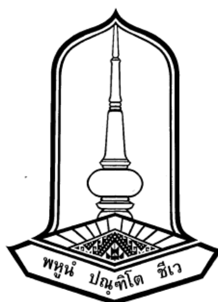

SELENIUM-ENRICHED PROBIOTIC BACTERIA AND APPLICATION OF HOME-MADE ELECTRODIALYSIS APPARATUS FOR SELENIUM SEPARATION

WITCHAPOL THOSAIKHAM

A dissertation submitted in partial fulfillment of the requirements for
the degree of Doctor of Philosophy in Chemistry
at Mahasarakham University
February 2016

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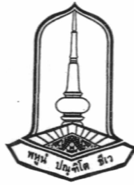

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Witphon Thosaikham

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TITLE Selenium-enriched probiotic bacteria and application of home-made electrodialysis apparatus for selenium separation

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ABSTRACT

The *L.casei subsp. rhamnosus* (TISTR No. 372) and *L. bulgaricus* (TISTR No. 451) strains were selected for producing Se-enriched probiotics. It could be observed that the *L. bulgaricus* strain could absorb and accumulate SeO_3^{2-} ion more than the *L. rhamnosus* strain. However, the ability of *L.casei subsp. rhamnosus* strain for transforming SeO_3^{2-} ion to be organic Se compounds was better than the *L.bulgaricus* strain. Therefore, the *L. rhamnosus* strain was selected to further study.

The conditions of Se-enriched *L.casei subsp. rhamnosus* cultivation, Se-supplemented concentration and initiation time for adding SeO_3^{2-} ion into the culture were optimized. The cultivation of Se-enriched *L.casei subsp. rhamnosus* was achieved by uptake 2.5 mg Se L^{-1} in the form of SeO_3^{2-} into the culture medium at the lag phase of growth curve. *L.casei subsp. rhamnosus* could absorb SeO_3^{2-} ion, and synthesized to be Sec as a primary selenoamino acid though the biosynthetic pathway. Normally, Sec is incorporated in to peptide chain as selenoproteins, and accumulated in each part of *Lactobacillus* cells. However, the finding of this work indicated that the partial of Sec was also exported to the extracellular environment in the form of free amino acid. Moreover, *L.casei subsp. rhamnosus* would rapidly detoxify excess SeO_3^{2-} ion by reducing oxidation state of Se^{4+} to be elemental selenium (Se^0) when Se-supplemented concentration in MRS broth was greater than or equal to 4 mg Se L^{-1}. This detoxification of Se was also occurred when SeO_3^{2-} ion was added at the starting point of culture, during the range of log phase and the end point of log phase.

A newly designed home-made electrodialysis (ED) was developed and applied to separating Se compounds from remained-medium of Se-enriched probiotic cultivation. The home-made ED apparatus was designed and constructed based on

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separation efficiency of selenium species, invention cost, suitable material, easy operation and versatile application. It consisted of 2 main parts; ED stack and power supply system. The home-made ED apparatus was achieved for using to remove SeO_3^{2-} ion from the remained-medium. The optimum applied voltage for driving the SeO_3^{2-} ion from the sample unit to the anode unit was 100 volt. It could remove 50 % SeO_3^{2-} ion from the remained-medium within 15 min.

Key words: electrodialysis; Se speciation; Se-enriched probiotics

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ชื่อเรื่อง แบคทีเรียโปรไบโอติกที่เสริมซีลีเนียมและการประยุกต์ใช้เครื่องอิเล็กโทรไดอะ-ลิซิสที่ประดิษฐ์ขึ้นสำหรับแยกซีลีเนียม
ผู้วิจัย นายวิชญ์พล โถสายคำ
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บทคัดย่อ

ได้เลือกเชื้อแบคทีเรียแลคโตบาซิลลัสแรมโนซัส (TISTR No. 372) และเชื้อแบคทีเรียแลคโตบาซิลลัสบัลการิคัส (TISTR No. 451) เพื่อผลิตโปรไบติกส์เสริมซีลีเนียม พบว่า เชื้อแบคทีเรียแลคโตบาซิลลัส บัลการิคัส สามารถดูดซึมและสะสมซีลีไนท์ไอออน ได้มากกว่าเชื้อแบคทีเรียแลคโตบาซิลลัส แรมโนซัส อย่างไรก็ตาม เชื้อแบคทีเรียแลคโตบาซิลลัสแรมโนซัส สามารถเปลี่ยนซีลีไนท์ไอออนให้เป็นสารประกอบซีลีเนียมอินทรีย์ได้ดีกว่าเชื้อแบคทีเรียแลคโตบาซิลลัสบัลการิคัส ดังนั้น งานวิจัยนี้จึงเลือกเชื้อแบคทีเรียแลคโตบาซิลลัสแรมโนซัส เพื่อศึกษาการทดลองต่อไป ได้หาสภาวะที่เหมาะสมของระดับความเข้มข้นของซีลีเนียมที่เสริม และเวลาที่เหมาะสมในการเสริมซีลีไนท์ไอออนให้กับเชื้อแบคทีเรียแลคโตบาซิลลัส แรมโนซัส ซึ่งพบว่า สภาวะที่เหมาะสม คือ เสริมซีลีไนท์ไอออนที่ระดับความเข้มข้น 2.5 มิลลิกรัมต่อลิตร เข้าไปในช่วงแล็กเฟส (lag phase) ของกราฟการเจริญของเชื้อแบคทีเรียแลคโตบาซิลลัส แรมโนซัส ซึ่งซีลีไนท์ไอออนจะถูกดูดซึมเข้าไปในเซลล์ของเชื้อแบคทีเรียแลคโตบาซิลลัสแรมโนซัส และสังเคราะห์เป็นซีลีโนซิสเตอีน โดยปกติซีลีโนซิสเตอีน จะเข้ารวมกับสายเปิ๊ปไทด์เป็นซีลีโนโปรตีนและสะสมตามส่วนต่างๆ ของเซลส์แลคโตบาซิลลัส อย่างไรก็ตาม งานวิจัยนี้พบว่าซีลีโนซิสเตอีนบางส่วนจะถูกขับออกมานอกเซลล์ในรูปของกรดอะมิโนอิสระด้วย

