การเพิ่มธาตุสังกะสีอินทรีย์ใน *SPIRULINA PLATENSIS*

นายอาทิตย์ จตุพรพงศ์ชัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ENRICHMENT OF ORGANIC ZINC IN

SPIRULINA PLATENSIS

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ENRICHMENT OF ORGANIC ZINC IN *SPIRULINA PLATENSIS*

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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วัตถุประสงค์ของงานวิจัยนี้เพื่อศึกษาประสิทธิภาพการดูดซึมเกลือสังกะสีในรูปต่าง ๆ (สังกะสีซัลเฟต สังกะสีไนเตรท และสังกะสีคลอไรด์) ของสไปรูไลนา และระบุตำแหน่งของธาตุสังกะสีที่สะสมในเซลล์สไปรูไลนา เพื่อประเมินการพร้อมนำไปใช้ประโยชน์ (accessibility) และการนำไปใช้ประโยชน์ (availability) ของธาตุสังกะสีอินทรีย์ในสไปรูไลนาโดยวิธีการจำลองระบบการย่อยในระบบทางเดินอาหารในหลอดทดลอง (*in vitro*) และรวมถึงประเมินการเปลี่ยนแปลงและการเก็บรักษาสไปรูไลนาสด จากการทดลองพบว่า สไปรูไลนาที่เลี้ยงในอาหารเลี้ยงดัดแปร Zarrouk's ด้วยธาตุสังกะสีซัลเฟต (MZS) มีค่าชีวมวล (biomass) ปริมาณธาตุสังกะสี และปริมาณโปรตีนของเซลล์สูงที่สุด และแตกต่างอย่างมีนัยสำคัญ (p<0.05) จากอาหารเลี้ยงที่เติมเกลือสังกะสีชนิดอื่น นอกจากนี้สไปรูไลนาที่เลี้ยงในอาหารเลี้ยงดัดแปร Zarrouk's ด้วยธาตุสังกะสีเข้มข้น 1.6 ไมโครโมลของสังกะสีซัลเฟต พบว่ามีธาตุสังกะสีมากที่สุดคือ 69.55±0.27 ไมโครกรัมต่อกรัม (น้ำหนักแห้ง) โปรตีนร้อยละ 63.11±1.40 (น้ำหนักแห้ง) อัตราการเจริญจำเพาะ (μ) 0.46 ต่อวัน อัตราการผลิตของเซลล์ (cell productivity) (P_x) 0.55 กรัมต่อลิตร-วัน (น้ำหนักแห้ง) และเวลาที่เซลล์เพิ่มจำนวนเป็น 2 เท่า (doubling time) (t_d) 1.50 วัน

ในส่วนของไซโทพลาซึม (cytoplasm fraction) จากเซลล์สไปรูไลนา พบว่ามีธาตุสังกะสีร้อยละ 72.8 (60.50 ไมโครกรัมต่อกรัมน้ำหนักแห้ง) เปรียบเทียบกับส่วนของผนังเซลล์ (cell wall fraction) มีธาตุสังกะสีร้อยละ 27.2 (22.62 ไมโครกรัมต่อกรัมน้ำหนักแห้ง) จากผลการทดลองดังกล่าวอธิบายโดยนัยว่าธาตุสังกะสีสามารถเข้าไปอยู่ในไซโทพลาซึม โดยการขนถ่ายสารแบบใช้พลังงาน (active transport) ผ่านเยื่อหุ้มเซลล์ นอกจากนี้ค่าสเปกตรา (spectra) จากการวิเคราะห์ด้วยเทคนิค fourier transform infrared (FT-IR) spectroscopy ช่วยยืนยันผลการเปลี่ยนแปลงของหมู่เอมีนในสไปรูไลนาที่เกิดขึ้นหลังจากมีการจับกับธาตุสังกะสี

จากการจำลองการพร้อมนำไปใช้ประโยชน์ (accessibility) และการนำไปใช้ประโยชน์ (availability) ของธาตุสังกะสีอินทรีย์ในสไปรูไลนา พบว่าสไปรูไลนาที่เลี้ยงในอาหารเลี้ยงดัดแปร Zarrouk's (SPM) หลังจากการย่อยผ่านไป 2 ชั่วโมง มีค่าการพร้อมนำไปใช้ประโยชน์ของสังกะสี (Zn accessibility) สูงที่สุด โดยภายหลังการย่อยของกระเพาะอาหารร้อยละ 55.20±0.57 และภายหลังการย่อยของลำไส้เล็กร้อยละ 63.55±0.21 ความสามารถนำไปใช้ประโยชน์ของธาตุสังกะสี ของสไปรูไลนาที่เลี้ยงในอาหาร SPM มีค่ามากที่สุดเช่นกันคือ ภายหลังการย่อยของกระเพาะอาหารร้อยละ 62.94±1.75 และภายหลังการย่อยของลำไส้เล็กร้อยละ 34.63±0.95

ในส่วนของเซลล์สไปรูไลนาสด การเก็บรักษาเซลล์ด้วยกลีเซอรอลร้อยละ 5 (5% glycerol) ที่อุณหภูมิ 4 องศาเซลเซียส ให้ผลการทดลองที่ดีที่สุด เมื่อเปรียบเทียบกับการเก็บรักษาที่อุณหภูมิ 20 และ 30 องศาเซลเซียส การเปลี่ยนแปลงของปริมาณโปรตีน และธาตุสังกะสีของเซลล์สไปรูไลนาสด ซึ่งจากการทดลองเป็นไปตามปฏิกิริยาอันดับศูนย์ (zero-order reaction) โดยสามารถทำนายการเก็บรักษาเซลล์สไปรูไลนาสดที่อุณหภูมิ 4 องศาเซลเซียส ด้วยกลีเซอรอลร้อยละ 5 โดยมีโปรตีนร้อยละ 55 มีอายุการเก็บ 50 วัน และธาตุสังกะสีในเซลล์ 54.89 ไมโครกรัมต่อกรัม (น้ำหนักแห้ง) ในขณะที่การเก็บรักษาเซลล์สไปรูไลนาสดที่อุณหภูมิ 4 องศาเซลเซียส ไม่เติมกลีเซอรอล มีอายุการเก็บ 22 วัน มีโปรตีนร้อยละ 55 และธาตุสังกะสีในเซลล์ 70.34 ไมโครกรัมต่อกรัม (น้ำหนักแห้ง)

สาขาวิชาเทคโนโลยีอาหาร ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2556 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ARTHIT JATUPORNPONGCHAI : ENRICHMENT OF ORGANIC ZINC IN *SPIRULINA PLATENSIS*. THESIS ADVISOR : ASST. PROF. SUWAYD NINGSANOND, Ph.D., 119 PP.

SPIRULINA PLATENSIS/ORGANIC ZINC/AVAILABILITY/ACCESSIBILITY/ STORAGE LIFE/ZERO-ORDER REACTION

The objectives of this study were to investigate the Zn uptake performance of *Spirulina platensis* using different Zn salts (zinc sulfate, zinc nitrate, and zinc chloride), and locate Zn deposit in cells to assess accessibility and availability of organic Zn in *Spirulina platensis* using *in vitro* simulated gastrointestinal digestion, and to evaluate changes and storage life of fresh *Spirulina platensis*. *Spirulina platensis* cultured in modified Zarrouk's medium with zinc sulfate (MZS) had significantly highest cell biomass, Zn, and protein among different various salts of Zn fortified media ($p<0.05$). In addition, the modified Zarrouk's medium with 1.6 µmol Zn of MZS provided cells with the highest Zn content of 69.55±2.70 µg/g dry weight, protein content of 63.11±1.40% dry weight, specific growth rate (µ) of 0.46 /day, cell productivity (P_x) of 0.55 g/L.day dry weight and doubling time (t_d) of 1.50 day.

Cytoplasm fraction obtained from *Spirulina platensis* cells had 72.8% (60.50 µg/g dry weight) Zn compared with 27.2% (22.62 µg/g dry weight) in cell wall fraction. This implied that Zn entered to cytoplasm via active transport across to the cell membrane. Fourier transform infrared (FT-IR) spectra indicated that the binding of Zn^{2+} apparently occurred at the amide groups.

Regarding simulation of accessibility and availability of organic Zn in *Spirulina platensis*. *Spirulina platensis* cultured in modified Zarrouk's medium (SPM) at 2 h digestion had the highest released Zn at 55.20±0.57% after the gastric digestion and 63.55±0.21% after the small intestinal digestion. Available Zn in the *Spirulina platensis* cells cultured in SPM was at 62.94±1.75% after the gastric digestion and at 34.63±0.95% after the small intestinal digestion.

For fresh *Spirulina platensis* cells, storage the cell with 5% glycerol at 4°C gave the best result compared with storage at 20°C and 30°C. Changes in protein and Zn contents of *Spirulina platensis* cells followed the zero-order reaction. The storage life of fresh *Spirulina platensis* cell at 4°C was predicted to be 50 days with 5% glycerol, estimating 55% protein and 54.89 µg/g dry weight Zn in the cells and to be 22 days without glycerol, having 55% protein and 70.34 µg/g dry weight of Zn in cell.

School of Food Technology Student's Signature Arthit

Academic Year 2013 Advisor's Signature S. Ningsanond

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Arthit Jatupornpongchai

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LIST OF ABBREVIATIONS

AAS	=	Atomic Absorption Spectrometry
ABC	=	ATP-binding cassette
Af	=	Accuracy factor
ANOVA	=	Analysis of variance
AOAC	=	Association of Official Chemists
ATR-FTIR	=	Attenuated Total Reflectance Fourier Transformed Infrared Spectrometer
Bf	=	Bias factor
C	=	Carbon
°C	=	Degree Celsius
$C_3H_8O_3$	=	Glycerol
C_i	=	Zn concentration at time *i* (µg/g dry weight)
C_0	=	Initial Zn concentration (µg/g dry weight)
cm	=	Centimeter
CO_2	=	Carbon dioxide
Cr	=	Chromium
DMRT	=	Duncan's multiple range test
FT-IR	=	Fourier transform infrared
g	=	Gram
h	=	Hour

LIST OF ABBREVIATIONS (Continued)

H	=	Hydrogen
HNO_3	=	Nitric acid
HCl	=	Hydrochloric acid
$HClO_4$	=	Perchloric acid
IR	=	Infrared spectra
IZiNCG	=	Zinc Nutrition Consultative Group
k	=	Concentration rate constant
kDa	=	Kilo Dalton
L	=	Liter
LDPE	=	Low density polypropylene
LOG	=	Logarithm
MTs	=	Metal-binding proteins
MZC	=	Modified Zarrouk’s medium with zinc chloride
MZN	=	Modified Zarrouk’s medium with zinc nitrate
MZS	=	Modified Zarrouk’s medium with zinc sulfate
µg	=	Microgram
µmol	=	Micromole
µm	=	Micrometer
mg	=	Milligram
mL	=	Milliliter
min	=	Minute
M	=	Molar

LIST OF ABBREVIATIONS (Continued)

μ	=	Specific growth rate
n	=	Number of observations
N	=	Nitrogen
$NaHCO_3$	=	Sodium hydrogen carbonate
nm	=	Nanometer
NZM	=	Non-Zn Zarrouk's medium
O	=	Oxygen
O.D.	=	Optical density
%	=	Percentage
PA	=	Phytic acid
P_0	=	Initial protein concentration (% dryweight)
P_i	=	Protein concentration at time *i* (% dryweight)
PZP1	=	periplasmic zinc-binding protein
RMSE	=	Root mean square
rpm	=	Rounds per minute
S.D.	=	Standard deviation
Se	=	Selenium
SH	=	Sulfhydryl
SPC	=	Commercial *Spirulina platensis* samples
SPM	=	*Spirulina platensis* samples cultured in modified Zarrouk's medium
SPZ	=	*Spirulina platensis* samples cultured in Zarrouk's medium

LIST OF ABBREVIATIONS (Continued)

t_d	=	Doubling time
t_i	=	Time interval between X_0 and X_i (day)
t_{xo}	=	Initial time (day)
t_{xi}	=	Time i (day)
X_0	=	Initial biomass density (mg/L dry weight)
X_i	=	Biomass density at time i (mg/L dry weight)
ZM	=	Zarrouk's medium
Zn	=	Zinc
$ZnCl_2$	=	Zinc chloride
$Zn(NO_3)_2$	=	Zinc nitrate
$ZnSO_4$	=	Zinc sulfate

CHAPTER I

INTRODUCTION

1.1 Introduction

Spirulina platensis is a filamentous prokaryotic cyanobacteria and commonly used as food, feed, dietary supplement and function due to its high protein content with low nucleic acids and good balance of nutritional values (Ciferri, 1983; Castenholz, 1984; Campanella, Crescentini, and Avino, 1999; Mosulishvili, Kirkesali, Belokobylsky, Khizanishvili, Frontasyeva, Pavlov, and Gundorina, 2002; Caballero, Trugo, and Finglas, 2003; Gershwin and Belay, 2008). *Spirulina platensis* can be utilized for the production of health foods commanding a high market value because some chemicals are unique to the alga. In addition, novel foods from *Spirulina platensis* have been developed with added nutrition value. Examples are biscuits, pasta, noodles, salad dressing, drinks, and pudding (Gouveia and Empis, 2003; Raymundo, Gouveia, Batista, Empis, and Sousa, 2005; Fradique, Batista, Nunes, Gouveia, Bandarra, and Raymundo, 2010; Batista, Nunes, Raymundo, Gouveia, Sousa, Cordobes, and Franco, 2011). Moreover, cells of *Spirulina platensis* contain functional groups such as carboxyl, hydroxyl, sulphate, and other charged groups which are responsible for metal binding (Li, Guo, and Li, 2003; Seker, Shahwan, Eroglu, Yilmaz, Demirel, and Dalay, 2008). Therefore, *Spirulina platensis* may be suitable an intermediated for minerals enrichment and may be used as dietary supplement.

Zinc (Zn) is an essential trace element for humans, plants, animals and involved in a wide variety of biochemical functions (Compano, Grima, Izquierdo, and Prat 1989; McClung and Bobilya, 1999; Al-Kindy, Al-Bulushi, and Suliman, 2008). An estimation of 31% of the world's population faces Zn deficiency, especially in African, Eastern Mediterranean, South Asian, and South-east Asian countries. International Zinc Nutrition Consultative Group (IZiNCG) has suggested the daily dosages of supplemental zinc for the adult men 13-19 mg/day and adult women 7-9 mg/day (Hotz and Brown, 2004; Black, Allen, Bhutta, Caulfield, Onis, Ezzati, Mathers, and Rivera, 2008; Pinkaew, Wegmuller, and Hurrell, 2012). Zn deficiency leads to several disorders, these include, a retardation of growth and development in children, retarded genital development and hypogonadism, dermatitis and delayed wound healing, alopecia, poor pregnancy outcomes and teratology. Further, this may result in decreased immune function with an increased susceptibility to infections, and metabolic disorders associated with neurological diseases which are Alzheimer's disease, Parkinson's disease, epilepsy hypoxia-ischemia and prostate cancer (Gyorkey, Min, Huff, and Gyorkey, 1967; Welch, 2002; Flinn, Hunter, Linkous, Lanzirotti, Smith, Brightwell, and Jones, 2005; Franklin and Costello, 2007; Haase, Overbeck, and Rink, 2008). However, Zn absorption is inhibited in human body by phytic acid which is found in cereal grains, legumes, nuts, oil seeds, and tubers (Hunt, 2003; Dost and Tokul, 2006; Abebea, Bogalea, Hambidgeb, Stoecker, Baileyd, and Gibsond, 2007; Moazedi, Ghotbeddin, and Parham, 2007; Karunaratne and Amerasinghe, 2008). As a result, phytic acid forms Zn-complex that cannot be absorbed in human body. To evaluate the absorption, availability of food nutrients is considered as a parameter.

Although, many foods are good sources of Zn, the availability of Zn in foods is limited by its bioavailability. A principle of bioavailability is the evaluation of nutrient effectiveness which is absorbed from gut to the systemic circulation (McDougall, Dobson, Smith, Blake, and Stewart, 2005; Fernandez, Carvajal, and Perez, 2009). *In vivo* digestion is performed to determine the bioavailability, which is simulation of gastrointestinal digestion. The *in vitro* method study is a simple predictive technique to estimate the potential bioavailability of food compounds. Previous researchers have studied the bioavailability of iodine and bromine in different types of edible seaweed. The results show that the *in vitro* method has a good accuracy evaluation of the bioavailability (Romaris, Garcia, Barciela, Dominguez, Moreda, and Bermejo, 2011). Therefore, the *in vivo* digestion method could be used to assess the availability of Zn in *Spirulina platensis*.

Autolysis is a biochemical mechanism of microalga self-digestion, which caused by lytic enzyme to decompose the cells. The autolysis leads to the breakdown of cell membrane, which release enzyme store in vacuole (Lahoz, Reyes, and Leblic, 1976; Harvey, McNeil, Berry, and White, 1998; Lewis, 2000; Ngwenya, 2007). However, this mechanism could be retarded by decreasing the temperature which is one of the most important factors in the postharvest of fresh produces by regulating the rate of all associated physiological and biochemical processes. As a result, the biochemical reaction rates would be reduced and the shelf-life of products would be increased. (Salunkhe and Desai, 1984; Robertson, 1993). The optimum storage condition of microalgae biomass has been studied and shown that the harvested biomass stored at chilled temperature could extend the shelf life. (Harith, Yusoff, Shariff, and Ariff, 2010). Furthermore, the use of cell protectants with temperature controlled techniques

can preserve quality of microalga cells (Montaini, Zittelli, Tredici, Grima, Sevilla, and Perez, 1995). Consequently, the storage temperature with cell protectants could inhibit cell autolysis and extend the storage life.

Postharvest technique does not only extend the storage life, but it also can maintain the quality of foods. In addition, the storage life can be predicted using a kinetics reaction model. Many researchers have studied the storage life of fresh vegetable to preserve the quality of produces, such as, asparagus, mushroom, and lettuce. It has been shown that the predicting storage life from the predictive model provides the accurate results under the different storage conditions which can be applied to the industry (Ares, Parentelli, Gambaro, Lareo, and Lema, 2006; Sanchez, Perez, Flores, Guerrero, and Garrido, 2009; Posada, Perez, Lopez, Allende, Gil, and Zurera, 2014). However, few studies have shown that the storage life of fresh *Spirulina platensis* is limited.

Therefore, the objectives of this study were to investigate Zn absorption of *Spirulina platensis* in different zinc salts media, to determine absorption and availability of organic zinc from *Spirulina platensis in vitro* simulating gastrointestinal tract, and the storage life of fresh *Spirulina platensis* during storage will be provided.

1.2 Research objectives

The objectives of this study were:

1. To study the absorption of different zinc salts by *Spirulina platensis*.
2. To locate zinc deposition in *Spirulina platensis* cells.
3. To study the availability of organic zinc of *Spirulina platensis*.
4. To evaluate the storage life of fresh *Spirulina platensis*.

1.3 Research hypotheses

The hypotheses of this study were:

1. *Spirulina platensis* could absorb zinc into the cells and transform to organic zinc.

2. Different sources of zinc affect zinc uptake in the *Spirulina platensis* cells.

3. Organic zinc in *Spirulina platensis* will be available.

4. Proper postharvest treatments would extend fresh *Spiralina platensis* storage life.

1.4 Scopes of the study

The scopes of this study were:

1. The cultivation of *Spirulina platensis* in different types of media with high levels of zinc from $ZnCl_2$, $Zn(NO_3)_2$ and $ZnSO_4$ will be carried out. The zinc concentrations will be measured using atomic absorption spectrometry.

2. *Spirulina platensis* cells will be disrupted and separated to obtain cell envelops, and remaining soluble fraction for zinc determination.

3. The availability of organic zinc will be tested using *in vitro* digestion.

4. Cold storage together with a cell protectant will be used to extend storage life of fresh *Spirulina platensis.*

1.5 Expected results

This research will be able to increase Zn content and lead to better understandings about the absorption of organic zinc by *Spirulina platensis*. Organic zinc enriched in

Spirulina platensis will be available. Additionally, the knowledge about postharvest stability and storage life evaluation of fresh *Spirulina platensis,* will be gained.

1.6 References

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CHAPTER II

LITERATURE REVIEWS

2.1 *Spirulina platensis*

Spirulina platensis is a photosynthetic filamentous cyanobacterium, spiral-shape, multicellular and blue-green algae which has been used as food, food supplement, nutraceuticals, biomedical research, and cosmetics industry (Ciferri, 1983; Leema, Kirubagaran, Vinithkumar, Dheenan, and Karthikayulu, 2010; Jeamton, Dulsawat, Laoteng, Tanticharoen, and Cheevadhanarak, 2011). *Spirulina platensis* can grow in tropical and subtropical water which high levels of carbonate and bicarbonate and high pH up to 11. In addition, *Spirulina platensis* is a widely distributed species, mainly found in Africa, as well as in Asia and South America. Nowadays, it is cultivated in several countries such as, USA, China, Japan, Myanmar, Taiwan, and Thailand (Vonsak, 2002; Henrikson, 2009)

2.1.1 Morphology of *Spirulina platensis*

Spirulina platensis, observed by scanning electron microscopy, showed filaments of cylindrical cells arranged in unbranched, helicoidal trichomes (Figure 2.1). The filaments are motile and gliding along their axis. The helical shape of the trichome is characteristic of the genus but the helical parameters (i.e., pitch length and helix dimensions) vary with the species or may be induced by changing the environmental conditions. The morphological cell walls exhibit similarly an outer gram-negative bacteria envelope. Trichomes are surrounded with a thin sheath about 0.5 μm thickness

and has a fibrillar. The sheath material, excreted through pores situated on the cell wall, has been thought to be involved in the filament motion (Van Eykelenburg, 1977; Gershwin and Belay, 2008; Vonsak, 2002). The multilayered cell wall is thin, about 40–60 nm, and has a sensitive electron-dense layer corresponding to the peptidoglycan. The general trichome widths vary about 3-12 µm, the helix open spiral with diameters ranging about 35-60 µm, and length about 200-500 µm (Ciferri,1983; Vonsak, 2002).

