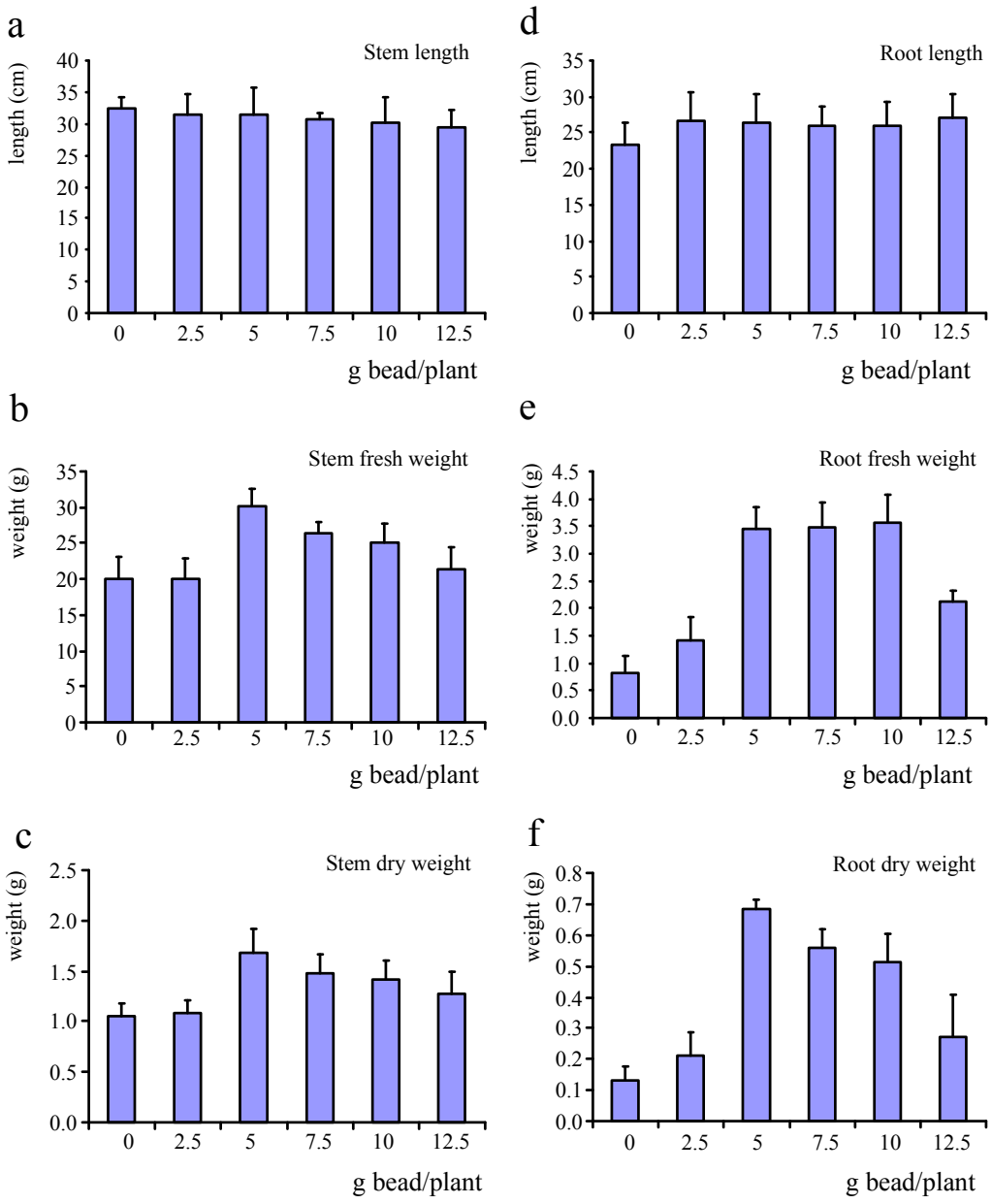

Figure 4.14 Growth parameter of Choy sum (*Brassica chinensis var parachinensis*) in light room applied with immobilized *Azotobacter* sp. 2.5-12.5 g bead/plant, temperature was 25^{o}C, nutrient solution pH 6.5, EC = 22 mS cm^{-1}; (a) stem length, (b) stem fresh weight, (c) stem dry weights, (d) root length, (e) root fresh weight and (f) root dry weight.

Table 4.5 Element analysis of Choy sum applied with immobilized *Azotobacter* sp. 2.5-12.5 g per plant in light room**,** temperature 25 ^{o}C nutrient solution pH 6.5, EC = 22 mS cm^{-1}.