นอกจากนี้ เชื้อแบคทีเรียแลคโตบาซิลลัส แรมโนซัส จะลดความเป็นพิษของซีลีไนท์ไอออนโดยการลดค่าออกซิเดชันจาก Se^{4+} ให้กลายเป็นธาตุซีลีเนียม (Se^{0}) เมื่อ มีการเสริมซีลีเนียมเข้าไปในอาหาร MRS broth มากกว่าหรือเท่ากับ 4 มิลลิกรัมต่อลิตร และยังพบว่า เชื้อแบคทีเรียแลคโตบาซิลลัสจะลดความเป็นพิษของซีลีไนท์ไอออน เมื่อมีการเสริมเข้าไปที่จุดเริ่มต้นของการเลี้ยง ระหว่างช่วงล็อกเฟส และจุดสิ้นสุดของล็อกเฟสของกราฟการเจริญของเชื้อแบคทีเรียแลคโตบาซิลลัสแรมโนซัส

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ได้พัฒนาเครื่องอิเล็กโตรไดอะลิซิสรูปแบบใหม่ สำหรับแยกสารประกอบของซีลีเนียมในน้ำเลี้ยงที่เหลือจากกระบวนการผลิตแบคทีเรียโปรไบโอติกส์เสริมซีลีเนียม ซึ่งการออกแบบและสร้างเครื่องอิเล็กโทรไดอะไซลิซิสนั้น คำนึงถึงประสิทธิภาพการแยกสารประกอบซีลีเนียม ต้นทุนการประดิษฐ์ วัสดุที่เหมาะสม ใช้งานง่ายและประยุกต์ได้อย่างเหมาะสม เครื่องอิเล็กโทรไดอะลิซิสที่ประดิษฐ์ขึ้นประกอบด้วยอุปกรณ์หลัก 2 ส่วน คือ หน่วยอิเล็กโทรไดอะลิซิส และระบบจ่ายกระแสไฟฟ้า เครื่องอิเล็กโทรไดอะไลซิสที่ประดิษฐ์ขึ้น สามารถนำมาประยุกต์ใช้กำจัดซีลีไนท์ไอออนออกจากน้ำเลี้ยงเชื้อได้ โดยใช้ค่าความต่างศักย์ที่เหมาะสมคือ 100 โวลท์ ซึ่งสามารถกำจัดซีลีไนท์ไอออนออกจากน้ำเลี้ยงเชื้อได้ 50 เปอร์เซ็นต์ ภายในเวลา 15 นาที

คำสำคัญ: อิเล็กโทรไดอะลิซิส รูปแบบจำเพาะของซีลีเนียม แบคทีเรียโปรไบโอติกส์เสริมซีลีเนียม

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List of Abbreviations

BSA	1-butanesulfonic acid
cps	Count per second
DTT	Dithiothreitol
ED	Electrodialysis
HPLC-ICP-MS	High performance liquid chromatography – Inductively coupled plasma – mass spectrometry
RM	Remained-medium
Sec	Selenocysteine
SeM	Selenomethionine
SeMC	Se-methylselenocysteine

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CHAPTER 1

INTRODUCTION

1.1 Background

Selenium (Se) is a trace element and accepted as an essential nutrient for animals including human. However, its intake of high concentration per day could be toxic effect for health (Klein, 2004). The recommended intake of human is 55-70 μg/day (Burk and Levander, 2006). Moreover, the toxicity or benefit of selenium depends on the chemical form and concentration of selenium in environmental and biological systems (Brown and Arthur, 2001). Generally, the inorganic species of selenium such as selenate ion (SeO_4^{2-}) and selenite ion (SeO_3^{2-}) have high toxicity at large doses (Templeton *et al*., 2000). Meanwhile, the organic species of selenium, selenocysteine (Sec), Se-methyl selenocysteine (SeMC) and selenomethionine (SeM) are amino acid forms of selenium in organisms and have several benefits for health (Böck *et al*., 1991; Hartikainen, 2005).

Nowadays, supplementation of inorganic Se into food stuff has been increasingly interested for providing organic Se source for human. However, there are only bacteria, archaea and eukaryotes, which can produce Sec (Böck, 2000). Various genus of bacteria, *Bifidobacterium, Enterococcus, Lactobacillus and Streptococcus*, have also used for producing organic Se source for human (Yang *et al*., 2009; Zhang *et al*., 2009 ; Pieniz *et al*., 2011; Andreoni *et al*., 2000). Most kinds of bacteria, the genus *Lactobacillus* has been mostly attended because of its probiotics property and daily consumption (Pophaly *et al*., 2014).