(a) (b)

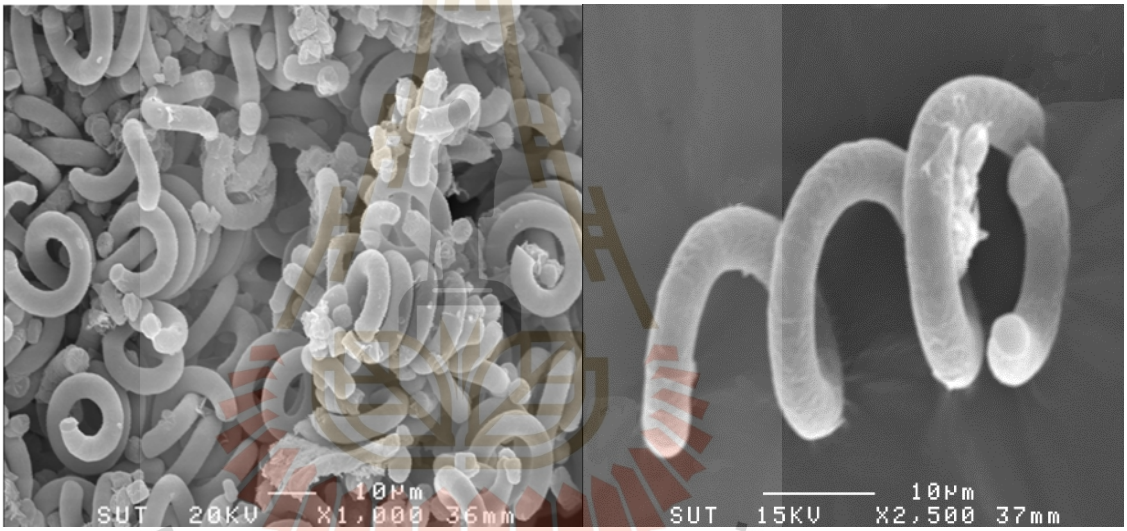

Figure 2.1 Scanning Electron Microscope of *Spirulina platensis*, IFRTD1208 at 1,000× (a) and 2,500× (b).

The cell wall of *Spirulina platensis* is divided into four layers that are LI, LII, LIII, and LIV layers (Figure 2.2). The outer membrane layer (LIV) is composed of lipopolysaccharides arranged linearly in parallel with the trichome axis. Layer III (LIII) is composed of protein fibrils coiled helically around the trichomes, whereas the peptidoglycan-containing layer (LII) folds towards the inside of the filament. The LI

layer contains β-1,2-glucan, which presented in septum wall to separate cells. The septum wall includes in LI and LII layers. In addition, plasmalema is the origin of thylakoids system which is important of cytoplasmic structure (Van Eykelenburg, 1977; Gershwin and Belay, 2008).

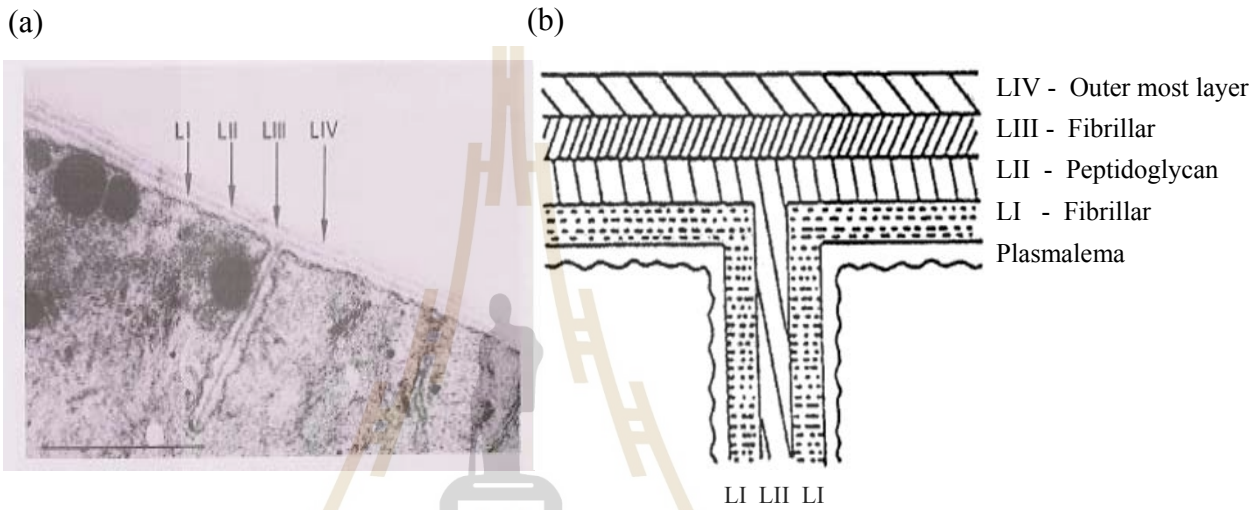

Figure 2.2 Cell wall of *Spirulina platensis*; the section through the wall (a) and the scheme cell wall model (b). The black bar indicates 500 nm.

Source: Van Eykelenburg (1977).

2.1.2 Life cycle of *Spirulina platensis*

A principal reproductive system of *Spirulina pltensis* includes three stages which are: 1) cell maturation; 2) cell division; 3) cell amplification (Figure 2.3). In the first stage, a mature trichome fragments into several pieces through the formation of specialized cells called “necridia”. In the second stage, the necridia divides itself to form short and motile filaments called “hormogonia”. In the last stage, the hormogonia cells

will go through the amplification and maturation processes (Ciferri, 1983; Gershwin and Belay, 2008).

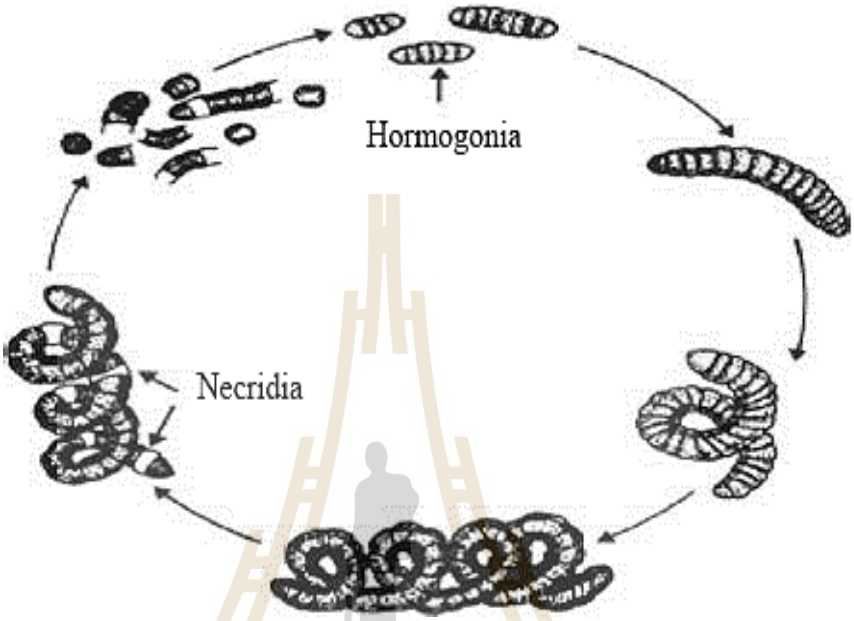

Figure 2.3 Life cycle of *Spirulina platensis*.

Source: Ciferri (1983).

2.1.3 Metal ion absorption of *Spirulina platensis*

Cell walls of *Spirulina platensis*, cyanobacterium, are those of similar to the gram-negative bacteria, which contain polysaccharides, lipids, and proteins. The absorption/binding of metal ions by cyanobacteria started from the coordination of the ions with functional groups in/on the cyanobactrium cell. These functional groups are carboxyl, hydroxyl, amines, sulfhydryl, imidazole, phosphate and other charged groups (Gardea-Torresdey, Becker-Hapak, Hosea, and Darnall, 1990; Chen, Shi, Chen, Xu, Chen, Wang, and Hu, 2007; Seker, Shahwan, Eroglu, Yilmaz, Demirel, and Dalay, 2008). The absorption mechanism of metal ions depends on the type of metal ions and

cyanobacteria (Gardea-Torresdey et al., 1990). The metal ions are generally transported into cells by specialized protein channel, which implicate 3 steps (Figure 2.4). Firstly, metal solution diffuse to the cell surface, while the complexion reaction occur rapidly. Secondly, absorption/surface complexion of the metal occurs at passive binding sites on the outer surface of the plasma membrane, and this interaction occurs by ligand exchange reaction (M–X cell). Lastly, the metal transports across the plasma membrane through the protein channel into cell interior (Sunda, Huntsman, 1998; Campbell, Errecalde, Fortin, Hiriart-Baer, and Vigneault, 2002). In addition, the Zn(II) uptake system in prokaryotes includes ABC (ATP-binding cassette) transporters, which allow the transport of a specific substrate from one side of the membrane to the other side, form a specific channel in the cell membrane. The ATP-binding regions play an important role to provide the energy for the substrate transports from ATP hydrolysis (Blencowe and Morby, 2003). This could explain the mechanism of metal absorption into the *Spirulina platensis* cell. Therefore, the enrichment of minerals could be possibly used *Spirulina platensis* as an intermediate.

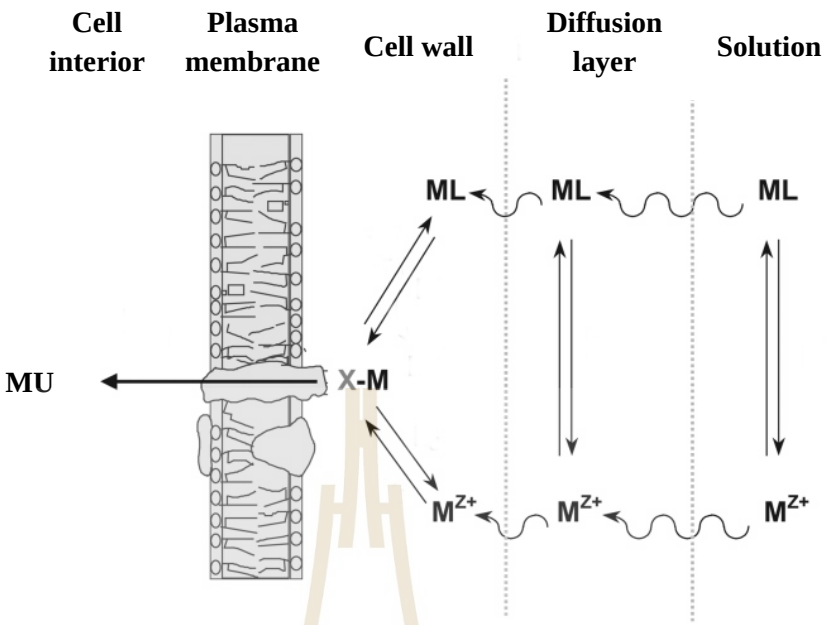

Figure 2.4 Conceptual model of cyanobacteria interactions. M^{Z+}, free-metal ion; ML, metal complex in solution; M–X membrane, surface metal complex; MU, metal uptake.

Source: Campbell et al. (2002).

2.1.4 Nutritional values, quality standards and applications of *Spirulina platensis*

Spirulina platensis is high in protein and could be applied for the production of several products e.g., food, feed, fertilizer, cosmetics, biomedical research, and dietary supplement, (Ciferri, 1983; Leema et al., 2010; Jeamton et al., 2011), when compared with the protein from other sources (Table 2.1). *Spirulina* powder has the highest protein among different protein sources (fish, egg, beer yeast, skimmed powdered milk, eggs, cheese, beef, and poultry). The chemicals composition of *Spirulina* have been reported as in Table 2.2. Currently, about 70% of *Spirulina* products have been used for human consumption. Therefore, the quality standard requirements for *Spirulina* products in Europe, Japan and United States Food and Drug

Administration's (FDA) have been established to control the quality of *Spirulina* products (Table 2.3).

Table 2.1 Protein contents in different sources.

Food type	**Crude Protein (% dry weight)**
Spirulina powder	65
Chicken egg	47
Poultry	24
Beer yeast	45
Skimmed powdered milk	37
Cheese	36
Beef	22
Fish	22

Modified from: Switzer (1982) and Ciferri (1983).

Table 2.2 Chemical components of *Spirulina* powder.

Compositions	Per 100 g (dry weight)
Total fat	4.3 g
- Saturated fat	1.95 g
- Polyunsaturated fat	1.93 g
- Monounsaturated fat	0.26 g
- Cholesterol	<0.1 mg
Total carbohydrate	17.8 g
- Dietary fiber	7.7 g
- Sugars	1.3 g
Protein	63 g
Calcium	468 mg
Iron	87.4 mg
Phosphorus	961 mg
Magnesium	319 mg
Zinc	1.45 mg
Copper	0.47 mg
Manganese	3.26 mg
Potassium	1,660 mg
Sodium	641 mg

Source: Gershwin and Belay (2008).

Table 2.3 Quality standards for *Spirulina*.

Standard	Country			
	France	**Sweden**	**Japan**	**USA**
Protein	55-65%	55-65%	≥50%	55-65%
Total Carotenoids	-	-	>100 mg	300 mg/100g
Chlorophyll-a	>500 mg %	-	>500 mg %	900 mg/100g
Phycocyanin	-	-	>2,000 mg %	8,000 mg/100g
Moisture	-	-	<7 %	<7 %
Standard Plate Count	<100,000/g	<1,000,000/g	<200,000/g	<200,000/g
Mold	-	<1,000/g	-	<100/g
Coliform bacteria	<10/g	<100/g	negative	negative
Total heavy metals (Lead, Mercury, Cadmium, Arsenic)	-	-	<20.0 ppm	<2.1 ppm
Insect fragment	-	-	-	<30 pcs/10 g
Rodent hair	-	-	-	<1.5 pcs/150 g

Definitions; - = No set standard, < = less than, > = greater than,
≥ = greater than or equal to.

Source: Koru (2012).

Spirulina platensis cells contain various functional groups with negative charged, namely, carboxyls, hydroxyls, sulfates, amines and other charged groups (Li, Guo and Li, 2003; Seker et al., 2008). These functional groups can be bound with

metals; thus, the changes of *Spirulina platensis* cells were examined using fourier transform infrared spectroscopy (FT-IR).

FT-IR is a simple, rapid, nondestructive, and effective technique applied to characterize the carboxyl groups of the cyanobacteria (Chen et al., 2007; Liu, Xu, Zhou, Wanga, MingLi, Ha, and Sun, 2013). The vibration infrared extends from 4000 to 400 cm^{-1} which corresponds to the wavelengths of 2.5 to 25 μm. The infrared (IR) spectrum (or the pattern of absorption) is used to investigate information of the molecule structure. The absorption of each type of bond (N-H, C-H, O-H, C-X, C=O, C-O, C-C, C=C, C≡C, C≡N, and others) is commonly detected in a small portion of the vibrational infrared region. However, the small range of absorption can be defined for each type of bond. The principal components have their own infrared characteristic peaks. Approximate regions of common types of bonds absorption are shown in Figure 2.5. (Pavia, Lampman, and Kriz, 1996).

Liu et al. (2013) have found that IR spectra of *Spirulina* powder sample are complex and contain several bands from different functional groups such as proteins, saccharides, and others nutrients (Figure 2.6). The main absorption bands at 1658 and 1541 cm^{-1} characterizing the vibrations of amines I and II, are from proteins in *Spirulina* powder. The IR band at 3302 cm^{-1}, assigned for O-H or N-H vibrations, is from saccharides or proteins. The IR bands at 1152, 1079, and 1051 cm^{-1}, which represent C–O or C–C vibrations, are from saccharides. The intensity of the characteristic band at 1658 cm^{-1}, representing the C=O bond in proteins is the strongest band because *Spirulina* contains protein more than 60% of the total weight. These results are consistent with the study by Finocchio, Lodi, Solisio, and Converti (2010) indicating the strong IR spectrum at 3450 cm^{-1} for starching vibration modes of O-H and the band

at 3300 cm^{-1} for NH_2 of primary amides or amine compounds. Additionally, the strong and complex IR spectrum at 1650 cm^{-1} associates to the amine I band of protein. The main IR spectra of cyanobacteria and detail spectral bands are presented in Table 2.4.

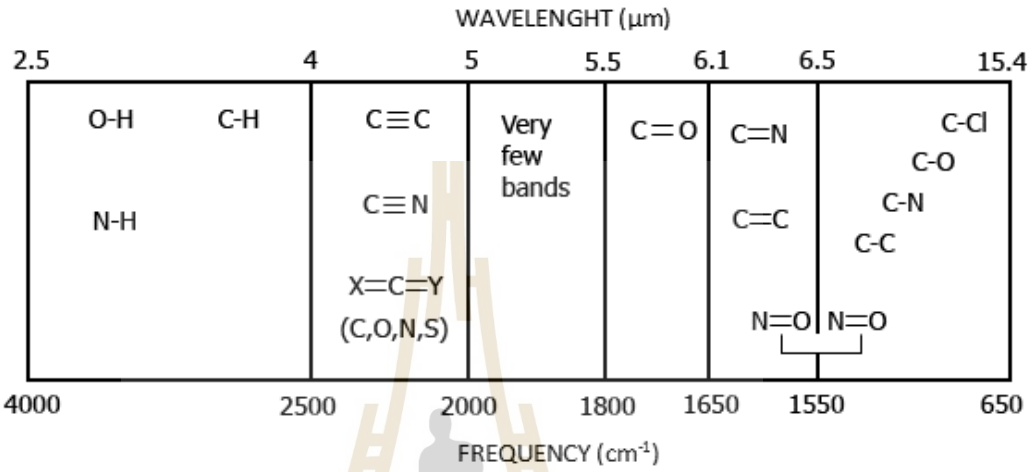

Figure 2.5 The approximate regions of various common types of bonds absorption (stretching vibration only; bending, twisting, and other types of bond vibrations have been omitted for clarity).

Source: Pavia, Lampman, and Kriz (1996).

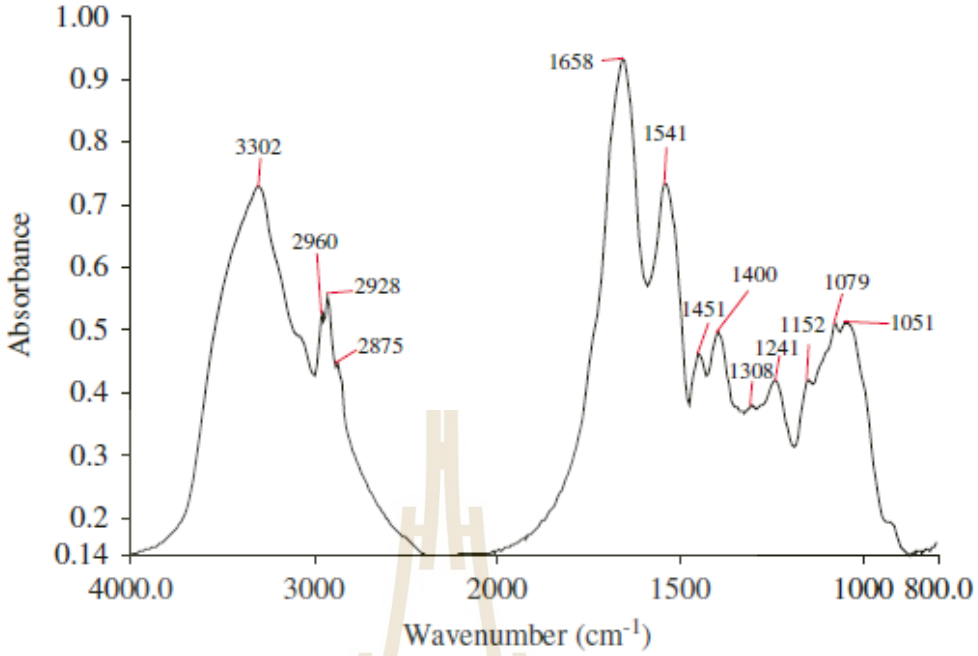

Figure 2.6 Infrared spectra of *Spirulina* powder (4000 to 400 cm^{-1}).

Source: Liu et al. (2013).

Table 2.4 Major infrared spectra and functional groups of cyanobacteria.

Frequency (cm^{-1})	Functional groups	Main attribution
3000-2800	$-CH_2$, $-CH_3$	Protien, lipid
1658-1650	C=O, C-N	Protein
1545-1540	N-H, C-N	Protein
1455-1451	$-CH_3$	Protein, lipid, polysaccharide
1155-1030	C-O, C-C, C-O-C	Polysaccharides

Modified from: Yee, Liane, Phoenix, and Ferris (2004); Chen et al. (2007); Pistorius, DeGrip, and Egorova-Zachernyuk (2009); Finocchio, Lodi, Solisio, and Converti (2010); Liu et al. (2013).

2.1.4.1 Applications of *Spirulina platensis*

Several studies have shown that *Spirulina platensis* could be applied in several novel food products to enhance nutritional quality and provide higher antioxidant activity for examples, pasta (Rodriguez De Marco, Steffolani, Martinez, and Leon, 2014), concentrated juice (Morist, Montesinos, Cusido, and Godia, 2001), and alternative vegetarian foods. The addition of *Spirulina* biomass to vegetable gelled deserts could provide good thermal stability to the products (Batista, Nunes, Fradinho, Gouveia, Sousa, Raymundo, and Franco, 2012). Therefore, application of *Spirulina platensis* in foods would provide novel alternative products to the market niche (Gouveia, Batista, Raymundo, and Bandarra, 2008). These research works supported that the *Spirulina platensis* could be used in innovative food products.

2.2 Zinc-essential micromineral

Major energy sources of human are carbohydrates, protein, and lipid. Minerals are also important for growth, maintenance, metabolic processes, and reproduction of body system. The minerals can be classified into two categories which are macrominerals and microminerals (trace elements). In general, the body needs macrominerals (i.e., calcium, phosphorus, magnesium, sodium, chloride, and potassium, and sulphur) more than 0.01% of body weight. Likewise, the body needs microminerals (i.e., iron, zinc, iodine, selenium, copper, manganese, chromium, fluorine, and molybdenum) less than 100 mg/day or less than 0.01% of body weight (Groff and Gropper, 1999; Benardot, 2000). Although, the microminerals are needed in a little amount, they play an essential role in metabolism and synthesis processes of the body system. At present, many

countries have focused on the retardation of growth and development in children due to inadequate zinc intake.