Beads (g/plant)	% Elements			
	N	P	K	Ca
0	5.09^{b}	0.45^{b}	2.63^{c}	0.77^{b}
2.5	5.12^{b}	0.41^{c}	3.13^{b}	0.91^{a}
5	4.42^{c}	0.40^{c}	3.11^{b}	0.91^{a}
7.5	5.06^{b}	0.45^{b}	2.73^{c}	0.73^{b}
10	4.39^{c}	0.41^{c}	3.16^{ab}	0.66^{c}
12.5	5.70^{a}	0.48^{a}	3.25^{a}	0.77^{b}

Values followed by the same letter are not significantly different at $P \leq 0.05$ by DMRT

4.7.2 Applied with IAA solution 10-100 µM

4.7.2.1 Parameter of plant growth

Growth parameters of plant in light room that applied with 1 ml IAA solution 10-100 µM per week per plant disclose that stem fresh weight, root fresh weight, stem dry weight and root dry weight were significant higher than control. Whereas stem length and root length were not significant different from control at P value ≤ 0.05. Applied with 1 ml IAA solution 80 µM per week per plant was showed highest for stem dry weight, for root dry weight applied with 1 ml IAA solution 60 µM per week per plant was highest.

4.7.2.2 Elements analysis

Plant element analysis of Choy sum applied with 1ml IAA solution 10-100 µM per plant per week were showed in Table 4.6. Nitrogen concentration were higher than control in applied with 1ml 40, 80 µM per plant per week, when potassium concentration was significant higher than control. Also in calcium concentration was significant higher than control except in applied with 1 ml 10 µM per plant per week was lower than control.