Cultivation of Se-enriched *Lactobacillus* has been chiefly carried out by up taking selenite ion (SeO_3^{2-}) from selenite salt into culture medium. SeO_3^{2-} ion is absorbed through cell membrane of *Lactobacillus*, synthesized to be Sec as a primary seleno amino acid, and incorporated into protein through the metabolism of *Lactobacillus* (Calomme, 1998a; Calomme, 1998b). The obtained Sec-containing selenoproteins in intracellular fraction of *Lactobacillus* are aqueous soluble protein, and their molecular weight were about 30-70 kDa (Alzate *et al*., 2007; Galano *et al*., 2013).

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Even though, Se-enriched *Lactobacillus* has been intensively researched; however, it has been mainly focused on accumulation and biotransformation of Se in biomass of *Lactobacillus*. The attention of extracellular Se species in the remain-medium from the culture has been less mentioned although it is the largest part of the culture. Therefore, biotransformation and distribution of selenium in *Lactobacillus* culture needs to evidence that organic Se is only synthesized and accumulated in cells or also exported in to extracellular environment.

Electrodialysis (ED) is a process for transferring ions from aqueous solution through a semi-permeable membrane under influence of an electric potential (Goldstein and Mass, 1985). In general application of ED, it is mainly used in desalination of water (Korngold *et al*., 1978). Furthermore, the use of ED in food, drug, and chemical industries has been studied quite extensively (Strathmann, 1992). The most application of ED for selenium is elimination selenium from Se-contaminated water (Murphy and Colo, 1990). Utilization of ED for separation of Se in biological sample has not been reported, therefore this work interested to develop and apply ED for separating Se compounds from remained-medium of Se-enriched cultivation.

1.2 Purposes of the Research

The purposes of this study were as follows:

1.2.1 To develop the process of Se-enriched probiotic bacteria cultivation.

1.2.2 To study the biotransformation of selenium in Se-enriched probiotic bacteria.

1.2.3 To develop home-made electrodialysis and apply to separation of Se compounds in Se-enriched probiotic bacteria.

1.3 Hypothesis of the Research

The hypotheses of each experiments of this study were as follows:

1.3.1 Development of Se-enriched probiotic bacteria

1.3.1.1 The difference Se-supplemented concentrations should affect on biotransformation of selenium in probiotic bacteira.

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1.3.1.2 The difference initiation time for adding SeO_3^{2-} ion should affect on accumulation biotransformation of selenium in probiotic bacteria.

1.3.2 Application of ED apparatus to separation of selenium species in Se-enriched probiotic bacteria

1.3.2.1 The difference applied voltage should affect on separation of sele-nium compounds.

1.3.2.2 The difference electrodialysis time should affect on separation of selenium compounds.

1.4 Benefits of the research

The benefits of this study were obtained as follows:

1.4.1 Know the optimum conditions for Se-enriched probiotic bacteria culti-vation.

1.4.2 Know the biotransformation of selenium in Se-enriched probiotic bacteria and can apply this data to other research.

1.4.3 Obtain the novel design of ED apparatus and its optimum conditions for separation of selenium species in Se-enriched probiotic bacteria.

1.5 Scope of the research

The scope of this research was classified as 3 main parts as follows;

1.5.1 Development of Se-enriched probiotic bacteria cultivation.

The optimization of conditions for Se-enriched probiotic cultivation was followed as;

1.5.1.1 Growth rate of Se-enriched probiotic bacteria.

1.5.1.2 The effect of Se-supplemented concentrations on Se content on probiotic bacteria.

1.5.1.3 The effect of initiation time for adding SeO_3^{2-} ion on Se content in probiotic bacteria.

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1.5.2 Development of home-made ED apparatus.

1.5.3 Application of the home-made ED apparatus to Se-enriched probiotic bacteria.

1.6 Definition of Terms

Se-enriched foods: Foods are supplemented with selenium by some procedures. Thus, these foods must contain selenium more than normal food.

Selenium species or Selenium speciation: Any chemical forms of selenium occurred in environmental or biological system such as isotopes, oxidation state, inorganic compounds, organic compounds and proteins.

HPLC-ICP-MS: A hyphenated technique between high performance liquid chromatography and inductively coupled plasma - mass spectrometry using for elemental speciation analysis

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CHAPTER 2

LITERATURE REVIEWS

2.1 Selenium

Selenium is a chemical element which was discovered by a Swedish Chemist, Jöns Jakob Berzelius, in 1817. Berzelius found the unknown substance with properties like those of tellurium, while studying a method for the production of sulfuric acid (Subcommittee on Selenium, National Research Council U.S.A, 1983). He gave it the name selenium from the Greek word *selene*, which signifies the moon, while *tellus* is the name of earth (Reilley, 1996). Since selenium was discovered, it has been variously used in several industrial applications such as the photo copying process of xerography, which depended on the light sensitivity of thin films of amorphous selenium, the decolorization of glasses tinted by the present of iron compounds, and used as a pigment in plastics, paints, enamels, glass, ceramics and ink (Krebs *et al*., 1994).

The effect of selenium on health was firstly recommended as a high toxic agent when it is contaminated in environment. The toxicity of selenium was the first mentioned in the 13th century by Marco Polo who reported a problem with the damaged hooves of horses after grazing on certain plants in regions of China (Shao and Zheng, 2008). However, the toxicity of selenium was widely interested in the 1930s when it was found to cause poisoning of livestock in areas with high-selenium soil (O'Toole and Raisbeck, 1995; Burk and Levander, 2006).