Zinc (Zn) has been known to be an essential trace element for more than a hundred years and zinc deficiency may severely affect human health. Zn is found in all organs, tissues and fluids, which is a component of more than 300 enzyme from all six classes (Tuormaa, 1995; Haase et al., 2008). Zn can be found in several foods, and good sources of zinc are seafood, meat and poultry (Table 2.5). Zn from plant foods tends to be low, and is absorbed less than that from meat (Groff and Gropper, 1999). Zn deficiency has been estimated about 31% of the world's population, especially in the developing countries in Africa, the Eastern Mediterranean, and Southeast Asia. Zn deficiency leads to the problems of growth and maturation in children, poor wound healing, alopecia, night blindness, cancerous prostate, and associated with neurological diseases (Gyorkey Min, Huff, and Gyorkey, 1967; Stefanidou, Maravelias, Dona, and Spiliopoulou, 2006; Franklin and Costello, 2007; Haase, Overbeck, and Rink, 2008). International Zinc Nutrition Consultative Group (IZiNCG) has suggested the daily dosages of supplemental zinc 13-19 mg/day for adult men, and 7-9 mg/day for adult women (Hotz and Brown, 2004; Black, Allen, Bhutta, Caulfield, Onis, Ezzati, Mathers, and Rivera, 2008).

Previous research studies in human prostate gland showed that the zinc content in carcinoma cell nuclei was low, when compared with normal cell (Gyorkey, Min, Huff, and Gyorkey, 1967). As well, zinc supplementation produced positive responses in increasing of height and weight in children. This finding supports an increased intake of zinc in populations at risk of zinc deficiency (Brown, Peerson, Rivera, and Allen, 2002). It has been found that zinc therapy for wound healing is effective for patients

with low serum zinc (Haley, 1979). Moreover, it has been reported that oral administration of zinc chloride with 30 mg/kg/day after 2 weeks at the stage of pregnancy rat, can improve the working memory offspring (Moazedi, Ghotbeddin, and Parham, 2007).

Table 2.5 Zn content in foods.

Foods	**Zn (mg/100g dry weight)**
Seafood	
Oyster	17-91
Crabmeat	3.8-4.3
Shrimp	1.1
Tuna	0.5-0.8
Meat and poultry	
Chicken	1.0-2.0
Beef	3.9-4.1
Pork	1.6-2.1
Eggs and dairy products	
Eggs	1.1
Milk	0.4
Legumes	0.6-1.0
Grains and cereals	
Rice and pasta	0.3-0.6
Bread (white)	0.6-0.8
Vegetables	0.1-0.7
Fruits	<0.1

Source: United states department of agriculture (2011).

2.2.1 Absorption and availability of zinc in food

Zinc is released from food as free ions during digestion. The liberated ions may then bind to endogenously secreted ligands before their transport into enterocytes in the duodenum and jejunum (Sharma and Singh, 2009). Zn is absorbed into the enterocyte by active and passive movements. Zn is mainly absorbed in gastrointestinal tract which is in the segment jejunum of the small intestine. The jejunum shows the highest rate of Zn absorption when compared with duodenum and ileum segment of the small intestine (Lee, Prasad, Brewer, and Owyang, 1989). However, there are chemical complexes to inhibit the Zn availability absorption.

Zinc and micronutrients in foods could be bonded with phytic acid in small intestinal as phytate complexes. These phytate complexes are not absorbed across the intestinal mucosa resulting in low bioavailability of Zn (Thavarajah, Thavarajah, See, and Vandenberg, 2010). Moreover, previous it has been reported that Zn solubility in soy polysaccharide fiber decreased with increasing of calcium concentration. As the results, the competitive effect between zinc and calcium required the suitable proportion to get high solubility and more potential availability absorption (Corneau, Lavigne, Zee, and Desrosiers, 1996).

Zinc is a trace element of the greatest concern considering the nutritional value of vegetarian diets without meat and increasing intake of phytate-containing legumes and whole grains. Moreover, the adsorption of zinc is lower with vegetarian diet than non-vegetarian diets (Hunt, 2003). Therefore, there are attempts to create an alternative for zinc fortification because of several disadvantages of zinc compounds used for fortification, such as zinc oxide and zinc sulfate. In generally, zinc(II) ions are hydrophilic that cannot cross cell membranes by the passive diffusion. Metal-binding

proteins (MTs) containing amino acids, most of which are cysteine, are introduced to improve the absorption of zinc into cells. The MTs are important for Zn uptake, distribution, storage, and release (Stefanidou, Maravelias, Dona, and Spiliopoulou, 2006). The metals are absorbed into the cell having a specific metal transport channel to allow passive and active transports of metal ions across the cell membrane (Ma, Jacobsen, and Giedroc, 2009; Hudek, Rai, Michalczyk, Rai, Neilan, and Ackland, 2012). Salgueiro, Zubillaga, Lysionek, Sarabia, Caro, Paoli, Hager, Ettlin, and Boccio (2000) reported that the absorption of Zn gluconate stabilized with glycine (BioZn-AAS) diet in rat (male) was higher than that from other Zn sources. Gluconic acid and glycine in BioZn-AAS are considered as weak ligands which may react with hydroxyl group and inhibit zinc precipitation. Therefore, BioZn-AAS has high solubility. In addition, Zn enriched yeast and Zn gluconate supplements showed that Zn gluconate gave higher Zn concentrations in blood, and also higher losses in feces, whereas more available Zn enriched yeast increased in blood and less loss in feces (Tompkins, Renard, and Kiuchi, 2007).

Additionally, Corneau, Lavigne, Zee, and Desrosiers (1996) found that the availability of Zn supplementation could be enhanced by binding with organic compounds to reduce oxidative stress in quail. The results suggested that organic Zn (zinc picolinate) gave higher protective effects than inorganic Zn (feed-grade zinc sulfate) by reducing the negative effect of oxidative stress. These findings are consistent with the earlier studies. When the bioavailability of zinc-methionine was compared with zinc sulfate in chick feed, the zinc methionine had more bioavailable zinc than zinc sulfate, because the zinc sulfate diet contained phytate and fiber which inhibited the Zn absorption (Wedekind, Hortin, and Baker, 1992).

2.2.2 Determination of trace elements using atomic absorption spectrometry (AAS)

There are several techniques to analyze minerals or inorganic elements, e.g., absorption techniques, emission techniques, and fluorescence techniques. Atomic absorption spectrometry (AAS) is the most commonly used method to measure the qualitative and quantitative of around 70 elements with extreme sensitivity of electrothermal atomic absorption. In addition, AAS can be applied in many research areas such as water, food, animal feedstuffs, soils, and clinical analysis (Parsons, 1990; Rouessac and Rouessac, 2007).

The principal advantages of the AAS techniques are the high sensitivity in the case of graphite furnace atomic absorption spectrometry (GF-AAS) and simplicity and low cost in the case of flame atomic absorption spectrometry (F-AAS). The determination of one element at-a-time is the only disadvantage of AAS techniques. However, the plasma techniques have the advantages of high sensitivity and multi-element determination. Unfortunately, the complex condition setting for each element and the higher cost of plasma technique are disadvantage of the technique (Garcia and Baez, 2012: Boschettii, Rampazzo, Dessuy, Vale, Rios, Hertz, Manfroi, Celso, and Ferrao, 2013).

The quantification of elements is evaluated using a relating existence between the concentration and the intensity of the corresponding light absorption or emission. In addition, the calibration method, standard addition and internal standard are used to estimate the concentration of analytes (Rouessac and Rouessac, 2007; Siraj and Kitte, 2013). Several studies used the AAS to determine the elements such as copper, nickel, cadmium, zinc, and manganese in water (Brajter and Slonawska, 1988) and cadmium,

copper, and zinc in fish and mussel (Manutsewee, Aeungmaitrepimon, Varanusupakul, and Imyim, 2007).

2.2.3 Bioavailability of elements

It has been recognized that total content of essential elements present in food is not totally absorbed by the human body (Intawonse and Dean, 2006). Bioavailability is a key concept for nutritional effectiveness. It has several working definitions, depending upon the research area it is applied to. From the nutritional point of view, as defined by Fairweather-Tait (1993, p. 384) "bioavailability refers to the fraction of the nutrient or bioactive compound ingested that is available for use in physiologic functions or to be stored". In addition, Benito and Miller (1998, p. 586) define it as "bioavailability as the proportion of a given nutrient in a given food or diet that the body can actually utilize". Moreover, Schumann, Classen, Hages, Prinz-Langenhol, Pietrzik, and Biesalski (1997, p. 369) define it as "the fraction of an oral dose of a parent compound or active metabolic from a particular preparation that reaches the systemic circulation". Regarding bioaccessibility, Fernandez-Garcia, Carvajal-Lerida, Perez-Galvez (2009, p. 752) define it as "the fraction of a compound that is released from its matrix in the gastrointestinal tract and thus becomes available for the intestinal absorption (i.e., enters the blood stream)". Furthermore, Harden, Diaz, and Svanberg (2002, p. 426) define it as "bioaccessibility as the fraction of a nutrient available for absorption, i.e., the amount of a nutrient that is released from its food matrix during digestion and made accessible for absorption into mucosa".

Bioavailability of essential elements from foodstuff can be achieved by *in vitro* or *in vivo* methods. However, using animals is expensive, difficult, and limited

data in each experiment (Intawonse and Dean, 2006). Therefore, the *in vitro* testing can be performed in bioavailability with more advantages and it is simple, rapid, inexpensive, and to control (Romaris-Hortas, Garcia-Sartal, Barciela-Alonso, Dominguez-Gonzlez, Moreda-Pineiro, and Bermejo-Barrera, 2011). The *in vitro* method is usually involved two experimental steps which are the simulation of gastrointestinal and intestinal digestion of food. The gastrointestinal step is conducted for mirroring the physiochemical conditions of food that take place during the human digestion in mouth, stomach, and intestine. The second step consists of determining the amount of nutrient or bioactive compound digestion that is assimilated from the digesta by the intestinal mucosa (Fernandez-Garcia, Carvajal-Lerida, and Perez-Galvez, 2009). At this second step, different experimental options are available including cell culture–based models (Caco-2 cell line) and dialysis method for measuring the fraction of the element available for absorption. A monolayer of cells or membrane of dialysis tubing is applied as a filter separating the digesta from gastric digestive. The filtrate fraction, which is passed through the cell (bottom) or dialysis tubing (IN), is used to determine the absorption efficiency (Ikeda, 1990; Fazzari, Fukumoto, Mazza, Livrea, Tesoriere, and Marco, 2008). The dialysis method could be modified to assess the bioavailability of microminerals including calcium, zinc, magnesium, and others (Miller, Schricker, Rasmussen, and Van Campen, 1981; Etcheverry, Grusak, and Flegie, 2012).

The *in vitro* digestion with the Caco-2 cell models has been used to determine the iron bioavailability in plant (Glahn, Lee, Yeung, Goldman, and Miller, 1998). In addition, the bioavailability of calcium, iron, zinc and copper has been measured by using a combined *in vitro* digestion with the Caco-2 cell culture system as well (Camara,

Barbera, Amaro, and Farre, 2007). However, the *in vitro* model with the Caco-2 is not useful for estimating the zinc bioavailability from food sample with limitation of zinc concentration (< 10 µmol/L of Zn) (Cheng, Tako, Yeung, Welch, and Glahn, 2012).

The simulated intestinal digestion with the dialysis tubing method is established to determine bioavailability of trace elements in several plants and foods, such as iron, zinc, copper, calcium, and magnesium in meat-based weaning foods (Santaella, Martinez, Ros, and Periago, 1996), iron, zinc, magnesium, calcium, and phosphorus in fish-based weaning foods (Martinez, Santaella, Ros, and Periago, 1998), and selenium in seafood (Moreda-Pineiro, Moreda-Pineiro, Romaris-Hortas, Dominguez-Gonzalez, Alonso-Rodriguez, Lopez-Mahia, Muniategui-Lorenzo, Prada-Rodriguez, and Bermejo-Barrera, 2013). The *in vitro* digestion method with the dialysis tubing is used to estimate trace metals, i.e. aluminum, cadmium, cobalt, chromium, copper, iron, manganese, nickel, vanadium, and zinc, bioavailability of marine products (Moreda-Pineiro, Moreda-Pineiro, Romaris-Hortas, Dominguez-Gonzalez, Alonso-Rodriguez, Lopez-Mahia, Muniategui-Lorenzo, Prada-Rodriguez, and Bermejo-Barrera, 2012) and bioavailability of anthocyanins from raspberry extracts (McDougall, Dobson, Smith, Blake, and Stewart, 2005). Similarly, the bioavailability of phenolic compounds from five different cultivar of cherries (*Prunus avium* L.) has also been used this method (Fazzari et al., 2008).

The *in vitro* method (the simulated gastric and intestinal digestion/dialysis tubing) has been applied to *Himanthalia elongate, Saccorhiza polyschides*, *Palmaria palmate, Porphyra umbilicalis, Ulva rigida*, *Undaria pinnatifida,* and *Spirulina platensis* to evaluate iodine and bromine bioavailability. The molecular weight cut-off

of the dialysis tubing at 10 kDa indicated that the iodine and bromine bioavailability are not significantly different in dialyzability of the samples (Romaris-Hortas et al., 2011).

It has been shown that *in vitro* methods can predict nutrient values of food in human (Etcheverry, Grusak, and Fleige, 2012). However, *in vitro* studies require *in vivo* studies to confirm the results. Therefore, the *in vitro* method could be used a preliminary test to identify nutrient bioavailability of the food matrix.

2.3 Storage life of food

Foods are perishable and will change during processing and storage affecting quality attributes of that food (Singh, 2000). Shelf life of food products is usually defined as the period of time which the product remains acceptable safety and either characteristics including sensory perspectives when stored under recommended conditions (Gimenez, Ares, and Ares, 2013). Environmental factors such as temperature, humidity, oxygen, and light cause several reaction mechanisms that lead to food deterioration. Physical, chemical, and microbiological changes are the major modes of food degradation, resulting in rejection by the consumer or becoming harmful for consuming (Singh, 2000). Therefore, the chemical reaction and rate of chemical change are useful for determining storage life of food.

2.3.1 Kinetic reaction

The rate of chemical reaction is normally evaluated by monitoring the concentration of either the reactant consumed or the product produced. A general form of kinetic model is obtained by equation (1) below:

$$aA + bB \underset{k_b}{\overset{k_f}{\leftrightarrow}} cC + dD \qquad (1)$$

Where A and B are reactants; C and D are the products; *a, b, c,* and *d* are stoichiometric coefficients for reactants and products; and k_f and k_b are reaction rate constant for forward and backward. The rate at which reactant, example, reactant A, are given in equation (2):

$$-\frac{\mathrm{d}[A]}{\mathrm{d}t} = k_f[A]^{\alpha}[B]^{\beta} - k_b[C]^{\gamma}[D]^{\delta} \qquad (2)$$

Where [A], [B], [C], and [D] are the concentration of reactant and product; α, β, γ, and δ are the reactant orders; and *t* is time. Equation (1) and (2) are unsolvable because of too many unknown parameters. Therefore, the simple procedures are used by choosing either the forward or the backward reaction which is predominant. If the change of reactant B is unimportant and the backward rate constant will be smaller than the forward reaction rate reaction, then the rate of reaction can be represented by equation (3):

$$-\frac{\mathrm{d}[A]}{\mathrm{d}t} = k'_f[A]^n \qquad (3)$$

Where; k'_f is the pseudo forward rate constant and n is the reaction order. Equation (3) may be rewritten as equation (4): for more specific quality attribute that decreasing with time.

$$-\frac{\mathrm{d}[A]}{\mathrm{d}t} = k[A]^n \qquad (4)$$

Where; *k* is the rate constant.

However, the reaction rate of quality change depends on the order of reaction (Singh, 2000).

2.3.1.1 Zero-order reaction

Zero-order reaction is used to describe the reaction such as enzyme degradation, non-enzyme browning and lipid oxidation. The linear plot represents a zero-order reaction (n=0) using equation (5) derived from equation (4). The zero-order reaction is represented by equation (5) and the shelf-life is determined by equation (6):

$$-\frac{\mathrm{d}[A]}{\mathrm{d}t} = k[A] \quad (5)$$

$$\mathrm{A}_i = \mathrm{A}_0 - \mathrm{k}t_s \quad (6)$$

Where; A_i = concentration at time *i*

A_0 = initial concentration

k = the concentration rate constant

t_s = the shelf-life time

2.3.1.2 First-order reaction

First-order reaction usually indicates food deterioration reaction include vitamin, proteins losses, and microbial growth. The rate of loss in quality attributes depends on the amount of quality remaining which shows the exponential plot between quality and time represents as first-order reaction with n = 1, and equation (4) is modified for first-order reaction equation (7).

$$ln\frac{[A]_i}{[A]_0} = -kt_s \quad (7)$$

2.3.1.3 Second-order reaction

A few food deteriorations relate to the second-order reaction such as auto-oxidation of pigments in chili. The reaction depends on the double concentration of reactant ($[A]^2$) or the concentration of two reactants ([A] and [B]) or the concentration of three reactant ([A], [B], and [C]). The equation of second-order reaction is shown in equation (8):

$$\left[\frac{1}{[A]_i}\right] = \left[\frac{1}{[A]_0}\right] + kt_s \quad (8)$$

2.3.2 Predictive model and model performance

Because the shelf life determination of food requires factors which limit the shelf life evaluation. The factors including chemical, physical, and biological changes affect changes in food and food safety. However, the limitation of shelf life test is cost and time-consuming. Alternatively, predictive models of food allow shelf life study under various conditions such as, temperature, pH, and modified atmosphere. The development of predictive models are simple and inexpensive (Walker, 2000). The last stage of model development is to ensure that the obtained prediction is useful. Therefore, the validation should state that not only does the model accurately describe the used data, but it should also demonstrate performance of the model to prediction (Singh, 2000).

Accuracy factor (A_f) and bias factor (B_f) are used as a quantitative method to measure the performance of the models (Ross, 1996; Zhong Chen, Wang, Wu, Liao, and Hu, 2005). Equation (11) was suggested by McClure, Baranyi, Boogard, Kelly, and Roberts (1993) to measure the accuracy of models, the fitted models are compared by statistical and graphical means of the root mean square error (RMSE) values of the differences between the observed and predicted values. These can be defined by the following equations:

$$A_f = 10^{\Sigma\left(\frac{\left[\log\left(\frac{\text{predicted}}{\text{observed}}\right)\right]}{n}\right)} \quad (9)$$

$$B_f = 10^{\Sigma \log\left[\left(\frac{\text{predicted}}{\text{observed}}\right)/n\right]} \quad (10)$$

$$\text{RMSE} = \sqrt{\frac{\Sigma(\text{predicted}-\text{observed})^2}{n-1}} \quad (11)$$

Where; observed = observed values

predicted = predicted values

n = number of observations

B_f and A_f for the model are calculated from the predicted and observed values. If the B_f is 1.00, the model shows a perfect agreement with the observed values. An underestimation value leads to the B_f above 1.00, an overestimation gives the B_f factor below 1.00. Similarly, the A_f of 1.00 showed a perfect agreement between observed and predicted values. The RMSE at lower values presents the better fit model (Ross, 1996; Zhong et al., 2005; Wang, Ni, Hu, Wu, Liao, Chen, Wang, 2007; Bruckner, Albrecht, Petersen, and Kreyenschmidt, 2013).

Wang et al (2007) have reported that the loss kinetics of total amino acid concentration is well fitted using the first-order reaction and the zero-reaction. When compared with the zero-order reaction, the first-order reaction provided a perfect fit with better model performance parameters with 1.057 of A_f, 1.013 of B_f, and 53.51 of RMSE (less than that of the zero-order reaction). Moreover, the first-order reaction coefficient (R^2) (0.979) is greater than that (0.940) of the zero-order reaction. Therefore, in this case, the first-order reaction presented the better explanation for the experimental data than the zero-order reaction.

2.4 References

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CHAPTER III

ZINC UPTAKE AND ITS LOCALIZATION IN *SPIRULINA PLATENSIS*

3.1 Abstract

Spirulina platensis has high proteins, carbohydrates, lipids and minerals and has been widely used as a food supplement. However, *Spirulina platensis* has low concentration of Zinc (Zn) (approximately 20-30 µg/g dry weight). This study aimed to investigate the Zn uptake of *Spirulina platensis* using different media for Zn sources and to localize Zn deposition in the cell. The results revealed that *Spirulina platensis* cultured for 7 days in modified Zarrouk's medium 1.6 µmol Zn of $ZnSO_4$ (MZS) contained the highest Zn concentration of 69.55±2.70 µg/g dry weight with protein concentration of 63.11±1.40 % dry weight, specific growth rate (µ) of 0.46 /day, cell productivity (P_x) of 0.55 g/L.day dry weight and doubling time (t_d) of 1.50 days. The majority of Zn deposit was in cytoplasm which was 2.7 fold higher than that in cell walls. Fourier transform infrared (FT-IR) spectra showed that peak intensity of the amide groups decreased after the Zn uptake indicating an important role of these functional groups in binding Zn^{2+}.

Keywords: *Spirulina platensis*, Zinc, Zinc uptake, Fourier transform infrared spectroscopy

3.2 Introduction

Zinc (Zn) is an essential trace mineral for humans, plants, animals because of its involvement in a wide variety of biochemical functions. It can prevent free radical formation to protect biological structures from damage, correct the immune functions, enhance growth in children, prevent neurological diseases such as Alzheimer's disease and Parkinson's disease, and prevent in prostate cancer (Gyorkey, Min, Huff, and Gyorkey, 1967; Stefanidou, Maravelias, Dona, and Spiliopoulou, 2006; Franklin and Costello, 2007; Haase, Overbeck, and Rink, 2008). Thai RDI (1995) recommends a dietary allowance of Zn for Thais over 6 years old at 15 mg/day. International Zinc Nutrition Consultative Group (IZiNCG) has estimated that zinc deficiency affects 31% of the world's population, especially those in the developing countries in Africa, the Eastern Mediterranean and Southeast Asia (Black, Allen, Bhutta, Caulfield, Onis, Ezzati, Mathers, and Rivera, 2008). Plant foods tend to be rich sources of Zn, but the bioavailability of zinc dietary can be reduced by phytic acid in most cereal grains, legumes, nuts, oil seeds, and tubers (Hunt, 2003; Dost and Tokul, 2006; Karunaratne, Amerasinghe, and Ramanujam 2008). Zinc bioavailability correlates well with solubility in aqueous solution depending on Zn sources (Allen, 1998). Zinc sulfate, chloride, and acetate are soluble, whereas zinc carbonate and zinc oxide are insoluble. The bioavailability of supplemental zinc will be decreased if Zn conjunction with food containing phytate inhibitor occurs. Diets, therefore, play an important role in determining zinc bioavailability.