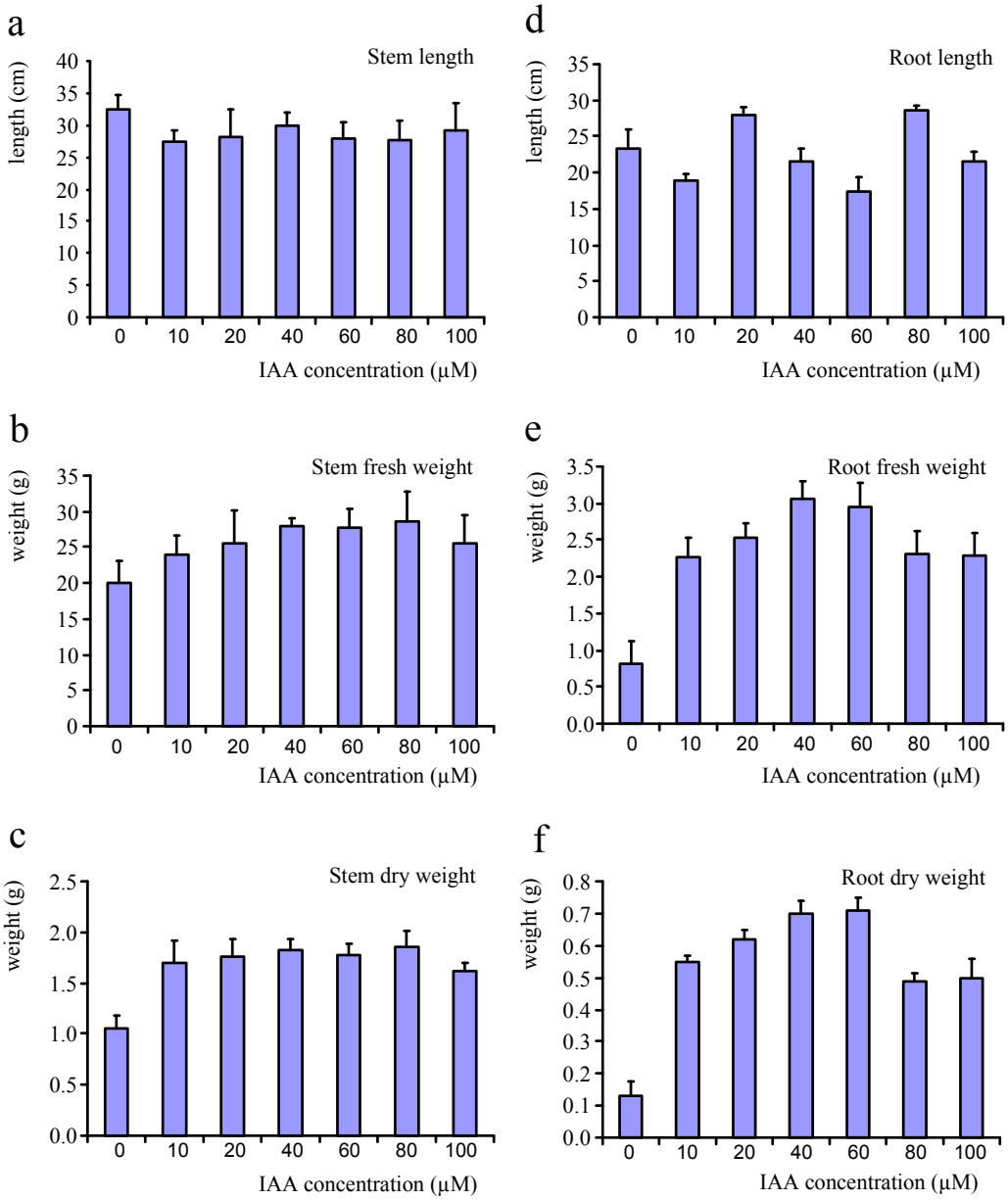

Figure 4.15 Growth parameter of Choy sum (*Brassica chinensis* var *parachinensis*) in light room applied with IAA solution in the range of 0-100 µM per week per plant, temperature was 25°C, nutrient solution pH 6.5, EC = 22 mS cm^{-1}; (a) stem length, (b) stem fresh weight, (c) stem dry weights, (d) root length, (e) root fresh weight and (f) root dry weight.

Table 4.6 Element analysis of Choy sum in light room applied with IAA solution in the range of 0-100 µM per week per plant, temperature was 25°C, nutrient solution pH 6.5, EC = 22 mS cm^{-1}**.**

IAA solution (µM)	% Elements			
	N	P	K	Ca
0	5.09^{ab}	0.45^{a}	2.63^{e}	0.77^{c}
10	5.03^{ab}	0.27^{c}	3.3^{c}	0.64^{d}
20	5.03^{ab}	0.45^{a}	3.23^{c}	0.78^{bc}
40	5.15^{a}	0.36^{b}	3.10^{d}	0.84^{abc}
60	4.62^{c}	0.32^{bc}	3.57^{a}	0.90^{a}
80	5.15^{a}	0.33^{bc}	3.40^{b}	0.78^{bc}
100	4.8^{bc}	0.42^{a}	3.02^{d}	0.86^{ab}

Values followed by the same letter are not significantly different at $P \leq 0.05$ by DMRT

In this study optimum condition for growth of Choy sum can summarized in Table 4.7. The first batch in hydroponic system revealed that applied with immobilized *Azotobacter* sp. 2.5 g bead per plant and IAA solution 100 µM (1 ml/plant/week) were the best condition for stimulated growth of Coy sum. Whereas, in the second batch, the best condition were showed in applied with immobilized *Azotobacter* sp. 5 g bead per plant but for IAA solution was similar to the first batch. In light room revealed that applied with immobilized *Azotobacter* sp. 5 g bead per plant and IAA solution 40-60 µM (1 ml/plant/week) were promoted plant growth at best.

Table 4.7 Summary of optimum condition for growth of Choy sum.

Growth system	Optimum condition for plant growth	
	Bead (g/plant)	IAA (µM) (at 1ml/plant/week)
Hydroponic (first batch)	2.5	100 µM (1 ml/plant/week)
Hydroponic (second batch)	5	100 µM (1 ml/plant/week)
Light room	5	40-60 µM (1 ml/plant/week)

Furthermore, from the result of plant growth found that when compare between the first batch and second batch, the first batch (performed in winter season) was showed the better in all parameter of plant growth than in second batch. Mikel et al., (2005) reported that growth season has effects on plant growth for the reason that different season result to diverse condition in plant system. In spring, temperature condition is softer, more acidic and had higher EC compare those grown in autumn. In addition, light in winter is less than light in summer, a plant's response to light will vary depending on the intensity, duration and wavelength of the light it receives.

Their morphological expression of etiolation is related to the effect of light on auxin distribution and synthesis (Bergman, 1985). Temperature was also effects o plant growth, an increase in temperature from 20-30 ^{o}C results in a marked increase in the photosynthetic rate (Mastalerz, 1977). As well in microorganism the different condition in various season may result to survival and activities of microorganism together with performance to promote plant growth, especially in IAA production because of its sensitivity to light.

From the data of plant growth found that two factors include of 1) immobilized *Azotobacter* sp. 2) IAA solution also can stimulated plant higher than control but was not significant different. This may explain by the available of nutrient in plant growth system especially in hydroponic system. However, some data were lower than control and the effect of two factors on plant were not constant. This may be because of the root exudates of plant result to ability to associated with plant roots and activities to promoted plant growth by microorganism. In this thesis found that in light room when root can generate through the lower side of pot that contain bead and nutrient, bead entrapped *Azotobacter* sp. were attach with root (Fig. 4.16). Plant roots able to release a wide range of organic and inorganic compounds into root environment. The majority of substrates for microbial activities in the rhizosphere are derived from the plant that depend on plant species and environmental conditions. An increase in the number and activities of microbes in the surrounding area of plant roots, a phenomenon known as the rhizosphere effect, was describes by Hiltner (1904). Furthermore, this study found that *Azotobacter* sp. can not synthesize IAA in the absence of glucose in culture medium. Several report revealed that, among the soluble components of roots exudate, sugars are often reported to be the major