The chronic selenosis is obtained by cirrhosis, lameness, hoof malformations, hair loss, and emaciation (Moxon and Rhian, 1943; Umesh *et al*., 1998). Extreme daily intakes of human as low as 1 to 3 mg per day can cause toxicity symptoms if taken for many mounts that result as hair loss, include a garlicky odor of the breath, nausea, diarrhea, fatigue and changes of fingernails toenails, rashes and cirrhosis of liver may also develop (Wardlaw, 2004).

The contrast understanding about the importance of selenium in human nutrition was firstly published in the 1950s (Wapnir, 1990). This function of selenium was expended after the biochemical basis of its essentiality was established in 1973 by

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identification of the enzyme glutathione peroxidase (GPx) and the first RDA for selenium was announced in 1989 (Stadtman, 1974; Drummond and Brefere, 2004; Watanabe, 2005). The homeostatic mechanisms appear to ensure that intake of selenium are in balance with losses over a wide range of intakes form 9 to 80 µg/day which selenium requirements depend on age and sex (Guthrie *et al*., 1995).

Deficiency of selenium associates several diseases such as Kashin-beck and Keshan disease which have been found in selenium deficiency region such as some part of China and New Zealand (Hinsenkamp, 2001). Kashin-Beck disease is an osteoarticular disorder of the wrists and fingers, elbows, knees and ankles (Nève, 1999). It is characterized by a range of bone and joint malformations that occur during childhood or pubertal growth and progress until growth ceases. Thus, the research of supplementing selenium in to food stuff for providing organic Se source for human since the late 1980s, (burk and Levander, 2006).

2.2 Biochemistry of selenium

Selenium in biological system is mostly presented in the form organic compound especially the forms of selenoamino acid and selenoproteins (Batley, 1990; B'Hymer and Caruso, 2006; Lobinski and Szpunar, 1999). Seleno amino acid is a kind of amino acid that contains selenium atom in a structure. Numerous selenoamino acids were identified in living organisms such as Se-methylselenocysteine, γ-glutamyl-Se-methyl selenocysteine and γ-glutamyl-Se-methyl selenomethionine, and selenomethionine (Chassaigne *et al*., 2000; Sugihara *et al*., 2004; Larsen *et al*., 2006; Kápolna *et al*., 2007; Cankur *et al*., 2006 and Ogra *et al*., 2009). In animal, selenocysteine (Sec) is a natural amino acid, and has been identified as the 21st essential amino acid (Behne *et al*., 1998). The chemical structure of Sec resembles cysteine (Cys) (Figure 2.1) by the position of sulfur atom is replaced with the selenium atom but Sec reacts faster than Cys (Mueller, 2009; Nauser *et al*., 2012). Moreover, Sec is also found in bacteria and archaea (Böck, 2000). Selenocysteine-containing proteins are the major selenoprotein in mammalian animals and bacteria (Behne *et al*., 1998; Klein, 2004; Allmang and Krol, 2006; Turanov, *et al*., 2011).

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The biosynthesis and specific incorporation of selenocysteine into protein in bacteria was demonstrated in Figure 2.2. The pathway of selenoprotein synthesis of bacteria requires the function of two cis and four trans elements. Sec is normally incorporated in to peptide chain as selenoproteins in response to UGA codons and Sec insertion sequence (SECIS) (Zhang and Gladyshev, 2005). The cis elements are a UGA codon determining the position of selenocysteine insertion into the nascent polypeptide and a secondary/tertiary structure within the mRNA, designated the SECIS element, following the UGA at its side (Burk, 2000).