Spirulina platensis is a filamentous cyanobacteria, known as micro algae. It has been widely used as a food supplement for promoting human health. It contains high essential nutrients such as 60-70% proteins, 12-16% carbohydrates, 9-14% lipid and

4-9% minerals dry weight, but low in Zn and it has not been reported to have toxic side effects (Ciferri, 1983; Castenholz, 1984; Salazar, Martinez, Madrigal, Ruiz, and Chamorro, 1998; Campanella, Crescentini, and Avino, 1999; Caballero, Trugo, and Finglas 2003; Gershwin and Belay, 2008). In addition, it can be used in nutraceuticals, biomedical research, food and cosmetics (Ciferri, 1983; Leema, Kirubagaran, Vinithkumar, Dheenan, and Karthikayulu, 2010; Jeamton, Dulsawat, Laoteng, Tanticharoen, and Cheevadhanarak, 2011). More than 70% of *Spirulina platensis* has been commercialized mainly as health food. It is cultivated in several countries such as, USA, China, Japan, Taiwan, and Thailand.

Active and passive uptakes are two processes for metal ions absorption into the algae cells and they vary significantly depending on the type of metal ions. Alternatively, the metal ions binding can occur on cell walls with different functional groups. These functional groups are carboxyls, hydroxyls, sulfates, amines, and other negative charge groups. The mechanisms responsible for metal binding are electrostatic interactions, ion exchange and ion complexion (Li, Guo, and Li, 2003; Chen, Shi, Chen, Xu, Chen, Wang, and Hu, 2007; Seker, Shahwan, Eroglu, Yilmaz, Demirel, and Dalay, 2008). These may have been used for zinc enrichment in *Spirulina platensis*.

Fourier transform infrared (FT-IR) spectroscopy is a non-destructive analysis method used for metal composition analyses in biological sources, e.g., cyanobacteria, yeast and bacteria and plants (Pistorius, DeGrip, and Egorova-Zachernyuk, 2009; Vazquez, Calvo, Sonia Freire, Gonzalez-Alvarez, and Antorrena, 2009; Finocchio, Lodi, Solisio, and Converti, 2010; Ferreira, Rodrigues, Carvalho, Lodi, Finocchio, Perego, and Converti, 2011; Liu, Xu, Zhou, Wang, Li, Ha, and Sun, 2013), which can identify. However, very little information is available for Zn absorption of the *Spirulina*

platensis cells. Therefore, FT-IR spectroscopy would be able to identify the functional groups involving metal ions binding.

The objectives of this study were to compare variety of zinc salts and their optimum concentration for growth, Zn absorption as well as location of Zn in the cell of *Spirulina platensis*.

3.3 Materials and Methods

3.3.1 Chemicals

Zinc chloride ($ZnCl_2$), Zinc nitrate $(ZnNO_3)_2$, and Zinc sulfate ($ZnSO_4$) were from Ajax Finechem (Scoresby, VIC, Australia). Standard Zinc was from Fisher Scientific Co. (Fair Lawn, NJ, USA). Nitric acid (HNO_3) and Hydrochloric acid (HCl) were from Merck KGaA (Darmstadt, Germany). Perchloric acid ($HClO_4$) was from Mallinckrodt Baker Inc. (Phillipsburg, NJ, USA).

3.3.2 Starter culture and preparation

Spirulina platensis IFRPD 1208 culture was from algae laboratory, Institute of Food Research and Product Development, Kasetsart University, Bangkok. The *Spirulina platensis* starter culture was grown indoors in 200 mL culture tubes supplied with Zarrouk's medium pH 8.5 ±0.2 at 30±2°C with 12 Klux light intensity (16h:8h; light:dark cycle), CO_2 flow rate 2 L/min. The starter cultures were grown for 6 days during which the growth was monitored from O.D. with a spectrophotometer at wavelength 560 nm.

The starter culture (O.D. = 0.2) was inoculated in culture tubes containing 150 mL of Zarrouk's medium (ZM), non-Zn Zarrouk's (NZM), modified Zarrouk's medium with zinc sulfate ($ZnSO_4$; MZS), zinc nitrate ($Zn(NO_3)_2$; MZN), and zinc

chloride ($ZnCl_2$; MZC) at concentrations of 0.4, 0.8, and 1.6 µmol of Zn. The inoculated culture tubes were maintained at 30 ± 2°C for 7 days before *Spirulina platensis* cell were filtered (nylon filter pore size 35 µm, Nylon mesh 140-T, Swiss Nybolt) and washed with 20 mL distilled water repeatedly 3 times to remove medium from the cells (Duangsee, Phoopat, and Ningsanond, 2009).

3.3.3 Growth measurement

Protein (Protein, %) was calculated from nitrogen in the samples the modified Kjeldahl analysis (AOAC, 2000; Lopez, Garcia, Fernandez, Bustos, Chisti, and Sevilla, 2010) and expressed as percent on dry basis. Biomass (X, g/L dry weight) was evaluated according to AOAC (2000), Doubling time (t_d, day), expressed in day, and Cell productivity (P_x), expressed in g/L.day, were calculated using equation (1) and (2), respectively.

$$\text{Doubling time } (t_d, \text{ day}) = \frac{ln2}{\mu} \quad (1)$$

Where; µ = Specific growth rate (/day)

$$\text{Cell productivity } (P_x, \text{ g/L.day}) = \frac{(X_i - Xo)}{t_i} \quad (2)$$

Where; X_0 = initial biomass density (mg/L dry weight)

X_i = biomass density at time *i* (mg/L dry weight)

t_i = time interval between X_0 and X_i (day)

Specific growth rate (µ), expressed in /day, was calculated according to equation (3):

$$\text{Specific growth rate } (\mu, \text{ /day}) = \frac{\ln X_i - \ln X_o}{t_i - t_o} \quad (3)$$

Where; X_0 = initial biomass density (mg/L dry weight)

X_i = biomass density at time *i* (mg/L dry weight)

t_o = initial time (day)

t_i = time *i* (day)

3.3.4 Determination of Zn

One hundred mg (dry weight) of the fresh *Spirulina platensis* cells were digested in 50 mL Erlenmeyer flask with 7 mL mixed 65% HNO_3: 85% $HClO_4$ (5:2), until clear solution was observed, and then the volume was adjusted to 25 mL. Zn in the *Spirulina platensis* was determined using Atomic Absorption Spectrometry (AAS), Perkin Elmer model PinAAcle 900F, MA, USA, as described by AOAC (2005).

3.3.5 Isolation of cell fractions

Cell fraction were prepared according to the method reported by Duangsee, Phoopat, and Ningsanond (2009); Vladimirescu (2010); Gan, Tang, Shi, Wang, Cao, and Zhao (2004). *Spirulina platensis* cells were filtered and washed with 20 mL distilled water for 3 times to remove medium remains with the sample, then was lyophilized and resuspened to 10 % solid with mixed solution 0.1 M Phosphate buffer pH 7.0 and 0.5% lysozyme at 35 °C and incubated for 4 h. The cells were collected by centrifugation at 10000g for 15 min. Supernatant and pellet were determined Zn concentration by AAS. Suspension was represented cytoplasm fractions and pellet was represented cell wall fractions. Extraction times of cells gave different Zn concentrations; thus, the cells were extracted until no significant differences in Zn concentrations between extraction times were found. Therefore, the fourth time was chosen to extract the Zn concentrations in *Spirulina platensis* cells.

3.3.6 Determination of cell functional groups

The *Spirulina platensis* cells (from section 3.3.2) was lyophilized in a Heto FD8 (Holten AS, Denmark) freeze dryer. The absorption of zinc in *Spirulina platensis* cells were investigated to seek the functional groups using Attenuated Total Reflectance Fourier Transformed Infrared (ATR-FTIR) Spectrometer (Bruker Tenser 27, GmbH). The FT-IR spectra were recorded in the region of 4000-700 cm^{-1}, the acquisition 64 scans and the resolution 4 cm^{-1}. Five mg of ground *Spirulina platensis* cells were used in the study. OPUS 7.0 (Bruker, GmbH) software was used for the intensity and frequency identification.

3.3.7 Statistical analysis

Results were statistically evaluated using analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) with the confidence level of 95% ($p<0.05$) in order to verify significance of different effects among the media.

3.4 Results and discussion

3.4.1 Growth and Zn uptake

Growth of *Spirulina platensis* cultured in ZM, NZM, MZS, MZN, and MZC media at concentrations of 0.4, 0.8, 1.6 µmol Zn for 7 days was present in Figures 3.1-3.3. The *Spirulina platensis* cultured in MZS (1.6 µmol Zn) had the highest biomass of 4.03±0.01 g/L dry weight, Zn 69.55 µg/g dry weight, and protein concentration 63.11% compared with NZM, MZC, and MZN. Zn in the media is not a main growth factor of *Spirulina platensis*, but nitrogen and carbon sources are. Growth of *Spirulina platensis* depends on nutrients of medium, especially balance between carbon and nitrogen in the cell. If the nitrogen and the carbon are not assimilated in the cell, the growth rate could

not reach in the maximum (Gordillo, Jimenez, Figueroa, and Niell, 1999; Rodigues, Ferreira, Concerti, Sato, and Carvalho, 2011).

However, medium without Zn (NZM) significantly showed the lowest biomass among cell treatments This indicate that Zn was essential for cell growth $ZnSO_4$ used in medium Zn enrichment at all concentrations was the best salts form to significantly increase Zn content in *Spirulina platensis* cells (showed provide reasons for this). Generally, increasing Zn in growth media did not give higher protein content in cells (Figure 3.3). Only $ZnSO_4$ at 0.8 and 1.6 µmol Zn provide cells with statistically same protein content as the standard medium (ZM). The rest of the media fortified with various Zn salts significantly resulted in low protein contents in the cell. These results showed similarity with cell biomass. The effects of $ZnSO_4$ on enhancing Zn uptake of *Spirulina platensis* cells while maintaining cell biomass and protein content may be important due to increases in protein bound sulfur, such as, cysteine (Cys), methionine (Met), histidine (His) and prokaryotic enzyme systems (Menon and Varma, 1982; Zander, Faust, Klink, Sanctis, Panjikar, Quentmeier, Bardisschewsky, Friedrich, and Scheidig, 2011).

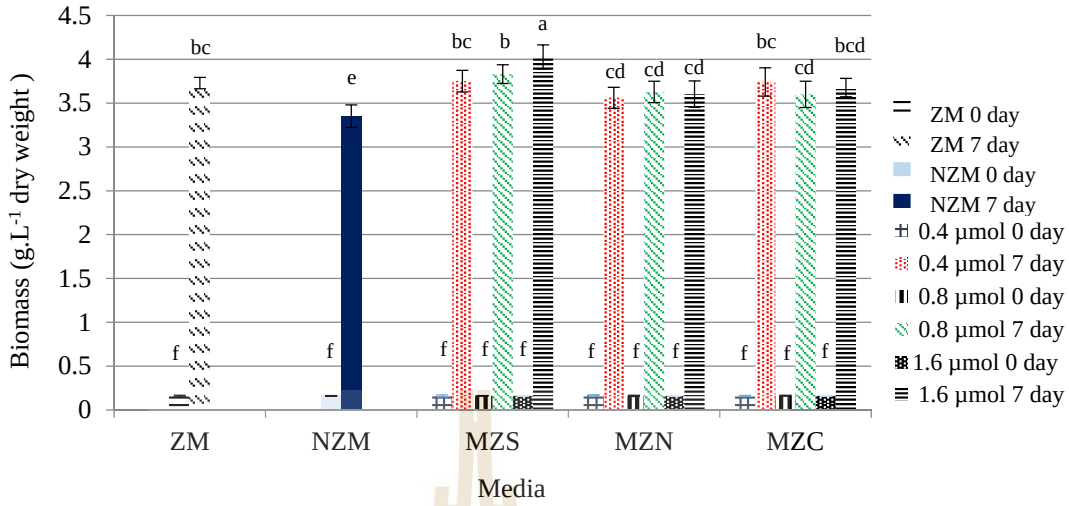

Figure 3.1 Biomass of *Spirulina platensis* cultured in ZM, NZM, MZS, MZN, and MZC media. Different letters indicate the significant differences (p<0.05). Values are the mean of triplicates (n=3).

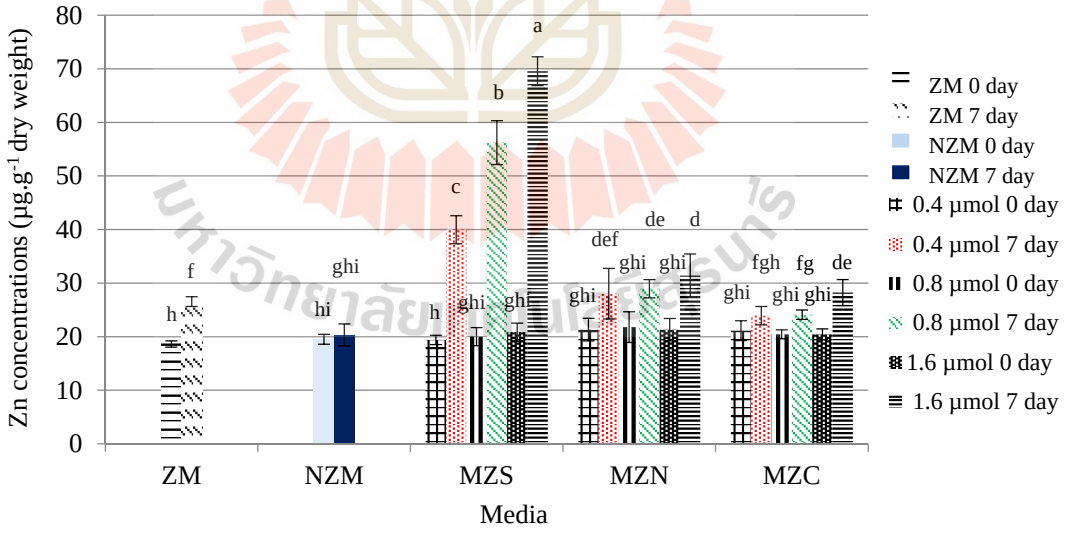

Figure 3.2 Zn in *Spirulina platensis* cultured in ZM, NZM, MZS, MZN, and MZC media. Different letters indicate the significant differences (p<0.05). Values are the mean of triplicates (n=3).

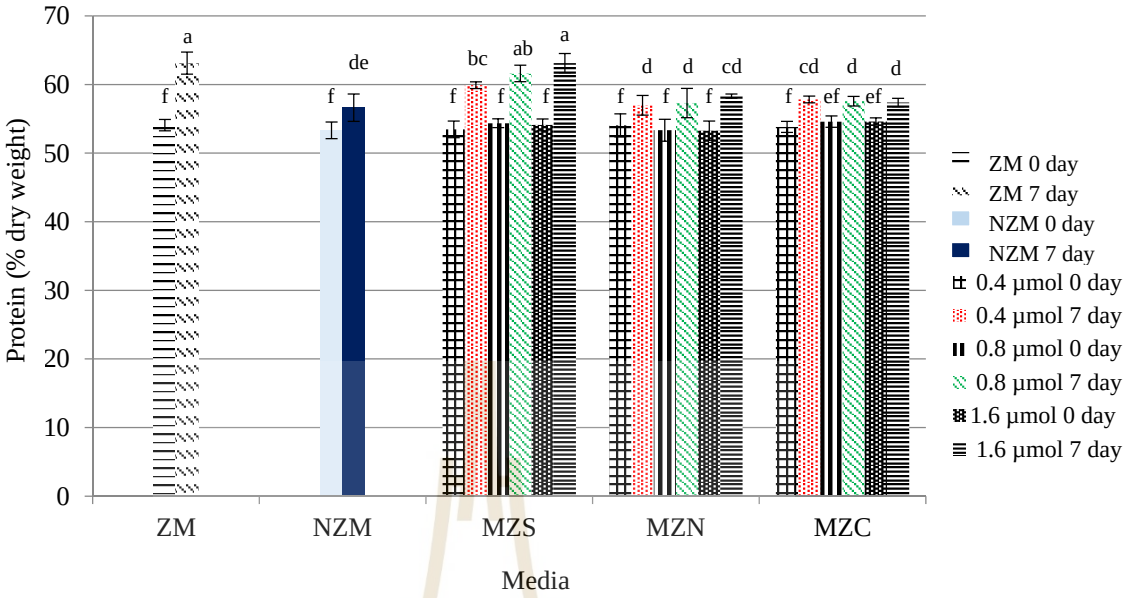

Figure 3.3 Protein in *Spirulina platensis* cultured in ZM, NZM, MZS, MZN, and MZC media. Different letters indicate the significant differences (p<0.05). Values are the mean of triplicates (n=3).

Table 3.1 showed specific growth rate (µ, /day), doubling time (t_d, day) and cell productivity (P_x, g/L.day) of *Spirulina platensis*. Zarrouk's modified 1.6 µmol Zn of $ZnSO_4$ exhibited significantly highest growth parameters; specific growth rate of 0.46 /day, doubling time of 1.50 days and cell productivity of 0.55 g/L.day dry weight.

The results presented $ZnSO_4$ form was absorbed in *Spirulina platensis* cell higher than $ZnCl_2$ and $Zn(NO_3)_2$ form because sulfate groups provided synthesis protein, which consist of amino acid groups in structure. Amino acid groups were cysteine and methionine, which can be bound Zn molecule in the cell.

Table 3.1 Specific growth rate (μ, /day), doubling time (t_d, day) and cell productivity (P_x,g/L.day) of *Spirulina platensis* cultured in ZM, NZM, MZS, MZN, and MZC media.

Media	μ (/day)	t_d (day)	P_x (g/L.day)
ZM	0.45±0.00^{ab}	1.53±0.01^{bc}	0.51±0.01^{bcd}
NZM	0.43±0.01^{c}	1.59±0.04^{a}	0.46±0.02^{e}
0.4 μmol Zn of MZS	0.45±0.01^{abc}	1.54±0.05^{bc}	0.51±0.02^{bcd}
0.8 μmol Zn of MZS	0.45±0.00^{ab}	1.53±0.02^{bc}	0.53±0.02^{ab}
1.6 μmol Zn of MZS	0.46±0.01^{a}	1.50±0.03^{c}	0.55±0.02^{a}
0.4 μmol Zn of MZN	0.44±0.00^{bc}	1.58±0.01^{ab}	0.48±0.02^{de}
0.8 μmol Zn of MZN	0.45±0.01^{abc}	1.56±0.04^{ab}	0.49±0.01^{bcd}
1.6 μmol Zn of MZN	0.44±0.01^{bc}	1.56±0.04^{abc}	0.49±0.03^{bcd}
0.4 μmol Zn of MZC	0.45±0.01^{abc}	1.54±0.03^{bc}	0.51±0.02^{bc}
0.8 μmol Zn of MZC	0.44±0.01^{bc}	1.57±0.02^{ab}	0.49± 0.02^{cd}
1.6 μmol Zn of MZC	0.45±0.00^{abc}	1.54±0.02^{abc}	0.50±0.02^{bcd}

Values with the different letters within a column are significantly different for $p<0.05$. Data are the mean of triplicates (n=3).

3.4.2 Location of Zn in *Spirulina platensis*

3.4.2.1 Zn in cell fractions

Spirulina platensis cells were separated into two fractions; the first fraction representing cytoplasm (supernatant) and the second fraction for cell wall (pellet). The results showed that Zn concentrated in cytoplasm in the amount of 60.50

µg/g dry weight (72.8%) and presented in cell wall in the amount of 22.62 µg/g dry weight (27.2%). Previous research studied the uptake of Zn, Cd, and Se in four species of phytoplankton, *Phaeodactylum tricornutum, Prorocentrum minimum, Tetraselmis levis,* and *Chlorella autotrophica*. The metals uptake in phytoplankton normally implicated an initial rapid surface absorption and the metals transport into the intracellular, respectively (Wang and Dei, 2001). The cell surface absorption incorporated complexion with algal extracellular organic compounds, e.g., extracellular polysaccharides. Inorganic selenite could be transformed into organic forms through binding with proteins, lipids, polysaccharides, and other cellular components in *Spilulina platensis* (Li, Guo, and Li, 2003).

3.4.2.2 FT-IR analysis of functional groups binding Zn

FT-IR spectra of lyophilized *Spirulina platensis* cells were normalized and obtained to evaluate the effects of MZS media on the functional group of cells. The FT-IR spectra in the 2,000-800 cm^{-1} region were shown in Figure 3.4. Band assignments are based on the references in Table 3.2. The bands at 1652 cm^{-1} can be assigned to amide I C=O and/or C-N groups of proteins. Amide II appeared at 1541 cm^{-1} can indicate the N-H and/or C-N groups of proteins. McLaughlin, Mulrine, Gresalfi, Vaio, and McLaughlin (1981) examined the mode of metal cations binding to membranes. The metal cations affected the stability and the structure of phospholipid bilayers, and modulated the binding proteins insertion. Infrared (IR) spectra showed the complex formation of the phosphate groups with Ba^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+}, Sr^{2+}, and Zn^{2+} ions that gave conformational change (Binder and Zschornig, 2002).

The FT-IR spectra band of amide I and amide II groups of modified Zarrouk's Zn of MZS decreased when compared with ZM. Intensity of amide I and

amide II groups decreased because Zn was absorbed in cells at these functional groups of protein. ZM showed the higher absorbance while 1.6 µmol Zn of MZS had the lower absorbance for amide I and amide II groups. Intensity absorbance of the free amide I and amide II functional groups was decreased by Zn^{2+}. This finding of the current study was consistent with those of Rodrigues, Ferreira, Carvalho, Lodi, Finocchio, and Converti (2012) who performed the Zn^{2+}, Ni^{2+}, and Pb^{2+} onto dry *Spirulina platensis* and *Chlorella vulgaris* using FT-IR spectroscopic method. The metals binding affected amide groups with a decrease in the bands intensities. Moreover, the metalloprotein or intracellular protein containing sulfhydryl group would also bind Zn^{2+}. Therefore, amide I and amide II played an important role in binding of Zn^{2+}.