component (Lochhead and Rouatt, 1955; Vancura, 1964; Vancura and Hovadik, 1965). The synthesis of phytohormones by rhizosphere bacteria often depends on the presence of phytohormone precursors in the root exudates of plants. Tryptophan is a metabolic precursor of IAA in almost all soil bacteria (Fallik et al., 1994; Sarwar et al., 1992). In the plant rhizosphere, the main source of tryptophan is the root exudates of plants (Kravchenko et al., 2002). The ability of *Azotobacter* sp. to survive in plant growth system may be result to its activities to plant growth. The absorbtion of nutrient into bead result to available of entrapped microorganism.

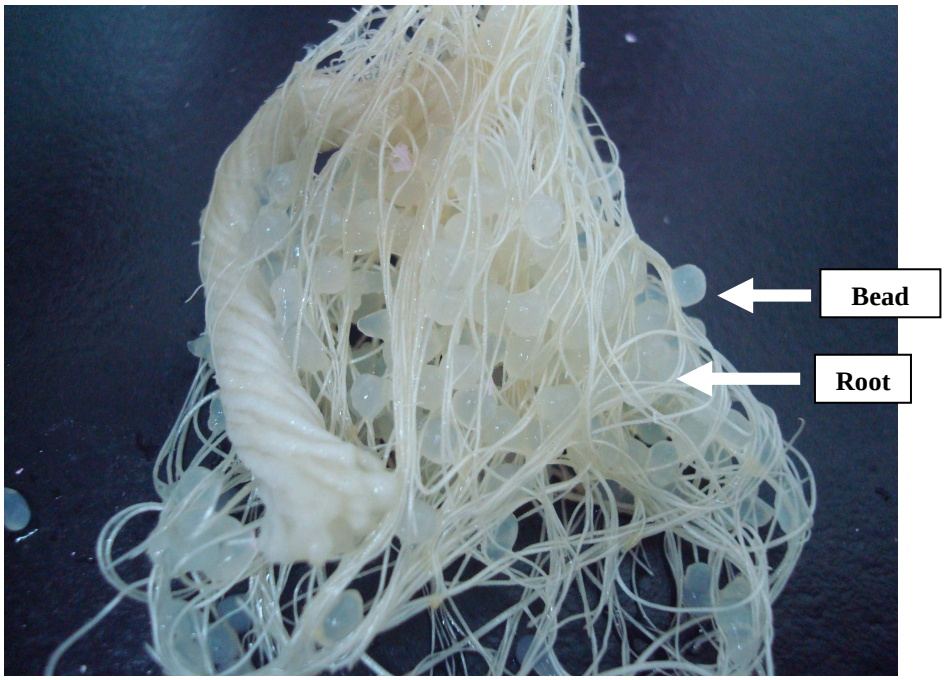

Figure 4.16 Beads entrapped *Azotobacter* sp. associated with plant root.

CHAPTER V

CONCLUSION

The optimum conditions for IAA production by immobilized *Azotobacter* sp. were preliminary studied. The immobilized *Azotobacter* sp. was able to produced IAA in LG medium containing L-tryptophan and produced higher in LG medium containing glucose and nitrogen. The optimal temperature for IAA production by immobilized *Azotobacter* sp. was 20 ^{o}C. The immobilized *Azotobacter* sp. able to survive in LG medium, initial pH 4-9 more than three weeks, only one week in initial pH 3.5 that does not happen in free cell.

This study observed parameters of plant growth in hydroponic system for two batch, two factors were tested in hydroponic system to estimated plant growth parameter; 1) immobilized *Azotobacter* sp. in the series 2.5-12.5 g bead/ plant, 2) IAA solution in concentration 10-100 μM was applied compare to control (no bead, no IAA). Choy sum (*Brassica chinensis* var *parachinensis*) was used as a model to study effect of immobilized *Azotobacter* sp. on plant growth. The parameter of plant growth included stem wet weight, root wet weight, stem dry weight, root dry weight, stem length, root length and element analysis (N, P, K and Ca) were investigated. Results showed that all of two treatments can promote plant growth better than control but not significant different ($P \leq 0.05$). However, optimum condition for growth of Choy sum in the first batch in hydroponic system showed that applied with immobilized *Azotobacter* sp. 2.5 g bead per plant and IAA solution 100 μM (1 ml/plant/week)

were exposed the best conditions for stimulated growth. Whereas, in second batch the best condition was showed in applied with immobilized *Azotobacter* sp. 5 g bead per plant but for IAA solution was similar to the first batch. In growth chamber revealed that applied with immobilized *Azotobacter* sp. 5 g bead per plant and IAA solution 40-60 μM (1 ml/plant/week) were promoted plant growth at best.

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