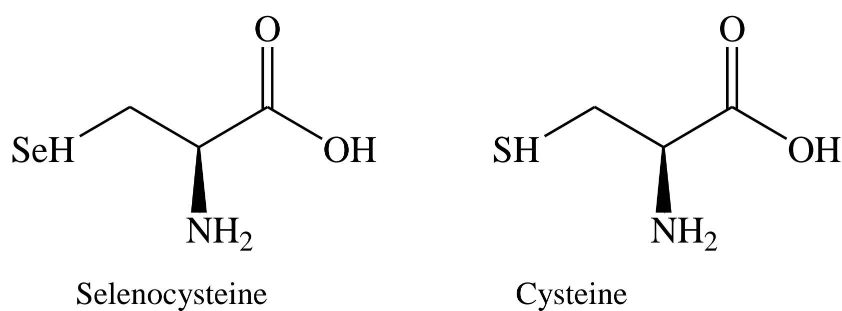

Figure 2.1 Chemical structures of selenocysteine and cysteine

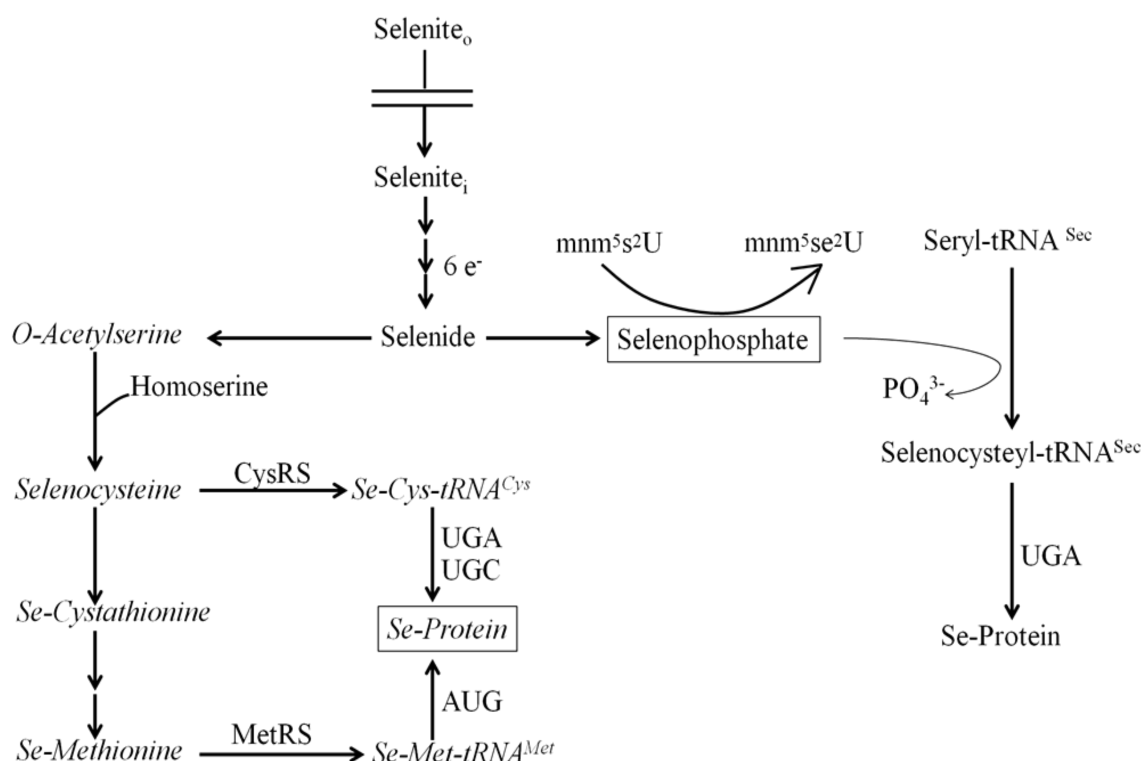

Figure 2.2 The biosynthesis of selenoprotein in bacteria (Burk, 2000)

2.3 Selenium-enriched food production

Selenium contents of foods relate to geographical area which each region has different selenium concentration in soil. Therefore, selenium contents in foods also are varied by the region (Burk and Levander, 2006). Plant foods are the major dietary sources and transfers to another living organism in food chain. Animals eat grains or plants which are grown in selenium-rich soil; they would have high levels of selenium in their muscle. In addition, people living in the high levels of selenium region have higher selenium intakes than people living in the lower levels of selenium region (Drummond and Brelere, 2004).

The toxicity or benefit of selenium absolutely depends on the chemical form and concentration of selenium in environmental and biological systems (Brown & Arthur, 2001; Clement *et al*., 2000; Frankenberger and Benson, 1994). Generally, the inorganic species of selenium such as selenate (SeVI) and selenite (SeIV) present high toxicity at large doses (Mazan *et al*., 2002; Orero *et al*., 2004). Meanwhile, the organic

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species of selenium, selenocysteine (SeC), Se-methylselenocysteine (SeMC) and selenomethionine (SeM) are amino acid forms of selenium in organisms and have several benefits for health (Bock *et al*., 1991; Combs *et al*., 1997; Hartikainen, 2005). Thus, the production of Se-enriched food by supplementing inorganic selenium into organisms such as yeast, mushroom and bacteria for providing an organic selenium source for humans has received wide interest around the world (Alzate *et al*., 2007; Casiot *et al*., 1999; Larsen *et al*., 2006; Rayman *et al*., 2008; Yoshida *et al*., 2005).

Plant is one of food stuff which has been mostly provided dietary Se in areas of Se deficiency, and to clean up Se pollution from seleniferous areas (Pilon-Smits and LeDuc, 2009). Therefore, production of selenium-enriched plant has been increasingly interested for using as dietary supplement. In Se-enriched plant process, the solution of inorganic selenium such as selenate and selenite ions are added for plant cultivation by treating in to soil or hydroponic medium (Li *et al*., 2008; Sugihara *et al*., 2004; Maneethong *et al*., 2013; Thosaikham *et al*., 2014). It is then absorbed through the root, accumulated in each part of plant and converted to organic selenium (Yathavakilla *et al*., 2005; Zayed *et al*., 2008; Li *et al*., 2015).

Several kinds of plant such as *Allium*, *Brassiceae* and *Astragarus* families have been utilized for studying their accumulation and biotransformation of selenium (Kotrebai *et al*., 1999; Wróbel *et al*., 2004; Pyrzynska, 2009). The major organic selenium compounds in Se-enriched plant are Se-methylselenocysteine, selenomethionine and γ-glutamyl-Se-methylselenocysteine (Montes-Bayón *et al*., 2002; Sugihara *et al*., 2004; Whanger, 2004; Ogra *et al*., 2009).

Currently, Se-enriched yeast is mostly recognized as the most successful commercial Se-enriched food product, which has high concentration of organic Se species, especially selenomethionine (Dumont *et al*., 2006; Rayman, 2004; Shia *et al*., 2010). Consumption of Se-enriched yeast has been recognized as a beneficial and safe form of Se for health (Schrauzer, 2000; Papp *et al*., 2007). The manufacture of Se-enriched yeast has usually utilized sodium selenite as an inorganic Se source for addition into the medium of yeast fermentation (Aguilar *et al*., 2008; Schlosser *et al*., 1994). Selenite is highly absorbed, accumulated and transformed to be organic Se species through the metabolism of yeast cells (Aguilar *et al*., 2009; Reyes *et al*., 2006).

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Additionally, Se-enrich plant and yeast have been applied to be supplement diet in animal feed (Chantiratikul *et al*., 2008; Chantiratikul *et al*., 2011).