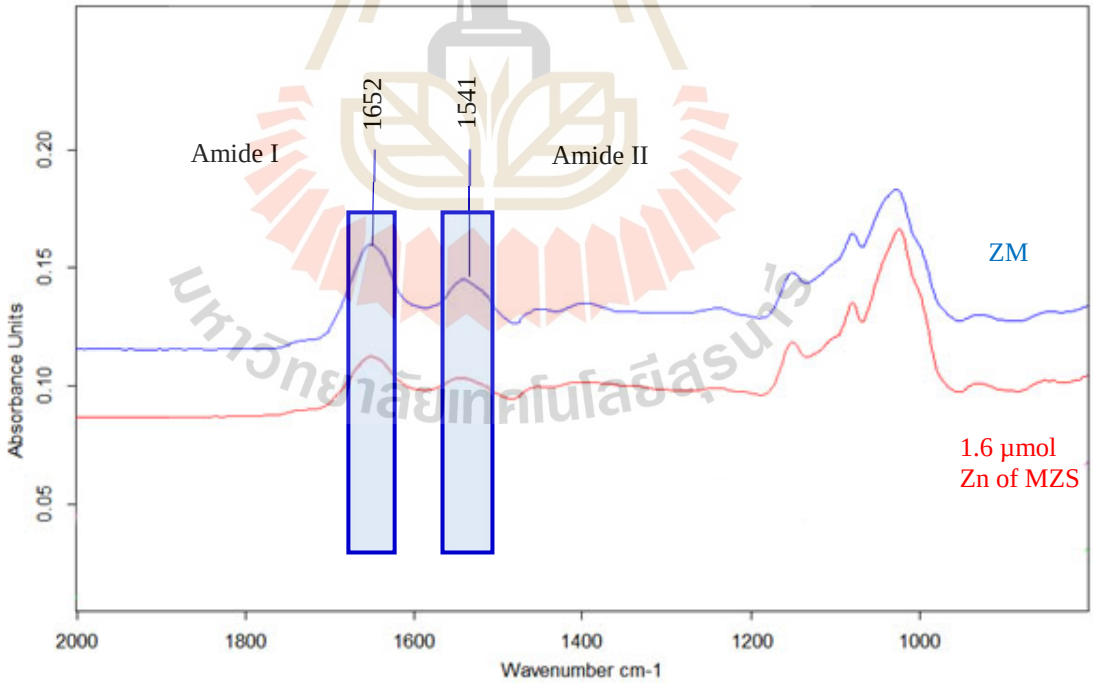

Figure 3.4 FT-IR spectra of *Spirulina platensis* cells cultured in; a) ZM, b) 1.6 µmol Zn of MZS media.

Table 3.2 Major absorption peaks in infrared spectra of *Spirulina platensis* sample.

Frequency (cm^{-1})	Functional groups	Main attribution
1658-1650	C=O, C-N	Amide I, Protein
1545-1540	N-H, C-N	Amide II, Protein

Modified from: Chen, Shi, Chen, Xu, Chen, Wang, and Hu (2007); Pistorius, DeGrip, and Egorova-Zachernyuk (2009); Finocchio, Lodi, Solisio, and Converti, (2010); Liu et al. (2013).

3.5 Conclusions

Spirulina platensis cultured in 1.6 µmol Zn of Zarrouk medium modified with $ZnSO_4$ (MZS) had the highest Zn concentration, protein, biomass, specific growth rate, doubling time and cell productivity. $ZnSO_4$ is appropriate form for Zn enrichment of *Spirulina platensis* cells. Zn uptake was higher in cytoplasm (72.8%) than in cell walls (27.2%). FT-IR spectra analysis and indicated that Zn^{2+} would bind to amide I and amide II functional groups of protein.

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CHAPTER IV

IN VITRO STUDY OF ORGANIC ZINC ACCESSIBILITY AND AVAILABILITY IN *SPIRULINA PLATENSIS*

4.1 Abstract

Health benefit of organic Zn in *Spirulina platensis* depends on its being accessible and available. Accessibility and availability of organic Zn in *Spirulina platensis* were investigated using *in vitro* digestion. Dry *Spirulina platensis* samples cultured in Zarrouk's medium (SPZ) and in modified Zarrouk's medium (SPM) were compared with a commercial sample (SPC). The results showed that SPM (starting with 98.07±0.59 µg Zn/g dry weight) at 2 h digestion gave the highest Zn accessibility at 55.20±0.57% in gastric simulation and 63.55±0.21% in small intestinal simulation. Zn concentrations in SPC (starting with 64.21±0.26 µg Zn/g dry weight) and SPZ (starting with 29.54±0.32 µg Zn/g dry weight) were accessible at 52.85±0.21% and 52.55±0.21% in gastric simulation whereas at 60.30±0.14% and 60.95±0.78% were accessible in small intestinal simulation, respectively. For the availability study, the simulation for 2 h was suitable. The results showed that Zn availability of SPM, SPC, and SPZ was 34.63±0.95, 31.68±0.07, and 31.43±0.63%, respectively. This indicated that organic Zn in SPM had the highest accessibility and availability.

Keywords: *Spirulina platensis*, Organic Zinc, Accessibility, Availability, Digestion

4.2 Introduction

Spirulina platensis is a filamentous cyanobacterium. It contains high amino acids, polyunsaturated fatty acids, vitamins, and pigments (Ciferri, 1983; Castenholz, 1984; Salazar, Martinez, Madrigal, Ruiz, and Chamorro, 1998; Campanella, Crescentini, and Avino, 1999; Caballero, Trugo, and Finglas 2003; Gershwin and Belay, 2008). The cyanobacteria cells consist of complex structures having unique cellular functional groups which can bind metals (Yee, Benning, Phoenix, and Ferris, 2004; Hudek, Rai, Michalczyk, Rai, Neilan, and Ackland, 2012). *Spirulina platensis* cells also have many functional groups, e.g., carboxyl, hydroxyl, sulphate, and other groups which can bind metals (Li, Guo, and Li, 2006; Seker, Shahwan, Eroglu, Yilmaz, Demirel, and Dalay, 2008).

Zinc (Zn) is an essential trace element which can plays a role in protect biological structures from damages, correct immune functions, and enhance growth in children, and prevent neurological diseases (Gyorkey, Min, Huff, and Gyorkey, 1967; Stefanidou, Maravelias, Dona, and Spiliopoulou, 2006; Franklin and Costello, 2007; Haase, Overbeck, and Rink, 2008). International Zinc Nutrition Consultative Group (IZiNCG) has suggested the daily dosages of supplemental zinc at 13-19 mg/day for the adult men and 7-9 mg/day for adult women. It has been estimated that zinc deficiency affects 31% of the world's population (Hotz and Brown, 2004; Black, Allen, Bhutta, Caulfield, Onis, Ezzati, Mathers, and Rivera, 2008). Therefore, Zn deficiency population can be reduced by consuming foods containing the bioavailable Zn element. The compounds having bioavailable properties in the tissue or organ, are called bioactive compounds (McDougall, Dobson, Smith, Blake, and Stewart, 2005).

Usefulness of bioactive compound depends on their accessibility and availability. Accessibility refer to the amount of the bioactive compound that is released from its food matrix during digestion and made accessible for absorption into mucosa (Harden, Diaz, and Svanberg, 2002). Availability defines as the fraction of bioactive compound ingested that is available for use in physiologic functions or to be stored (Fairweather-Tait, 1993). Zn is a cofactor of more than 200 enzymes. The Zn binding proteins have been categorized as metalloprotein which are intracellular proteins in living cells. Zn binding proteins play an important role in the immune system (Mocchegiani, Giacconi, Cipriano, Muzzioli, Fattoretti, Bertoni-Freddari, Isani, Zambenedetti, and Zatta, 2001; Stefanidou, Maravelias, Dona, and Spiliopoulou, 2006).

The assessment of mineral bioavailability using *in vitro* method has been proposed as an alternative to *in vivo* method. Most *in vitro* methods consist of a simulation of gastrointestinal digestion followed by determination of the element using dialysis through a membrane of a certain pore size. The *in vitro* methods may give higher precision and lower variability than that of the *in vivo* methods. Other advantages the *in vitro* methods are low cost and shorter time needed to obtain results. Additionally, the *in vitro* tests of minerals bioavailability, the *in vitro* dialysis model has usually been used together with the Caco-2 cell monolayers model. This method requires technical skills and research facilities. Therefore, the dialysis model remains an advantageous technique for particular research work by providing efficient experimental measurements and minimal research facilities (Argyri, Theophanidi, Kapna, Staikidou, Pounis, Komaitis, Georgiou, and Kapsokefalou, 2011). Previous studies showed that the *in vitro* procedure using dialysis tubing was developed to estimate availability in plants and foods, which are for example, legumes, nuts, grains, spinaches, carrots,

cherries, pears, juices, fish, and mollusk (Gil-Izquierdo, Gil, Tomas-Barberaan, and Ferreres, 2003; Sahuquillo, Barbera, and Farre, 2003; Bollinger, Tsunoda, Ledoux, Ellersieck, and Veum, 2005; Fazzari, Fukumoto, Mazza, Livrea, Tesoriere, and Marco, 2008; Tesroiere, Fazzari, Angileri, Gentile, and Liverea, 2008). This study adopted, *in vitro* accessibility and availability of organic Zn in cultivated *Spirulina platensis* along with the optimum times of gastric and small intestinal digestion to obtain Zn released from *Spirulina platensis* samples.

4.3 Materials and methods

4.3.1 Chemicals

Pepsin and Bile extract were from Sigma-Aldrich Co. (St. Louis, MO, USA). Pancreatin was from Acros Organics (Fair Lawn, NJ, USA). Sodium hydrogen carbonate ($NaHCO_3$) and Standard Zinc were from Fisher Scientific Co. (Fair Lawn, NJ, USA). Nitric acid (HNO_3) and Hydrochloric acid (HCl) were from Merck KGaA (Darmstadt, Germany). Perchloric acid ($HClO_4$) was from Mallinckrodt Baker Inc. (Phillipsburg, NJ, USA).

4.3.2 Samples preparation

Spirulina platensis cultured for 8 days in Zarrouk's medium (SPZ) and in Zarrouk's medium (SPM) modified were washed with deionized water for 3 times to remove media from the *Spirulina platensis* cells and then lyophilized in a Heto FD8 (Holten AS, Denmark) freeze dryer. The *Spirulina platensis* commercial sample (SPC) was purchased from The Royal Chitralada Project.

4.3.3 Zn accessibility simulation

The simulated accessibility study consists of two sequential steps: 1) simulated gastric digestion and 2) simulated small intestinal digestion.

4.3.3.1 Gastric digestion simulation

Five hundred mg of lyophilized *Spirulina platensis* samples were transferred into a 50 mL polypropylene tube, then 20 mL of deionized water was added, and pH was adjusted to 2.0 with 6 M HCl. Subsequently, 315 units/mL pepsin was added to the sample before incubation in a shaking bath set at 37 °C, 150 rpm for 0.30, 1, 1.30, 2, 2.30, 3, 3.30, and 4 h. Activity of enzyme was stopped by immersion of the tube in ice-water bath. The sample was centrifuged at 13000g at 4 °C for 15 min. The supernatant was collected for Zn concentration and the further use in simulated small intestinal digestion (McDougall et al., 2005; Fazzari et al., 2008; Moreda-Pineiro, Moreda-Pineiro, Romaris-Hortas, Dominguez-Gonzalez, Alonso-Rodriguez, Lopez-Mahia, Muniategui-Lorenzo, Prada-Rodriguez, and Bermejo-Barrera, 2012).

Accessibility of Zn after the simulated gastric digestion (Martinez, Santella, Ros, and Periago, 1998; Arkasuwan, Siripinyanond, and Shiowatana, 2011; Moreda-Pineiro et al., 2012), expressed as a percentage (% dry weight), was calculated as the following equation:

Accessibility of Zn after the simulated gastric digestion (% dry weight)

$$= \frac{\text{Zn after simulated gastric digestion}}{\text{Total Zn in sample}} \times 100 \qquad (1)$$

4.3.3.2 Small intestinal digestion simulation

Five mL of intestinal solution (4% panceratin and 2.5% bile salts dissolved in 0.1 M $NaHCO_3$) was added to the 50 mL tube containing digested sample

from the simulated gastric digestion and the mixture was adjusted to pH 7.5 with 1 M $NaHCO_3$. Then the sample was incubated in a shaking bath set at 37 °C, 150 rpm for 0.30, 1, 1.30, 2, 2.30, 3, 3.30, and 4 h. Enzymatic activity was stopped by immersion the tube in an ice-water bath. The sample was centrifuged at 13000g at 4 °C for 15 min and the collected supernatant was used for Zn concentration (Gil-Izquierdo et al., 2003; Sahuquillo, Barbera, and Farre, 2003; Tesroiere, Fazzari, Angileri, Gentile, and Liverea, 2008).

Accessibility of Zn after the simulated small intestinal digestion (Martinez, Santella, Ros, and Periago, 1998; Arkasuwan, Siripinyanond, and Shiowatana, 2011; Moreda-Pineiro et al., 2012), expressed as a percentage (% dry weight), was calculated as the following equations:

Accessibility of Zn after the simulated small intestinal digestion (% dry weight)

$$= \frac{\text{Zn after simulated small intestinal digestion}}{\text{Total Zn in sample}} \times 100 \qquad (2)$$

4.3.4 Zn availability simulation

The simulated availability study was conducted in two sequential steps: 1) gastric digestion and 2) small intestinal digestion.

4.3.4.1 Gastric digestion simulation

The simulated gastric digestion method was prepared as described in section 4.3.3.1. The supernatant was collected for Zn concentration and the further use in small intestinal digestion.

4.3.4.2 Small intestinal digestion simulation

Five mL intestinal solution (4% panceratin and 2.5% bile salts dissolved in 0.1 M $NaHCO_3$) containing digested samples into a 50 mL polypropylene tube was added. Dialysis tubing (molecular weight cut-off of 10-14 kDa; Fisher

Scientific Co., Pittsburgh, PA, USA) that contained sufficient $NaHCO_3$ to neutralize the sample (adjusted pH to7.5) was then placed into the polypropylene tube. Consequently, the tube was incubated in a shaking bath set at 37 °C, 150 rpm for 0.30, 1, 1.30, 2, 2.30, 3, 3.30, and 4 h after sealed with parafilm and screw-cap. The enzymatic activity was stopped activity by immersion the tube in an ice-water bath. The solution outside the dialysis tubing represented materials remained in the gastrointestinal tract. The solution entered the dialysis tubing represented materials entered the serum. The solution inside the dialysis tubing was used for Zn concentration (Gil-Izquierdo et al., 2003; McDougall et al., 2005; Fazzari et al., 2008; Moreda-Pineiro et al., 2012).

Availability of Zn after the simulated digestion (Martinez, Santella, Ros, and Periago, 1998; Arkasuwan, Siripinyanond, and Shiowatana, 2011; Moreda-Pineiro et al., 2012), expressed as a percentage (% dry weight), was calculated as the following equation:

Availability of Zn after the simulated digestion (% dry weight)

$$= \frac{\text{Zn after dialysis tubing digestion}}{\text{Total Zn in sample}} \times 100 \qquad (3)$$

4.3.5 Atomic absorption spectrometry (AAS) analysis

The determination of Zn accessibility and availability in samples obtaining from section 4.3.3 and 4.3.4.2, five mL digested sample was transferred to a 50 mL flask and 15 mL mixed HNO_3:$HClO_4$ (5:2) was added, before the sample was hydrolyzed to obtain clear solution and then volume was adjusted to 25 mL. Subsequently, the hydrolyzed sample was used for Zn content determination as described in section 3.3.4.

4.3.6 Statistical analysis

All experiments were prepared in duplicate. Analysis of variance (ANOVA) was performed on data obtained with the confidence level of 95% (p<0.05) in order to verify the significant difference among the samples.

4.4 Results and Discussion

4.4.1 Zn accessibility simulation

Pepsin was used to digest the samples in the simulated gastric digestion. Optimum time of the gastric digestion and the small intestinal digestion were examined by varying time of digestion (0.30, 1, 1.30, 2, 2.30, 3, 3.30, and 4 h). SPM in simulated gastric digestion showed the highest Zn concentration of 54.14±0.61 µg Zn/g dry weight, at 2 h (Figure 4.1). For longer digestion, Zn concentrations were not significantly different increase. Zn concentrations of SPC and SPZ after simulated gastric digestion were 33.59±0.88 and 15.53±0.05 µg Zn/g dry weight, respectively. Similar to the results of previous work, the optimum simulated gastric digestion time of 2 h was found in spinaches, carrots, cherries, tomatoes, and fruit beverages (Garrett, Failla, and Sarama, 1999; Fazzari et al., 2008; Cilla, Garcia-Nebot, Perales, Lagarda, Barbera, and Farre, 2009). Therefore, 2 h digestion was chosen in sample preparation for the simulated small intestinal digestion step.

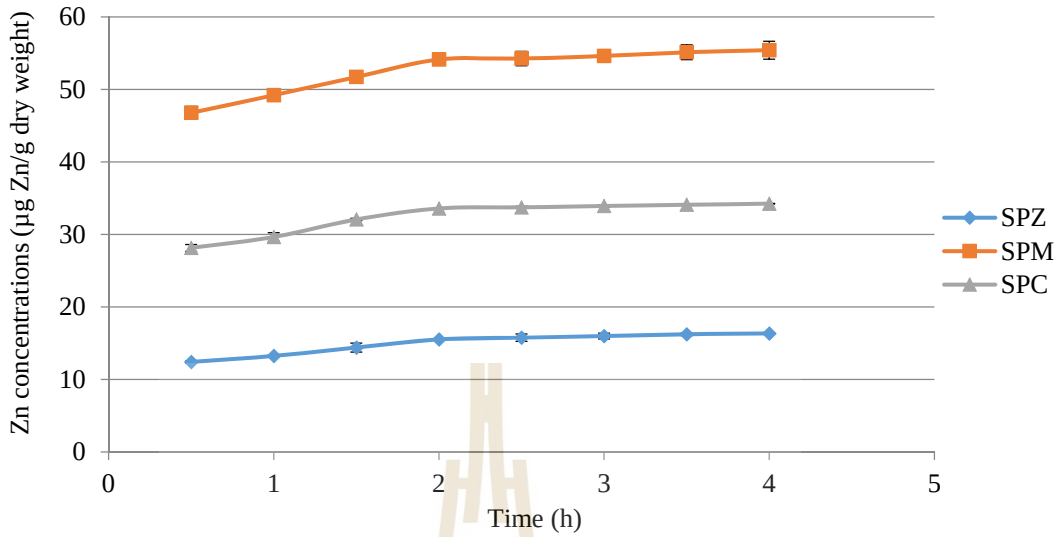

Figure 4.1 Zn concentrations of *Spirulina platensis* after the simulated gastric digestion.

The samples were digested in the simulated small intestine by pancreatin and bile salts. The optimum time was at 2 h, during which 2-4 h digestion showed no significant difference. SPM had the highest Zn concentration of 62.30±0.18 µg Zn/g dry weight whereas Zn concentration of SPC and SPZ were 38.48±0.30 and 18.01±0.24 µg Zn/g dry weight, respectively (Figure 4.2). This study indicated that the simulated small intestinal digestion had higher Zn concentration than the simulated gastric digestion because the activity of pancreatic enzymes and bile salts effectively released Zn from the matrix of *Spirulina platensis* cells. Pancreatin and bile salts contain many enzymes, such as trypsin, lipase, amylase, chymotrypsin, and carboxypeptidase (Young, Nau, Pasco, and Mine, 2011). Moreover, the mixture of pancreatic enzymes and bile salts is important for the micellarization of lipid (Hofmann and Borgstrom 1962; Monsbach, Newton, and Stevens, 1980).

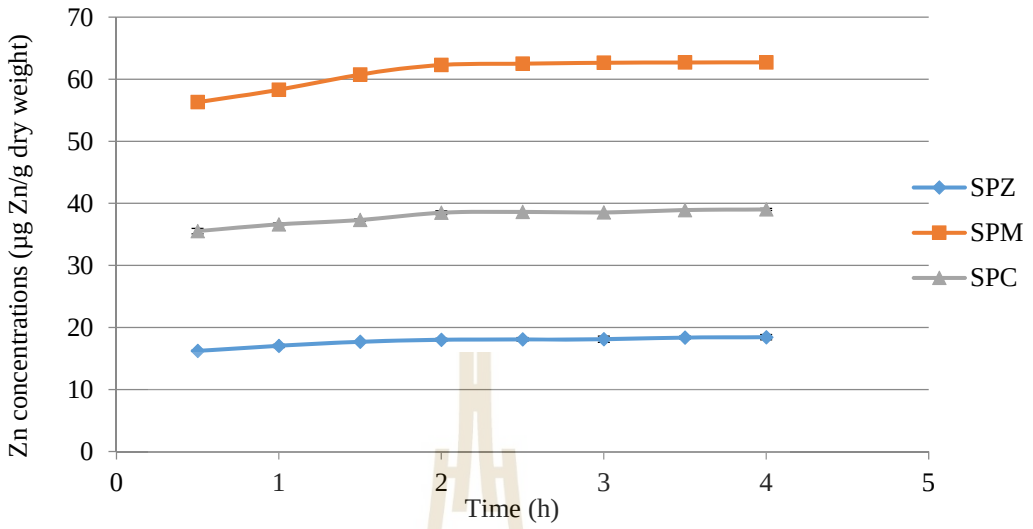

Figure 4.2 Zn concentrations of *Spirulina platensis* after simulated small intestinal digestion.

SPM (starting with 98.07±0.59 µg Zn/g dry weight) gave significantly higher Zn concentration after simulated gastric digestion (55.20±0.57%) and simulated small intestinal digestion (63.55±0.21%) than the other samples (p<0.05) (Figure 4.3). Accessibility after the simulated gastric digestion and simulated small intestinal digestion of SPC (starting with 64.21±0.26 µg Zn/g dry weight) were 52.85±0.21% and 60.30±0.14%, respectively, which was statistically similar to Zn concentration of SPZ (starting with 29.54±0.32 µg Zn/g dry weight) with 52.55±0.21% after gastric digestion and 60.95±0.78% after small intestinal digestion.