2.4 Probiotics

Probiotics are living microbial that beneficially affects the host through its effects in the intestinal system (Roberfroid, 2000). Health benefits of probiotics are reconzied as good bacteria that are similar to microorganisms obtained in the human gut (National Center for Complementary and Integrative Health, 2015). There are various microbial that have been discovered and defined as probiotics. In numerous of discovered probiotics, lactic acid bacteria is recognized as the most important probiotic microorganisms associated with the human gastrointestinal tract, and can glow and survive under acidic condition (Rolfe, 2000, Charalampopoulos *et al*., 2003; Ding and Shah, 2007). Thus, probiotics have been used as a supplement in human and animal for the control of gastrointestinal health (Sanders, 2009; Pan *et al*., 2011).

Several lactic bacteria are associated with various plant and animal niches, and play a key role in the production of fermented foods and beverages (Heller, 2001; Makarova *et at*., 2006). Traditionally, lactic acid bacteria have been classified on the basis of phenotypic properties, e.g., morphology, mode of glucose fermentation, growth at different temperatures, lactic acid configuration, and fermentation of various carbohydrates (Holzapfel *et al*., 2001). Currently, genera *Lactobacillus*, *Bifidobacterium* and *Enterococcus* have been mostly focused and applied in an industrial application. They could be classified with the physiology and taxonomic position of species (Klein *et al*., 1998).

The health effects attributed to the use of probiotics have been widely researched for enhancing the understanding about probiotics. Currently, probiotics have been used for the prevention and treatment of several diseases (Boyle et al., 2006). Various evidence of health effects through the use of probiotics were reported such as reduction of *Helicobacter pylori* infection, reduction of allergic symptoms, relief from constipation, relief from irritable bowel syndrome, beneficial effects on mineral metabolism, and particularly bone density and stability (Marteau *et al*., 2001). Moreover, the benefits of probiotics are included cancer prevention, and reduction of

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cholesterol and triacylglycerol plasma concentrations (Schrezenmeir and de Vrese, 2001).

2.5 Selenium-enriched probiotics

Supplementation of inorganic Se into food stuffs has been increasingly interested for providing organic Se source for human. However, only bacteria, archaea and eukaryotes can produce Sec, which is the most important selenoamino acid to be synthesized as selenoprotein in human (Böck, 2000). However, there are few research that have been reported about Se supplementation and biotransformation in bacteria especially probiotic bacteria (Calomme *et al*., 1995; Zhang *et al*., 2009).

The first application of bacteria was utilization of bacteria for removing soluble selenium from environment (Oremland *et al*., 1989; Siddique *et al*., 2006). Several bacterial species have been applied to elimination of selenium from the Se-contaminated environment such as *Desulfovibrio desulfuricans*, *Anabaena fitsaquae*, *Gammaproteobacteria*, *Deltaproteobacteria*, *Deferribacteres*, *Chrysiogenetes* and *Stenotrophomonas maltophilia* (Dungan *et al*., 2003; Tomie *et al*., 1995; Kiffney and Knight, 1990; Narasingarao and Haggblom, 2007).

Addition of inorganic Se in to probiotics cultivation was firstly studied in *Lactobacillus*, which was reported by Calomme, 1995. Nowadays, various bacteria genus such as *Bifidobacterium, Enterococcus and Streptococcus* has been widely studied their possibility and potential for producing as organic Se source for human (Yang *et al.*, 2009; Zhang *et al*., 2009 ; Pieniz *et al*., 2011; Andreoni *et al*., 2000). However, most kind of bacteria, *Lactobacillus* has been mostly attended because of its probiotics property and daily consumption (Pophalya *et al*., 2014).

Cultivation of Se-enriched probiotics has been chiefly carried out by up taking selenite ion (SeO_3^{2-}) from selenite salt into culture medium. Selenite ion is absorbed through cell membrane of *Lactobacillus*, synthesized to be SeC, and incorporated into protein though the metabolism of *Lactobacillus* (Calomme, 1995a; Calomme, 1995b; Robles *et al*., 1999). Selenoprotein distributes in each part of probiotic cells such as polysaccharide and the nucleic acid. (Zhang *et al*., 2009). Molecular weights of the obtained selenoproteins are in the range of 30–70 kDa (Alzate *et al*., 2007). Moreover,

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selenoproteins could decrease chaperone expression, which is the stress factor of *Lactobacillus* (Mangiapane *et al*., 2014).

Generally, probiotics could usually endure selenite at concentration lower than 2 μg L^{-1} in broth medium. In the case of excess selenite-supplemented uptakes concentration, probiotics would reduce a toxic of selenite (Se^{4+}) by reducing oxidation state of selenite (Se^{4+}) to insoluble elemental selenium (Se^{0}) granule, thereby depositing it both in the cytoplasm and in the extracellular space (Gerrard *et al*., 1974; Silverberg *et al*., 1976; Turner *et al*., 1998; Xia *et al*., 2007).

A study of Se-enriched fermented milk products was firstly presented by Alzate *et al*., 2007, the result showed that selenocystine and Se-methyl selenosysteine were obtained the major organic selenium species in Se-enriched yogurt. Otherwise, a study of biotransformation of selenite by *Lactobacillus* and *Saccharomyces* in lactic fermentation process of kefir obtained selenomethionine including selenocystine and Se-methyl selenosysteine. This could be discussed that selenomethitonine was synthesized by *Saccharomyces*, meanwhile it was not obtained in Se-enriched yogurt which was only fermented by lactic bacteria (Alzate *et al*., 2008).

Applications of Se-enriched probiotics have been also studied for evaluating its benefit on health. Several studies indicated that Se-enriched probiotics has high antioxdative activity. For instant, the protein fraction from Se-enriched *Bifidobacterium animalis* 01 presented a positive role in enhancing the antioxidant activity (Shen *et al*., 2010). Se-enriched exopolysaccharide is a water-soluble fraction, extracted from Se-enriched probiotics cells, and has high bioactivity (Jin *et al*., 2010). Recently, Guo *et al*., (2013) reported that the fraction of exopolysaccharide isolated from Se-enriched *Lactococcus lactis subsp. Lactis* showed stronger *in vitro* and *in vivo* antioxidant activity. Moreover, the *in vivo* study by Yazdi *et al*., (2013) demonstrated that Se nanoparticle-enriched *Lactobacillus brevis* could reduce the liver metastasis in metastatic form of mouse breast cancer. Based on antibacterial action, the selenium-enriched probiotics could strongly against pathogenic *Escherichia coli* (*E. coli*) *in vitro* and *in vivo* (Yang *et al*., 2009).

Se-enriched probiotics has increasingly interested for using as dietary supplement in animal feed. Pan *et al*., (2011) reported that supplementation of Se-enriched probiotic in laying hens feed could affect on egg quality, egg selenium content,

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and glutathione peroxidase (GPX) activity. Recent report by Lu *et al*., 2013 presented that supplementation of exopolysaccharide extract from Se-enriched *Enterobacter cloacae* Z0206 in to broiler feed could enhance antioxidant status and immune function. Furthermore, Se-enriched probiotics could increase Se concentration, glutathione peroxidase (GPX) activity and mRNA level in piglet (Gan *et al*., 2011).

2.6 *Lactobacillus casei subsp. rhamnosus*is

*L. casei subsp. rhamnosus*is is a species of gram-positive, rod-shaped bacteria and recommended as a probiotic bacteria. It originates natively in the human intestine (Salminen *et al*., 2002). The strain of *L. casei subsp. rhamnosus*could is stable, and survives in acidic pH of gastric juice (Corcoran *et al*., 2005). Several health effects of *L. casei subsp. rhamnosus*are informed including prevention of intestinal inflammation, and stimulation of immune responses of allergic symptoms (Pessi *et al*., 2000; Segers and Lebeer, 2014).

Based on supplementation of selenium in to *L. casei subsp. rhamnosus*culture, the first report by Andreoni *et al*., (2000) demonstrated that *L. casei subsp. rhamnosus* could transform Selenite to be organic Se, and accumulate high amount of Se in biomass.

2.7 *Lactobacillus bulgaricus*

Lactobacillus bulgaricus is a gram-positive bacteria and probiotic (Goldin *et al*., 1992). It could grow within the environment of the gastro-intestinal tract (Parvez *et al*., 2006). *L bulgaricus* appears to play several important roles as a soldier to defense the digestive tract from harmful bacteria (Fuller, 1992). *L. bulgaricus* is traditionally used as starter together with *Streptococcus thermophilus* (*S. thermophilus*) (Michaylov *et al*. 2007).

Previously, Alzate *et al*. (2008) demonstrated that *L. bulgaricus* could high absorb inorganic selenium and transform to be selenocysteine which is incorporated in selenoprotein. Additionally, Se-enriched lactobacillus could reduce CCl_4-induced liver injury in mice, decrease lipid-peroxidative damage in cells, inhibit excessive release of

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TNF-α and protect dramatic elevation of hepatocyte [Ca^{2+}_i] concentration (Chen *et al*., 2005).

2.8 Electrodialysis

2.8.1 Principal of electrodialysis

Electrodialysis (ED) is an electrochemical process for separation of the soluble ions; negative charge ion and positive charge ion from solution through semipermeable membranes under the electric field (Figure 2.3) (Xu, 2005; El-Ghonemy, 2011). For the structure of ED apparatus, membrane is the most important device for filtering the ions from solution. Therefore, several kinds of membranes have been developed for supporting the ED apparatus especially ions-exchange membrane. The semipermeable could be classified as cation and anion exchange membranes. These membranes are used to allow only the opposite charge ion prior to pass to the electrode. For instance, the anions can move through the anion-exchange membrane, but they can not pass by the cation-exchange membrane. Therefore, they are blocked and trapped in the solution. Similarly, cations can move in the opposite direction through the cation-selective membrane under a negative charge, and are trapped by the anion-selective membrane (Kariduraganavar *et al*., 2006).