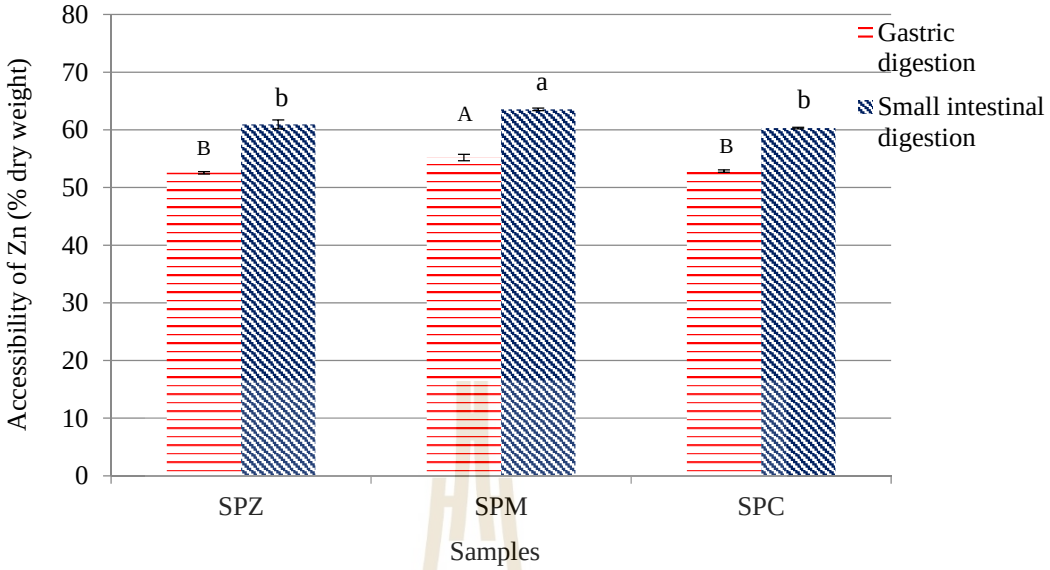

Figure 4.3 Simulated accessibility of Zn after *Spirulina platensis* digestion. Different letters indicate the significant differences (p<0.05).

4.4.2 Zn availability of simulation

Digested samples from the simulated gastric digestion were evaluated for Zn availability using dialysis tubing. SPM showed the highest available Zn concentration at 2 h of 33.96±0.93 µg Zn/g dry weight, while Zn availability of SPC and SPZ were 20.29±0.24 and 9.28±0.19 µg Zn/g dry weight, respectively (Figure 4.4). Digestion longer than 2 h showed no significant difference. Therefore, the 2-hour digestion time was selected for the Zn availability study.

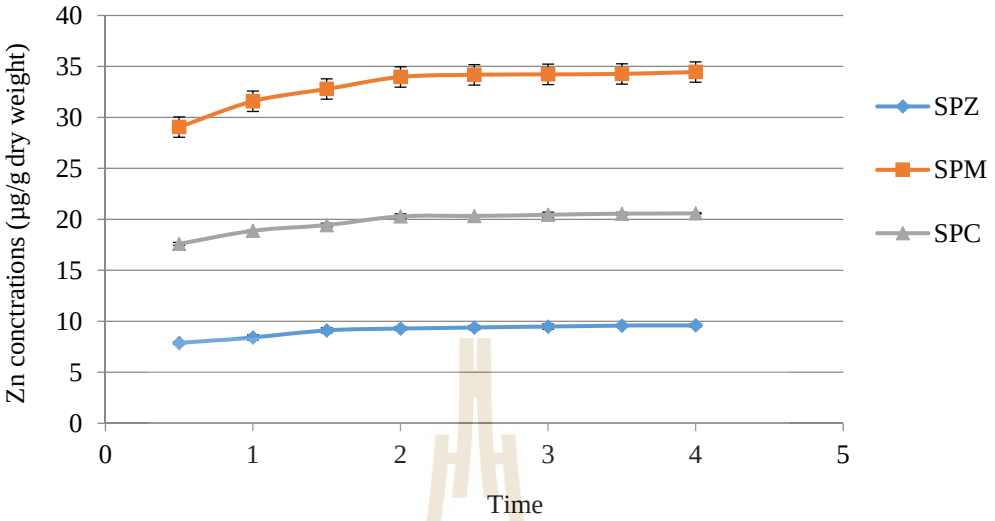

Figure 4.4 Zn concentrations of *Spirulina platensis* after simulated digestion.

Zn availability of samples after the post gastric digestion was illustrated in Figure 4.5. Availability of Zn of SPM, SPZ, and SPC were 62.94±1.75, 59.45±1.19 and 59.97±0.15 %, respectively. Surprisingly, the availability of all samples has lower Zn concentration than the gastric digestion. The availability of the samples of SPM was 34.63±0.95 followed by SPC of 31.68±0.07 and SPZ of 31.43±0.63 %.

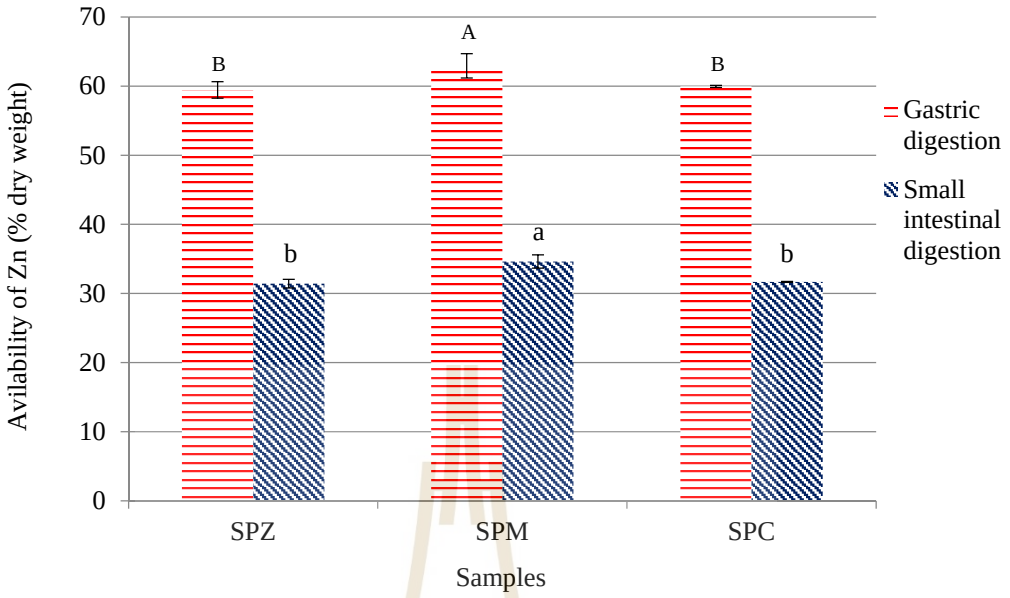

Figure 4.5 Simulated availability of Zn after simulated digestion with *Spirulina platensis.* Different letters indicate the significant differences (p<0.05).

The decreases in Zn availability could be the result of high chlorophyll in *Spirulina platensis* inhibiting pancreatic enzyme activity. Ferruzzi, Failla, and Schwartz (2001) reported that the chlorophyll derivatives from spinach puree showed the obstruction of pancreatic enzyme catalytic capacity because the porphyrin backbone of chlorophyll could be reacted with proteolytic of enzymes. The large molecular weight of Zn complex could also be formed between deprotonated form of bile acid and Zn which would not allow passage through the pore of dialysis tubing. The molecular weight of platonic cyanobacterium *Anabaena flos-aquae* protein was 20.6 kDa (Walker and Walsby, 1983).

Therefore, the coordination of Zn with constituents in *Spirulina platensis* such as, protein and pigments may increase molecular weight of organic Zn together with Zn-bile complex reduced Zn availability of *Spirulina platensis* after small

intestinal digestion. In addition, Zn in *Spirulina platensis* has been reported to be lower availability than accessibility because using the dialysis tubing method to mimic the small intestine cell wall could not mimic active transport mechanism. As the result, Zn in *Spirulina platensis* diffused into the dialysis tubing only by the passive transport mechanism (McDougall et al., 2005).

4.5 Conclusions

The optimum time of the simulated gastric and small intestinal digestion for Zn accessibility in this study was 2-hour. SPM had the highest accessibility which was 55.14±0.61 µg Zn/g dry weight (55.20±0.57%) in the simulated gastric and 62.30±0.18 µg Zn/g dry weight (63.55±0.21%) in the small intestinal simulation, respectively. The optimum time for Zn availability study was 2-hour. Zn concentration in SPM had the highest availability at 33.96±0.93 $\mu g.g^{-1}$ dry weight (34.63±0.95%). Simulated availability of Zn after small intestinal digestion was low due to pancreatic enzyme inhibited by chlorophyll and Zn-bile complex.

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CHAPTER V

STORAGE LIFE OF FRESH *SPIRULINA PLATENSIS*

5.1 Abstract

The objectives of this study were to evaluate changes and storage life of fresh *Spirulina platensis* packed in low density polypropylene (LDPE) with various glycerol concentrations (0, 5, and 10%) and stored at 4, 20, and 30°C. The results showed that storage of fresh *Spirulina platensis* 14 days was best at 4ºC and 5% glycerol. After protein and Zn contents were 60.89% dry weight and 83.34 µg/g dry weight, respectively. The kinetics reactions of protein and Zn changes and fitted well with the zero-order reaction. The storage life of fresh *Spirulina platensis* with 5% glycerol was then predicted using the zero-order reaction models to be about 50 days at 4ºC with 55% protein, and 54.89 µg/g dry weight Zn remaining. The storage life of fresh *Spirulina platensis* without glycerol as a protectant would be about 22 days, leaving cell with 55% protein and 70.34 µg/g dry weight Zn.

Keywords: Fresh *Spirulina platensis*, Storage life, Kinetics reaction, Zn

5.2 Introduction

Spirulina platensis, a filamentous cyanobacterium, has been widely used as food, dietary supplement, and functional food. It is one of the richest protein sources up to 74% dry weight. The general protein standard for Spirulina food requires ≥50% in Japan

and 55-65% in France, Sweden, and USA (Castenholz, 1984; Cohen, 1997; Campanella, Crescentini, and Avino, 1999; Ciferri, 1983; Caballero, 2003; Gershwin and Belay, 2008; Koru, 2012). Detrimental changes of fresh *Spirulina platensis* cells during storage start with autolysis.

The cell autolysis is a part of the development process, including, reproduction of cells, released of nutrients, and transfer of genetic materials. This begin with the breakdown of cell wall process by peptidoglycan hydrolase (Lewis, 2000; Ngwenya, 2007; Harvey, McNeil, Berry, and White, 1998). After harvested, however, storage life of microalgae with cell protectant can be extended at chilled temperature (Harith, Yusoff, Shariff, and Ariff, 2010).

Cell protectants e.g. glycerol, serum albumin, skimmed milk, peptone have been used in cold storage for microorganisms i.e. viruses, bacteria, fungi, algae, and protozoa. (Hubalek, 2003; Motham, 2009; Tan, Aziz, and Aroua, 2013). Among cell protectants mentioned above, glycerol has been widely used, because it has low toxicity, high solubility, no color, and no odor (Tan, Aziz, and Aroua, 2013). The glycerol mixed protein samples would enhance crystallization of proteins leading to stabilization of the protein structure (Hussels and Brecht, 2011).

Prediction of storage life and nutrient deterioration during processing and storage can be establish by the kinetics reaction model (Tiburcio, Galvez, Cruz, and Gavino, 2007; Ma, Yu, Frear, Zhao, Li, and Chen, 2013). However, there are very few reports on using kinetics model for storage life evaluation of fresh *Spirulina platensis.*

Therefore, this study aimed to evaluate storage changes and storage life of fresh *Spirulina platensis* as well as to estimate change from kinetics production.

5.3 Materials and Methods

5.3.1 Chemicals

Glycerol ($C_3H_8O_3$), Nitric acid (HNO_3), and Perchloric acid ($HClO_4$) were from Quality Research Chemical (New Zealand), Merck KGaA (Darmstadt, Germany), and Mallinckrodt Baker Inc. (Phillipsburg, NJ, USA), respectively. Standard Zinc was from Fisher Scientific Co. (Fair Lawn, NJ, USA).

5.3.2 Fresh *Spirulina platensis*

The *Spirulina platensis* starter was cultured as described in Chapter III (section 3.3.2), and then grow in 200 mL culture tube containing Zarrouk's medium fortified with 1.6 µmol Zn of $ZnSO_4$ (MZS) at 30 ± 2°C for 8 days (starting O.D. = 0.2). Then, *Spirulina platensis* cells were filtered (nylon filter pore size 35 µm, Nylon mesh 140-T, Swiss Nybolt) and washed with 20 mL distilled water for 3 times to remove growth medium.

5.3.3 Samples preparation

Fresh *Spirulina platensis* were used to evaluate the stability and shelf life evaluation. Three grams of washed *Spirulina platensis* cells were packed in a low density polypropylene (LDPE) zip lock bag size 9×13 cm. with addition of glycerol of 5 and 10%. The samples were kept out of daylight at 4, 20, and 30°C for 14 days. Samples were collected and filtered using Whatman, Grade 1 (Sigma-Aldrich Co.; St. Louis, MO, USA) every 2 days for protein and Zn analyze. All samples were evaluated in duplicate.

5.3.4 Determination of protein and Zn

Protein (Protein, %) was calculated as nitrogen in samples using modified Kjeldahl analysis (AOAC, 2000; Lopez, Garcia, Fernandez, Bustos, Chisti, and Sevilla, 2010) and expressed as protein percentage on dry basis.

Zn contents were determined using Atomic Absorption Spectrometry (AAS) as described in Chapter III (section 3.3.4). The results were expressed as μg/g (dry weight). All of the samples were evaluated in duplicate.

5.3.5 Prediction model from kinetics reaction

A general form of zero-order and first order reaction kinetic models was derived from kinetic chemical reactions (Carabasa-Giribet, and Ibarz- Ribas, 2000; Singh, 2000; Trifiro, Gherardi, Belloli, Saccani, and Aldini, 1990; Avila and Silva, 1999; Wang, Ni, Hu, Wu, Liao, Chen, and Wang, 2007) as the following equations:

Zero-order reaction:

$$C_i = C_0 - kt$$

First-order reaction:

$$C_i = C_0 \exp(-kt)$$

Where; C_i = concentration at time *i*

C_0 = initial concentration

k = the concentration rate constant

t = the time

5.3.6 Model performance

Accuracy factor (Af) and bias factor (Bf) were used as a quantitative method to measure the performance of the models by comparing predicted and observed values (Ross,1996; Zhong, Chen, Wang, Wu, Liao, and Hu, 2005; Wang et al., 2007; Bruckner, Albrecht, Petersen, and Kreyenschmidt, 2013).

The fitted models were compared by root mean square error (RMSE) value (Ross, 1996; Wang et al., 2007). These models were defined as the following equations:

$$\mathrm{Af} = 10^{\left(\Sigma\frac{|\log(\mathrm{predicted}_i/\mathrm{observed}_i)|}{\mathrm{n}}\right)}$$

$$\mathrm{Bf} = 10^{\Sigma\frac{\log(\mathrm{predicted}_i/\mathrm{observed}_i)}{\mathrm{n}}}$$

$$\mathrm{RMSE} = \sqrt{\frac{\Sigma(\mathrm{predicted}_i - \mathrm{observed}_i)^2}{\mathrm{n}-1}}$$

Where; $\mathrm{predicted}_i$ = predicted values at time *i*

$\mathrm{observed}_i$ = observed values at time *i*

n = number of observations

5.3.7 Statistical Analysis

All experiments were performed at least in duplicate. Analysis of variance (ANOVA) with confidence level of 95% (p<0.05) was performed on the obtained data.

5.4 Results and discussion

5.4.1 Changes of fresh *Spirulina platensis*

Fresh *Spirulina platensis* were packed in LDPE zip lock bags with 0, 5, and 10 % glycerol and kept at 4, 20, and 30ºC for 14 days. At 4ºC fresh *Spirulina platensis* highest protein contents (p<0.05). The protein concentrations were gradually decreased during storage. Protein contents of *Spirulina platensis* stored with reduced from

about 62% to 0, 5 and 10% glycerol at 4ºC of 58.88, 60.89, and 60.58%, respectively after 14 days. At 5 and 10% glycerol changes in protein contents were not significantly different (Figure 5.1a) for all storage temperatures. Decreased in protein contents were more pronounced at high temperature storage. Cell samples with no glycerol showed highest decreased in protein contents in all temperature storages. This clearly emphasized the role of glycerol as a good cell protectant. *Spirulina platensis* cells stored at 20 ºC for 14 days had protein contents of about 54% with glycerol and 52.8% without glycerol (Figure 5.1b) whereas the cells stored at 30ºC had protein about 47% with glycerol and 44.2% without glycerol (Figure 5.1c). Using Japanese standard for protein content in Spirulina of >50%, fresh cell would have storage life longer than 14 days if they were kept below 20ºC, but only 10 days without glycerol and 12 days with glycerol at 30ºC.

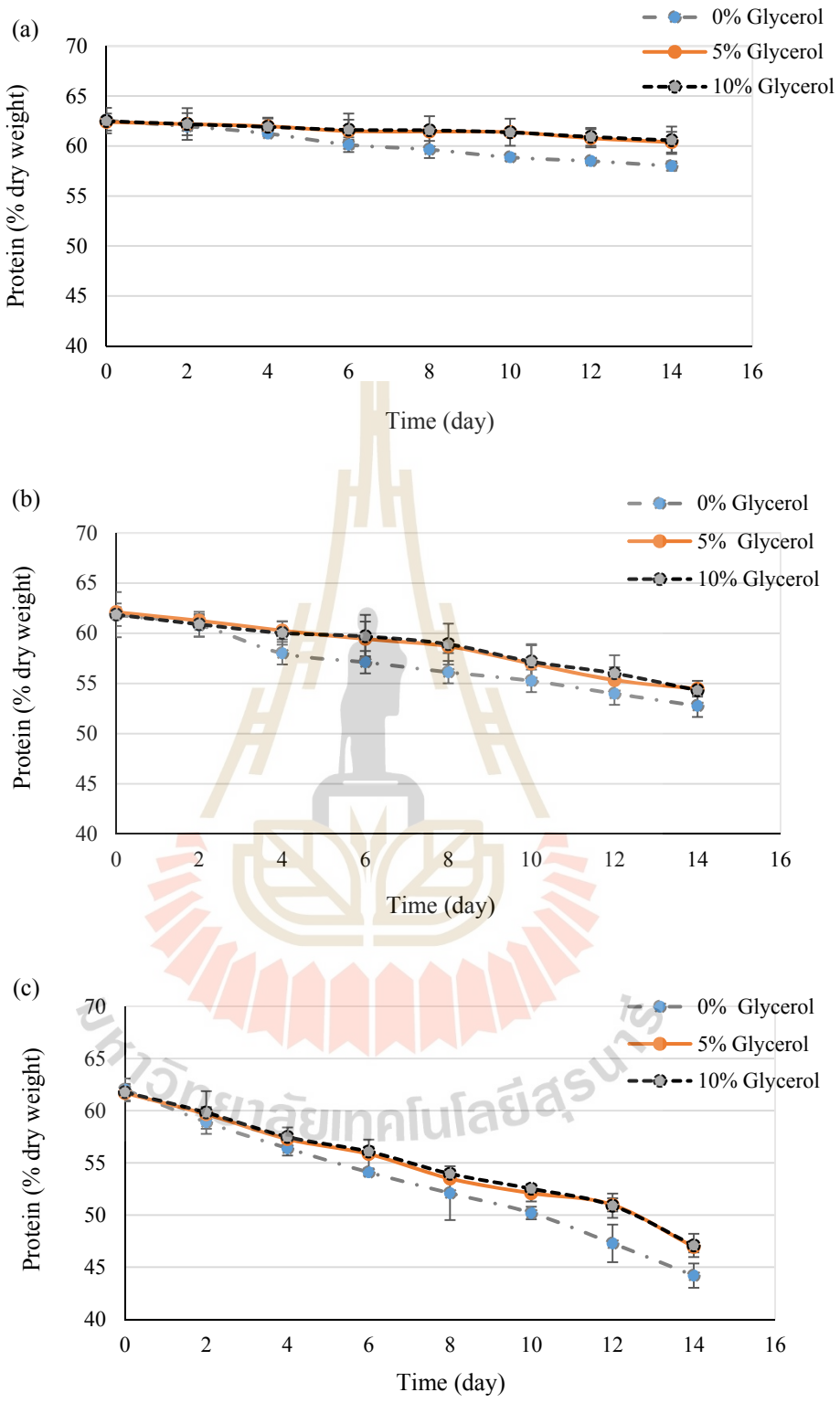

Figure 5.1 Protein contents of fresh *Spirulina platensis* with 0, 5, and 10% glycerol stored at 4ºC (a), 20ºC (b), and 30ºC (c).

Similarly, Zn contents of fresh *Spirulina platensis* store at 4ºC was highest during storage for 14 days. Zn contents decreased grater at higher storage temperature. Glycerol addition at 5 and 10% glycerol gave no significant changes in Zn contents of every storage temperature. The content of Zn remained after 14 days at about 83 µg/g dry weight with glycerol and 80 µg/g dry weight without glycerol (Figure 5.2a). The storage of fresh *Spirulina platensis* at 20ºC for 14 days showed Zn contents at about 72-73 µg/g dry weight with glycerol and 68.5 µg/g dry weight without glycerol (Figure 5.2b). The lowest Zn content (p<0.05) was found in the fresh *Spirulina platensis* samples stored at 30 ºC for 14 days without the 0% glycerol at 51.21 µg/g dry weight and at about 56. µg/g dry weight with glycerol (Figure 5.2c).

Decrease in protein and Zn contents were due to cell autolysis of fresh *Spirulina platensis* resulted from the action of lytic enzymes especially peptidoglycan hydrolase (Lahoz, Reyes, and Leblic, 1976; Harvey, McNeil, Berry, and White, 1998).

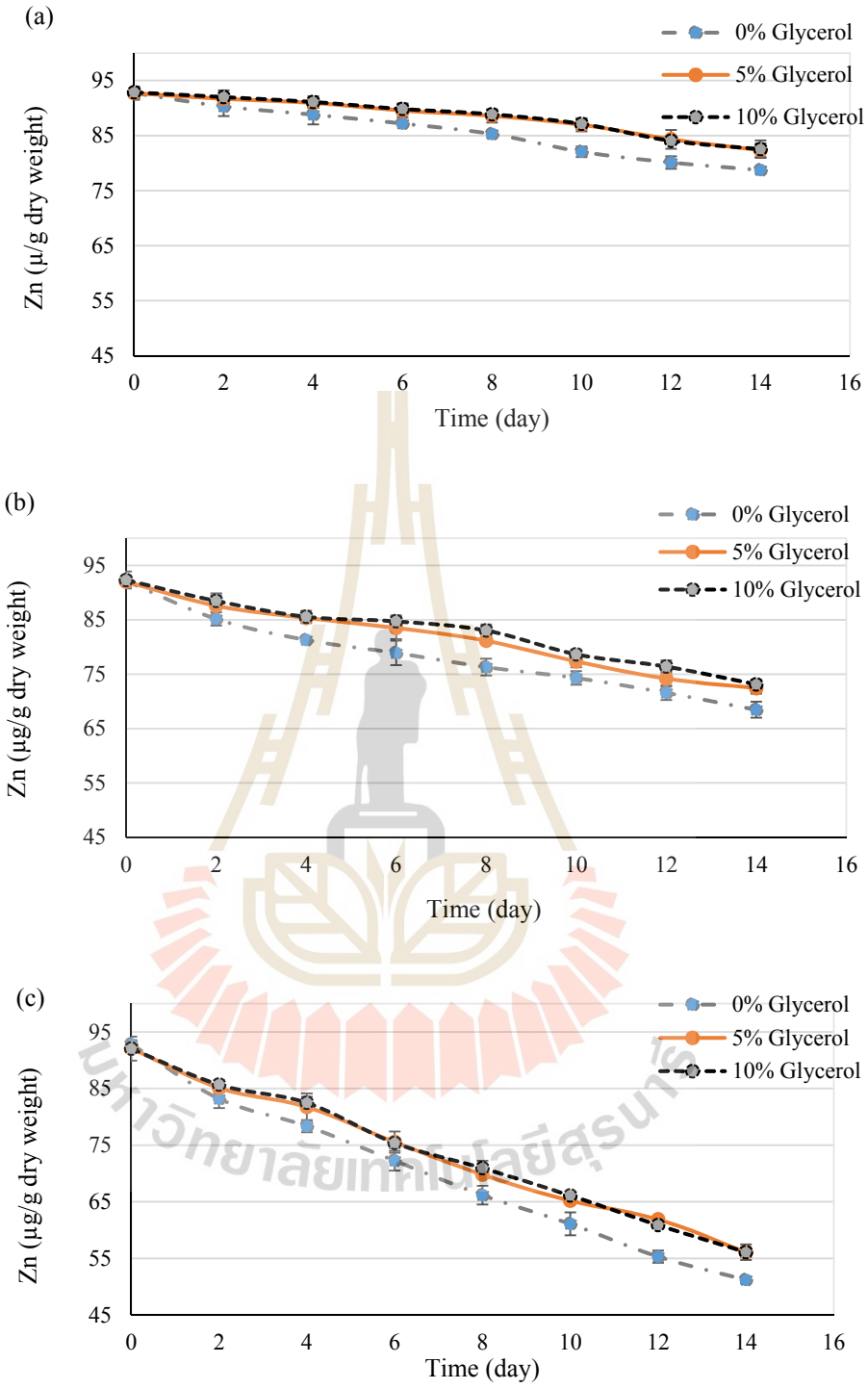

Figure 5.2 Zn contents of fresh *Spirulina platensis* with 0, 5, and 10% glycerol stored at 4ºC (a), 20ºC (b), and 30ºC (c).

Among storage life of different temperatures of this study, storage at 4ºC best extended storage of fresh *Spirulina platensis* with high remaining protein. Low temperature is the simple way for retaining the cell quality. The metabolic process, oxidative denature of vitamins and unsaturated fatty acids, autolysis, and microbial degradation are reduced at low temperature condition while the cell viability is maintained (Harith et al., 2010; Heasman, Sushames, Diemar, O'Connor, and Foulkes, 2001).

5.4.2 Kinetics reaction life prediction from fresh *Spirulina platensis*

Data of decreased protein and Zn contents were used to fit the kinetics reaction model. The zero-order and first-order kinetics reactions were considered to predict the storage life of fresh *Spirulina platensis* (See Appendix C). The correlation between the observed and predicted data obtained from the zero-order and first-order reaction models were constructed for protein (Figure 5.4) and Zn (Figure 5.5). The results showed that the fitted model was determined by regression values. The regression values of zero- and first-order reaction seem to be fitted both of the reactions. Previous researchers reported the degradation gamma linolenic acid in sun-dried *Spirulina platensis* at 45 and 55ºC followed first-order reaction (Tiburcio, Galvez, Cruz, Gavino, 2007) as well as the thermal degradation kinetics of phycocyanin extract from *Spirulina platensis* at 50-55ºC which related to first-order reaction (Antelo, Costa, and Kalil, 2008). Therefore, the model performance was used to evaluate the best fitted of zero- and first-order reactions. The accuracy factor (Af), bias factor (Bf), and root mean square error (RMSE) values of protein and Zn were compared (Table 5.1). The model could provide a better fit when the Af and Bf of performance model closed to 1 and a lower values of RMSE was obtained (Ross, 1996; Devlieghere, Belle, and Debevere,

1999; Wang et al., 2007; Bruckner, Albrecht, Petersen, and Kreyenschmidt, 2013). In this study, the kinetic reaction of protein was fitted zero-order reaction which Af, Bf, and RMSE of 1.0001, 0.9999, and 0.0419, respectively. Similarly, the kinetic reaction of Zn was also fitted zero-order reaction that Af, Bf, and RMSE values were 1.0000, 10000, and 0.034. Thus, the zero-order reaction was selected for this study. The zero-order reaction linear regression of protein at 4ºC with 5% glycerol was expressed as the equation; $Y = -0.1469X + 0.0843$. Zn at 4ºC with 5% glycerol was expressed as the equation; $Y = -0.7829X + 1.34$. According to the equations, the storage life of fresh *Spirulina platensis* with 5% glycerol was predicted to last about 50 days with the 55% protein (Base on quality standard of *Spirulina platensis*), and 54.89 µg/g dry weight Zn remained at the end of 50 days. The storage life of fresh *Spirulina platensis* at 4ºC without glycerol as a protectant would be about 22 days, and 70.34 54.89 µg/g dry weight Zn.

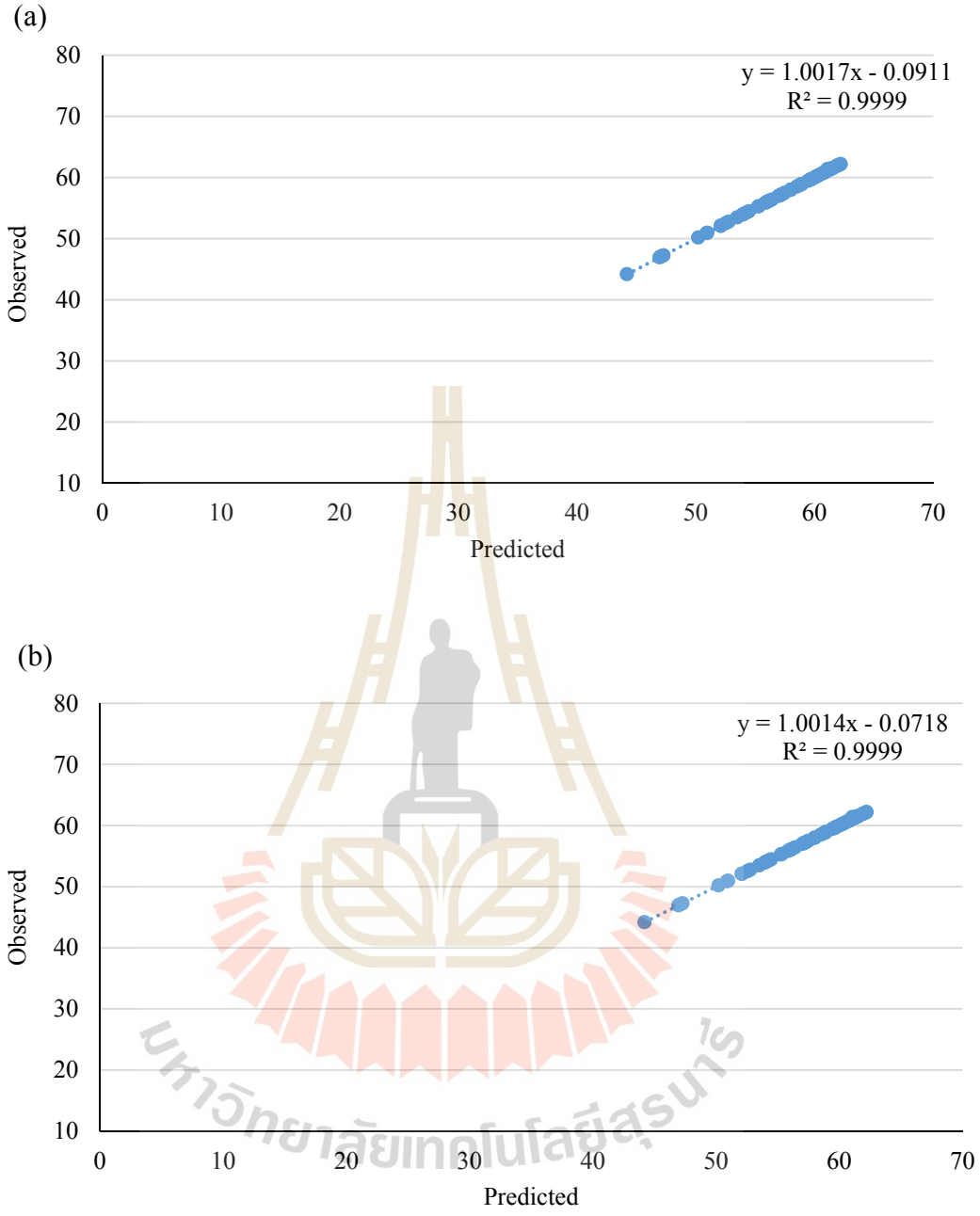

Figure 5.3 Correlation between the observed and the predicted data of protein contents in *Spirulina platensis* obtained with the zero-order reaction (a) and first-order reaction (b).

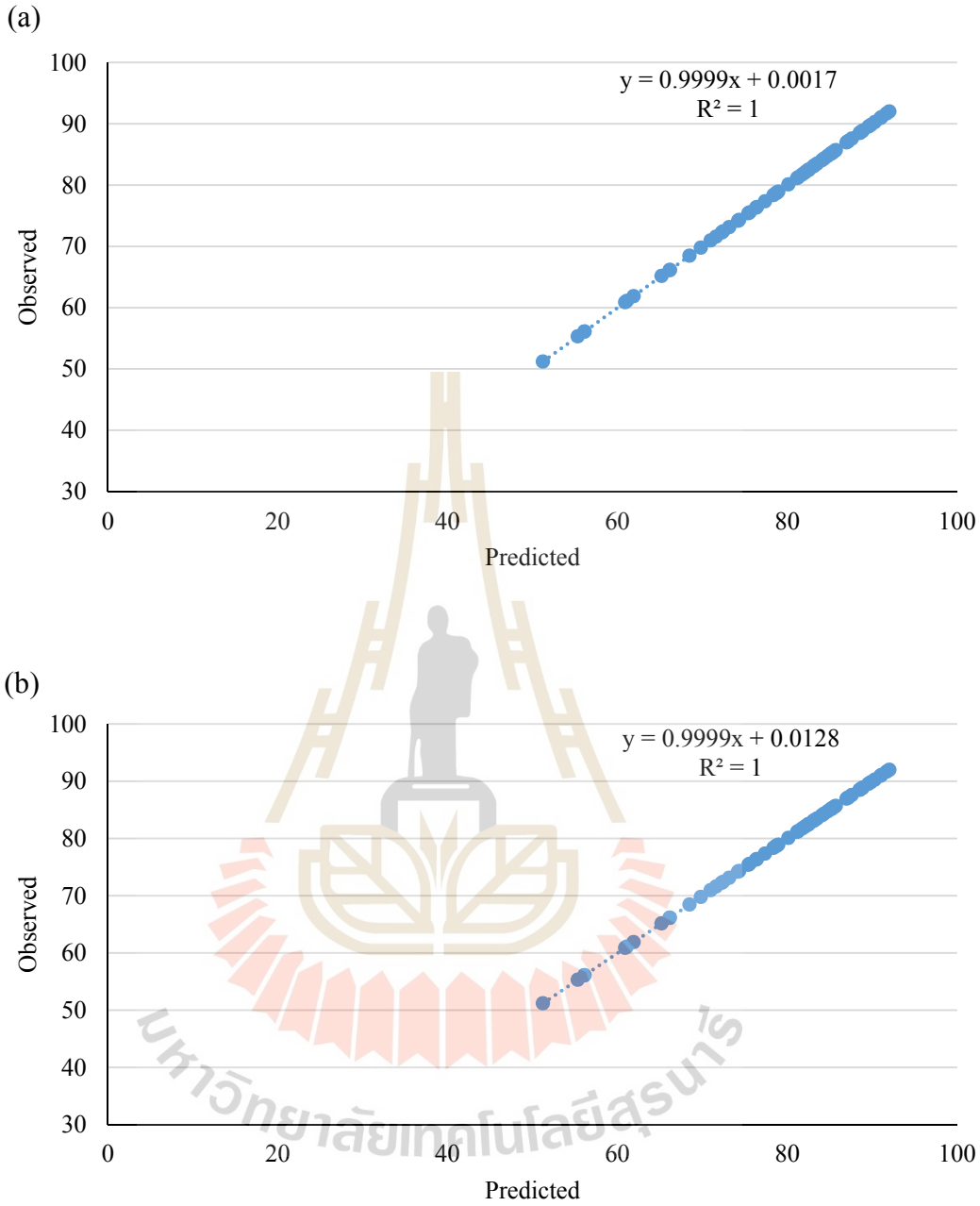

Figure 5.4 Correlation between the observed and the predicted data of Zn contents in *Spirulina platensis* obtained with the zero-order reaction (a) and first-order reaction (b).

Table 5.1 Model performance of Af, Bf, and RMSE values of zero-order and first-order reaction order for protein and Zn contents.

Model	**Protein**		**Zn**	
performance	**Zero-order**	**First-order**	**Zero-order**	**First-order**
Af	1.0001	1.0002	1.0000	1.0001
Bf	0.9999	0.9998	1.0000	1.0000
RMSE	0.0419	0.0468	0.0034	0.0054

5.5 Conclusions

Using glycerol as a cell protectant retarded these changes with statistically similar results between 5 and 10% application. Therefore, storage of fresh *Spirulina platensis* was best at 4ºC with 5% glycerol addition providing high protein and Zn content retention. Storage at higher temperature increased greater losses protein and Zn contents. The zero-order reaction was fitted the kinetic reaction of storage changes for fresh *Spirulina platensis*. The storage life of fresh *Spirulina platensis* was predicted to be about 50 days with the 55% protein, and 54.89 μg/g dry weight Zn.

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CHAPTER VI

SUMMARY

Zn uptake performance of *Spirulina platensis* was studied using different Zn salts media which include Zarrouk's medium (ZM), non-Zn Zarrouk's (NZM), modified Zarrouk's medium with zinc sulfate (MZS), zinc nitrate (MZN), and zinc chloride (MZC). *Spirulina platensis* cultured in 1.6 µmol Zn of MZS showed the highest Zn concentration of 69.55±2.70 µg/g dry weight with protein concentration of 63.11±1.40% dry weight. It also shows specific growth rate (µ) of 0.46 /day, cell productivity (P_x) of 0.55 g/L.day dry weight, and doubling time (t_d) of 1.50 days. Zn uptake was higher in cytoplasm 72.8% (60.50 µg/g dry weight) than that in cell walls 27.2% (22.62 µg/g dry weight). Decreased FT-IR spectra band intensity of the amide groups after Zn uptake at high concentrations suggested that the amide I and amide II groups played a crucial role in the binding of Zn^{2+}.

Zn accessibility and availability were evaluated using *in vitro* digestion. The results revealed that 2 h digestion of modified Zarrouk's medium (SPM) had the highest accessibility compared with Zarrouk's medium (SPZ) and commercial sample (SPC) which was 55.14±0.61 µg Zn/g dry weight (55.20±0.57%) in the simulated gastric and 62.30±0.18 µg Zn/g dry weight (63.55±0.21%) in the small intestinal simulation, respectively. The highest Zn availability of SPM after small intestinal digestion was at 2 h digestion with 34.63±0.95% (33.96±0.93 µg/g dry weight).

The storage life of fresh *Spirulina platensis* was studied using various glycerol concentration (0, 5, and 10%) and temperature (4, 20, and 30°C). Storage at the higher temperature increased greater losses of protein and Zn contents. The results showed that protein and Zn contents of the *Spirulina platensis* storage at 5% glycerol and 4ºC were maintained at the highest contents at 60.89% and 83.34 µg/g dry weight, respectively. The zero-order reaction well fitted with storage changes of fresh *Spirulina platensis*. The prediction of storage life at 4°C with 5% glycerol, providing 55% protein, was about 50 days with 54.89 µg/g dry weight Zn.

APPENDICES

APPENDIX A

ZARROUK'S MEDIUM PREPARATION

Zarrouk's medium 1 liter contains chemicals composition as the following:

Table A1 The chemicals composition of Zarrouk's medium.

Chemicals	Concentrations (g/L)
$NaHCO_3$	16.8
$NaNO_3$	2.50
K_2HPO_4	0.50
K_2SO_4	1.00
NaCl	1.00
$MgSO_4.7H_2O$	0.20
$CaCl_{2.}7H_2O$	0.04
EDTA	0.08
$FeSO_4.7H_2O$	0.01
A5	1 mL/L
B6	1 mL/L

Table A2 The chemicals composition of A5.

Chemicals	Concentrations (g/L)
H_3BO_3	2.86
$MnCl_2 \cdot 4H_2O$	1.81
$ZnSO_4.7H_2O$	0.22
$Cu_2SO_4.5H_2O$	0.08
MoO_3	0.01

Table A3 The chemicals composition of B6.

Chemicals	Concentrations (mg/L)
NH_4VO_3	22.96
$K_2Cr(SO_4).24H_2O$	96.00
$NiSO_4.7H_2O$	47.80
$NaWO_4.2H_2O$	17.94
$Co(NO_3)_2.6H_2O$	43.98

APPENDIX B

STANDARD CURVE OF *SPIRULINA PLATENSIS*

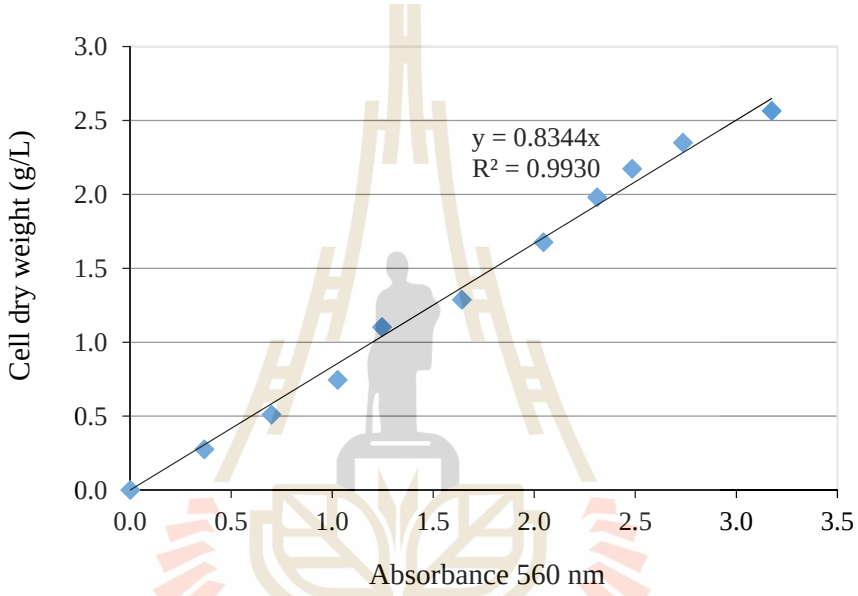

Figure B1 Standard curve of *Spirulina platensis* cell dry weight.

APPENDIX C

KINETICES REACTION MODEL OF FRESH *SPIRULINA PLATENSIS*

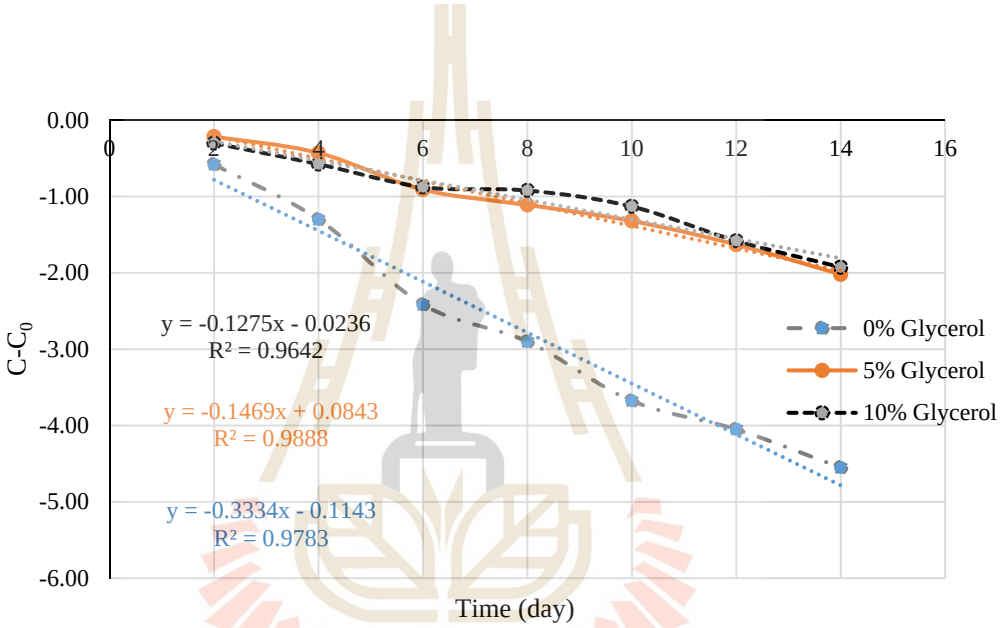

Figure C1 The plot of zero-order reaction of protein concentrations of fresh *Spirulina platensis* with various glycerol concentrations at 4ºC.

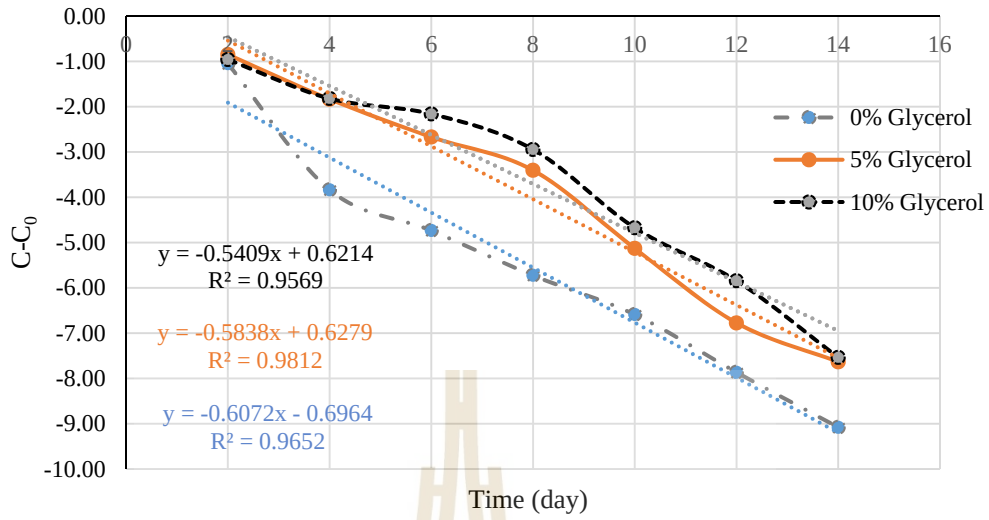

Figure C2 The plot of zero-order reaction of protein concentrations of fresh *Spirulina platensis* with various glycerol concentrations at 20ºC.

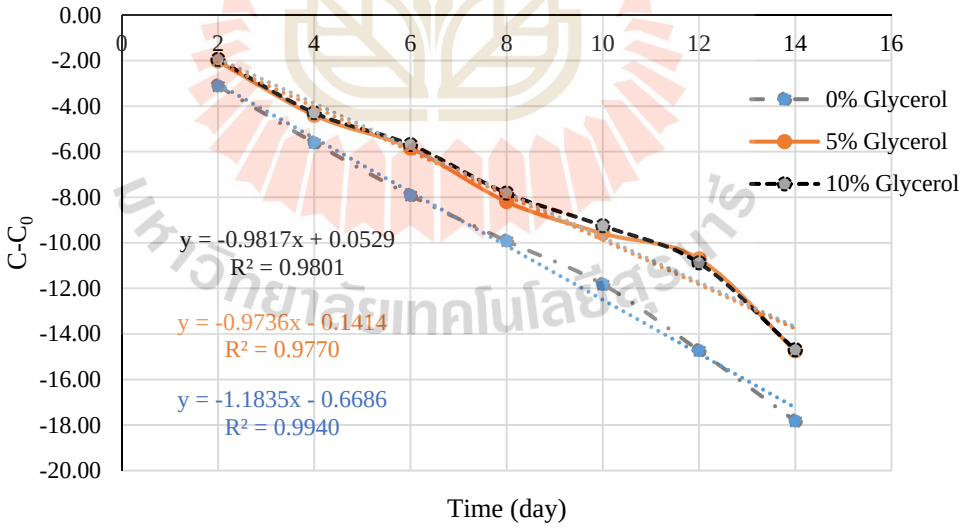

Figure C3 The plot of zero-order reaction of protein concentrations of fresh *Spirulina platensis* with various glycerol concentrations at 30ºC.

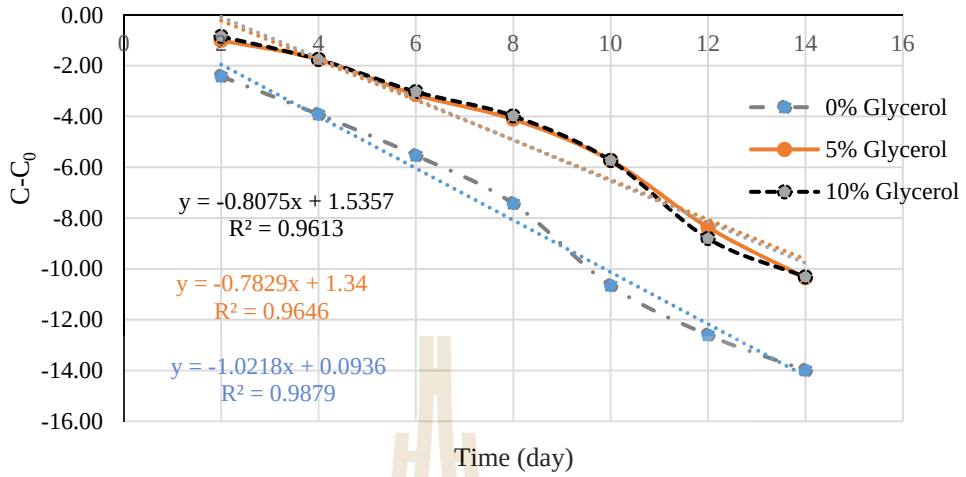

Figure C4 The plot of zero-order reaction of zinc concentrations of fresh *Spirulina platensis* with various glycerol concentrations at 4ºC.

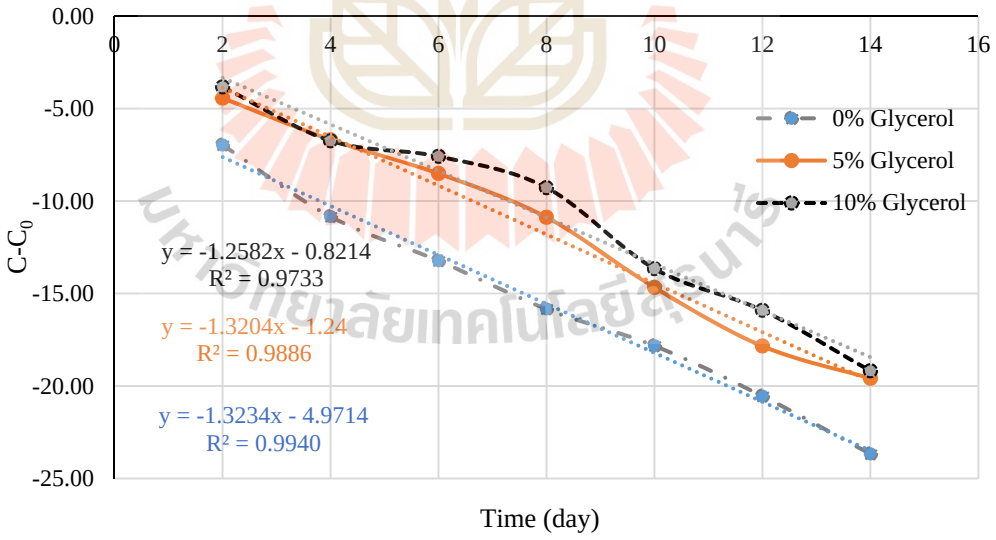

Figure C5 The plot of zero-order reaction of zinc concentrations of fresh *Spirulina platensis* with various glycerol concentrations at 20ºC.

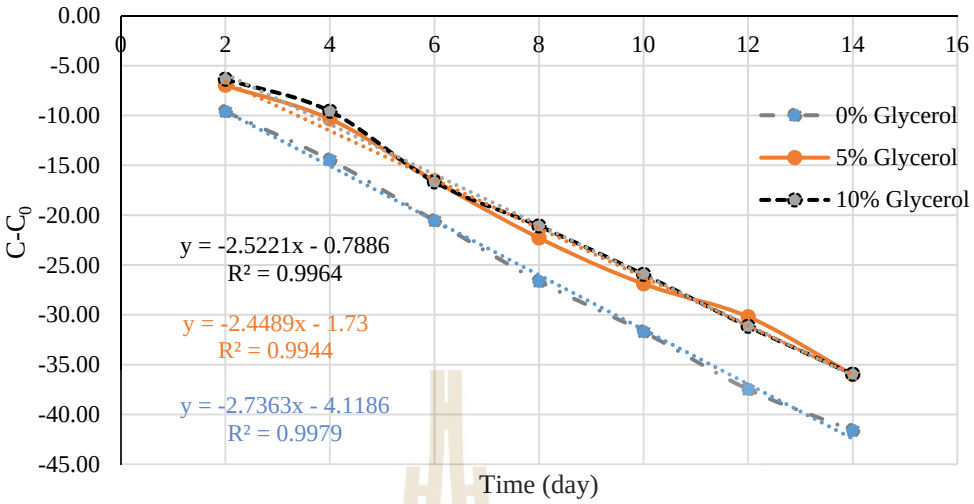

Figure C6 The plot of zero-order reaction of zinc concentrations of fresh *Spirulina platensis* with various glycerol concentrations at 30ºC.

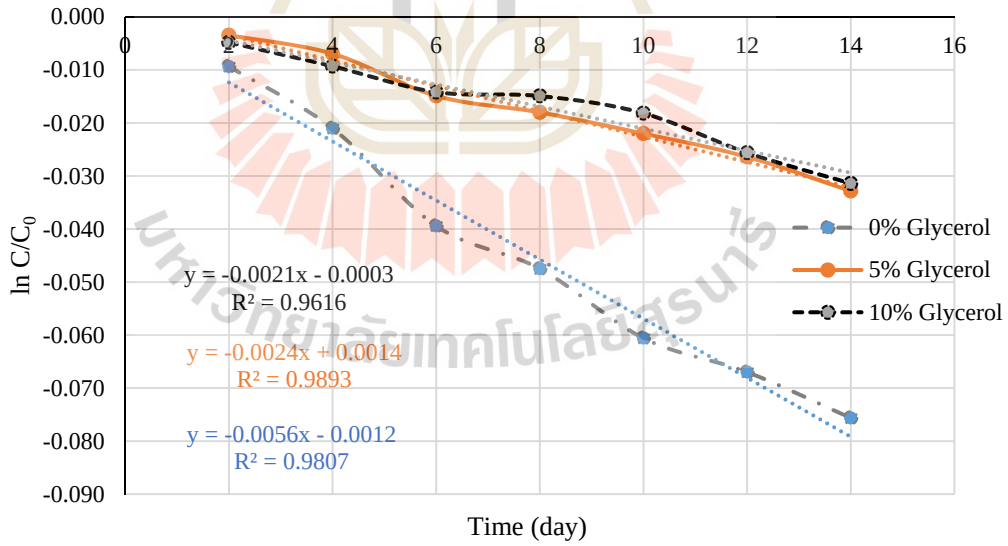

Figure C7 The plot of first-order reaction of protein concentrations of fresh *Spirulina platensis* with various glycerol concentrations at 4ºC.

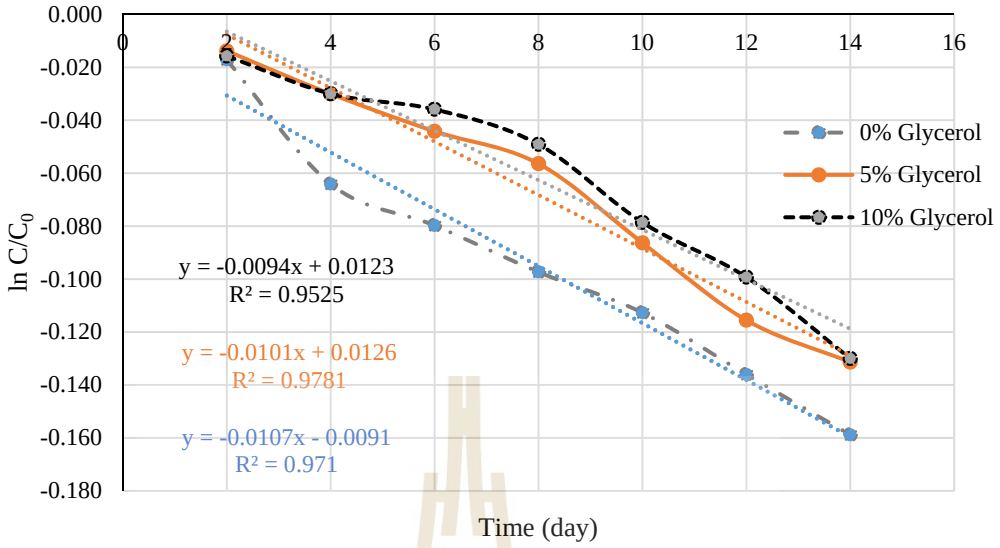

Figure C8 The plot of first-order reaction of protein concentrations of fresh *Spirulina platensis* with various glycerol concentrations at 20ºC.

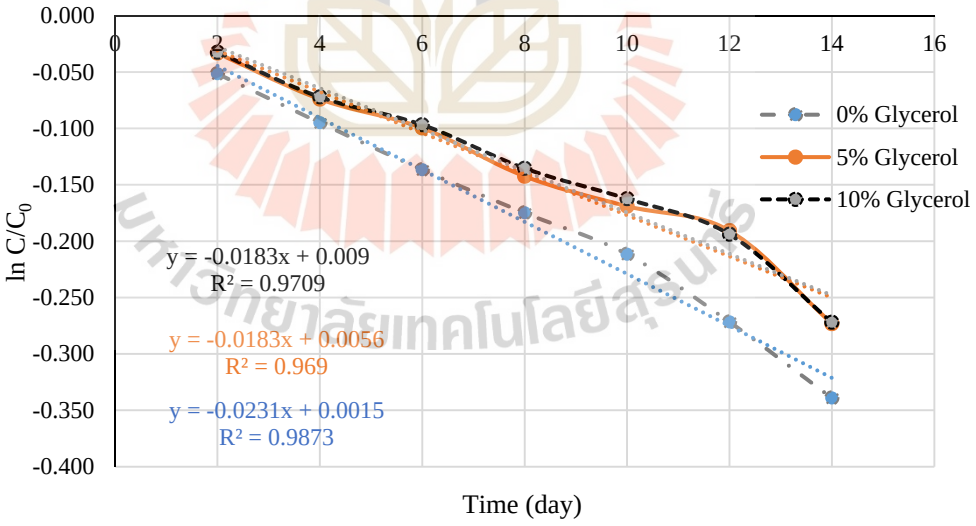

Figure C9 The plot of first-order reaction of protein concentrations of fresh *Spirulina platensis* with various glycerol concentrations at 30ºC.

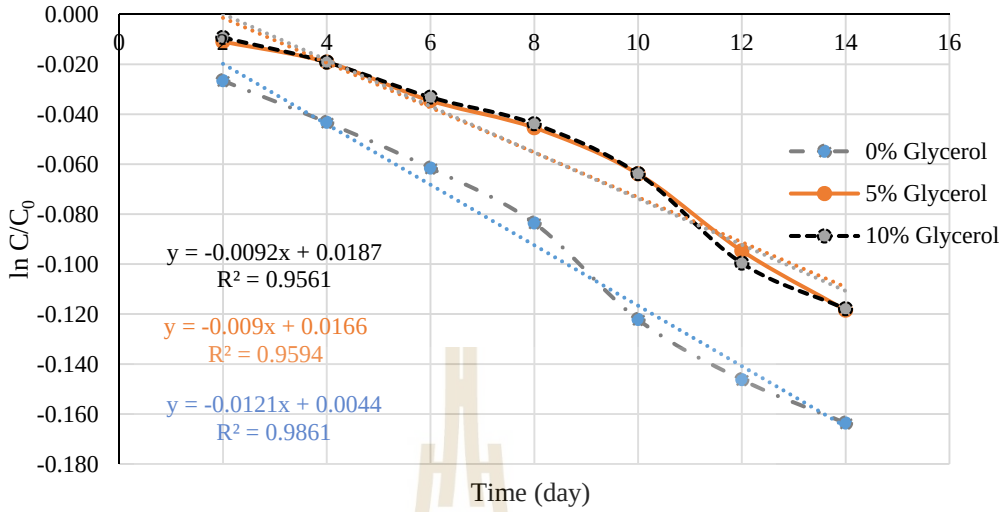

Figure C10 The plot of first-order reaction of zinc concentrations of fresh *Spirulina platensis* with various glycerol concentrations at 4ºC.

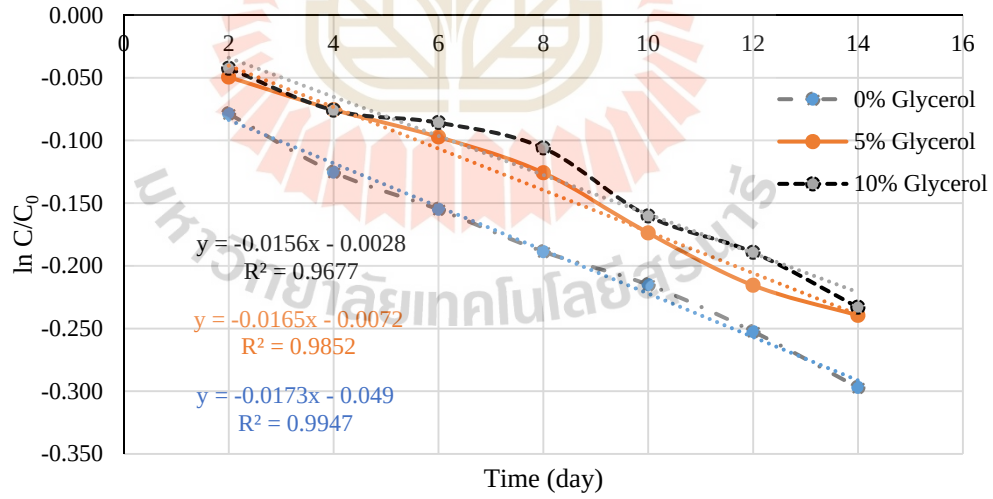

Figure C11 The plot of first-order reaction of zinc concentrations of fresh *Spirulina platensis* with various glycerol concentrations at 20ºC.

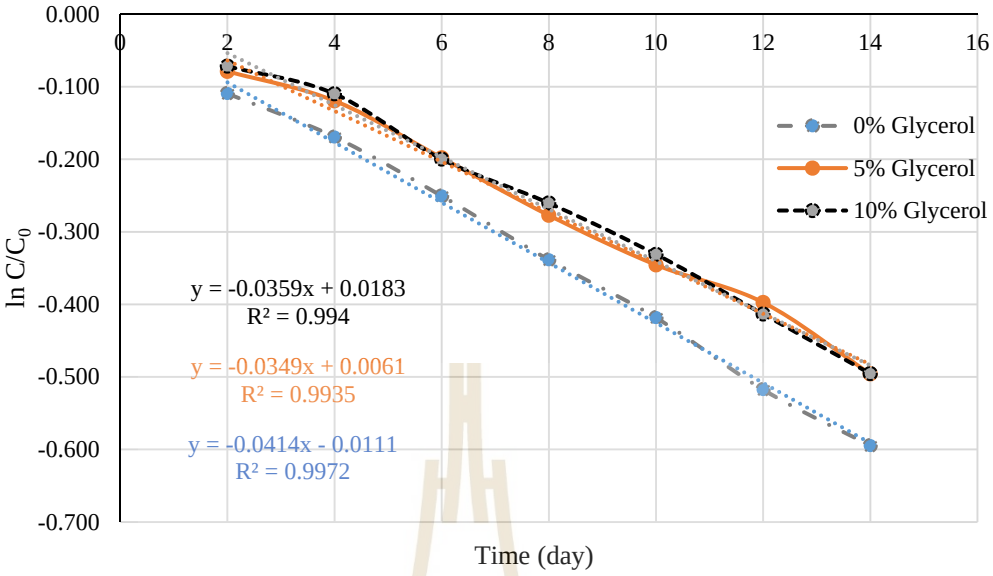

Figure C12 The plot of first-order reaction of zinc concentrations of fresh *Spirulina platensis* with various glycerol concentrations at 30ºC.

APPENDIX D

EXTRACTION TIMES OF *SPIRULINA PLATENSIS*

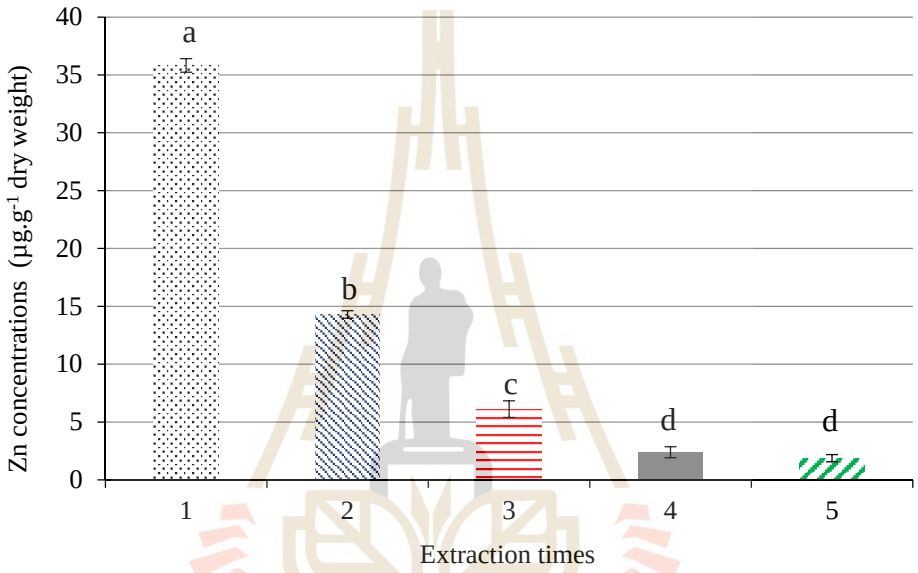

Figure D1 Zn in *Spirulina platensis* cytoplasm (supernatant) with extraction times. Different letters indicate the significant differences (p<0.05).

The concentration of Zn in each extraction time was the first extraction of 35.82 µg/g dry weight, the second extraction of 14.30 µg/g dry weight, the third extraction of 6.11 µg/g dry weight, the fourth of 2.39 µg/g dry weight, and the fifth extraction of 1.88 µg/g dry weight as illustrated in Figure D1. The extraction time results did not show a significant difference between the fourth and the fifth times.

BIOGRAPHY

Arthit Jatupornpongchai was born in Bangkok, Thailand. In 1999, he graduated a Bachelor's Degree of Science (Food Science and Technology) at Rajamangala Institute of Technology, Nakhon Si Thammarat, Thailand, and in 2004 he graduated a Master's Degree of Engineering (Chemical Engineering) at Prince of Songkla University, Songkhla, Thailand. Furthermore, in 2008, he received the One Research One Graduated student (OROG) scholarship from Institute of Research and Development, Suranaree University of Technology to study the Doctorate degree in Philosophy (Food Technology) at Suranaree University of Technology, Nakhon Ratchasima, Thailand.