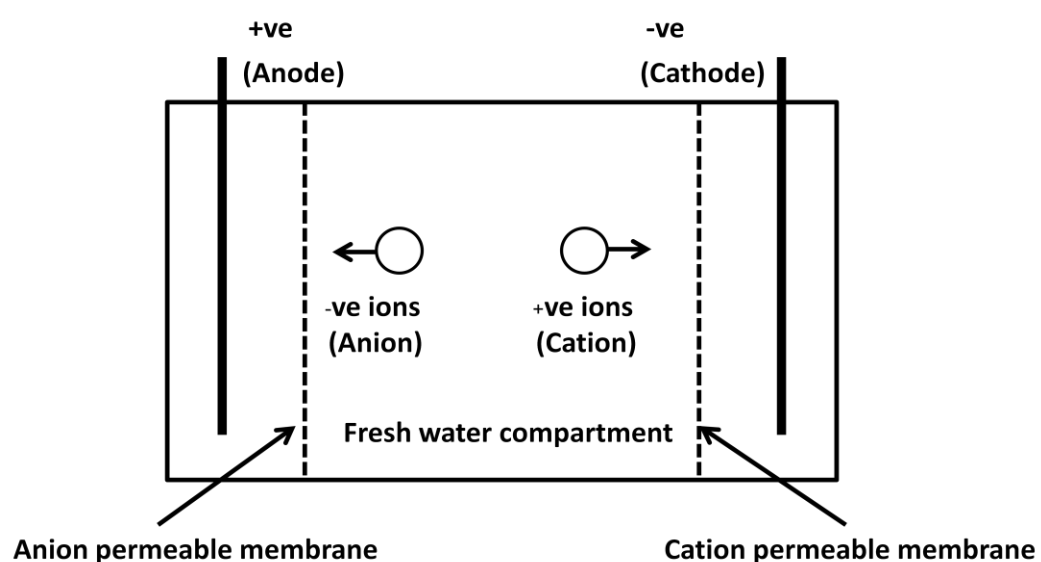

Figure 2.3 Principle of electrodialysis (ED) (El-Ghonemy, 2011)

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2.8.2 Application of electrodialysis

Base on application of ED for selenium, it has been usually applied to treat Se-contaminated water by removing inorganic ions selenium such as selenate and selenite from that water (Murphy and Colo, 1990). Nevertheless, the utilization of ED for studying biotransformation of selenium is not reported.

The concept of this work was considered from the metabolism of selenium in organism. The living organisms are usually treated with inorganic selenium such as selenate and selenite. They could absorb and transform these selenium species into various selenoproteins (SP). Moreover, partial of the inorganic selenium are transformed to be low molecular weight organic selenium species (LMOS) especially free seleno amino acid such as SeMC and gramma-glutamyl selenosysteine (Terry *et al*., 2000; Ellis *et al*., 2004; Johanson *et al*., 2005). Meanwhile, partial of accessed SIS concentration would not be transformed and remained as non-transformed inorganic selenium (NTIS) in the Se-enriched food culture system (Montes-Bayón *et al*., 2006). Normally, these selenium species could present their charge and in appropriate aqueous solution. Furthermore, they have different molecular weight which selenoproteins are macromolecules; their molecular weights are usually larger than 10 kD.

The SIS and LMOS are small molecules that their molecular weight is lower than 300 D (Szpunar, 2005). Generally, these selenium species could solute and present their charge in appropriate solution (Zheng *et al*., 2000). Thus, they could migrate through the permeable membrane under electric field of the ED. From the biotransformation mechanism of selenium and the property of each selenium species, they are the possible to design and construct the ED apparatus for pretreatment and fractionation selenium species in Se-enriched food.

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CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

1. Polyacrylic plastic (10 mm thickness), Pan Asia Industrial Co., LTD. Thailand.

2. Notebook silicone, Brand New Laptop, China

3. Graphite electrodes from pencil 2B type, QuanTum, DHA Siamwara LTD., Thailand

4. Cellulose membrane (45 mm flat width and 2000 Dalton nominal MWCO), Cellu·Sep H1, Membrane Filtration Products, Inc., USA.

5. The strains of *Lactobacillus casei subsp.rhamnosus* (TISTR No. 372) and *Lactobacillus bulgaricus* (TISTR No. 451), TISTR Culture Collection, Thailand Institute of Scientific and Technological Research, Pathum Thani, Thailand.

3.2 Chemical and reagents

All chemical and reagents used were of analytical, BioPerformance certified or HPLC grade. Sodium selenite, Tris hydrochloride, lysozyme from chicken egg white (~ 70000 U/mg solid and Protease XIV from *Streptomyces griseus* (≥3.5 U/mg solid) were obtained from Sigma-Aldrich (Germany). DL-selenomethionine, L-selenocystine, Se-methylseleno-L-cysteine, trifluoroacetic acid, 1-butanesulfonic acid sodium salt, tetramethylammonium hydroxide, and DL-1,4-dithiothreitol were purchased from Acros Organics (Belgium). Selenium standard solution, nitric acid, ammonia solution and methanol were obtained from Carlo Erba (Italy). Hydrochloric acid was purchased from BHD (UK). *Lactobacillus* MRS Broth used was obtained from HiMedia (India).

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3.3 Instrumentation

The ICP-MS instrument for Se determination was the Elan DRC-e (Perkin–Elmer SCIEX, Norwalk, USA), equipped with a double-pass Scott spray chamber fitted with a cross-flow nebulizer. The chromatographic system was carried out with isocratic system using the Dual pump KP-11 (Ogawa & Co., Ltd., Kobe, Japan), equipped with a syringe loading injector (Model 9725i, Rheodyne six-port injection valve) with a 100 µL sample loop. The reversed phase columns for separation of Se species utilized Inersil® C18 (GL Science Inc., Tokyo, Japan, 250 mm length × 4.6 mm I.D., 5 µm particle size). The chromatographic system was connected to the ICP-MS instrument by using PEEK capillary tubing (300 mm length × 0.25 mm I.D.).

Total selenium concentration in the electrodialyzed solution was determined by using hydride generation - atomic absorption spectrometry (HG-AAS). The atomic absorption spectrometer was Model 280FS AA (Agilent technology, USA). The production of selenium hydride was achieved by using the vapor generation accessory for model VGA 77 (Agilent technology, USA). The atomization of selenium hydride was carried out by using electrothermal controller (ETC 60, Agilent technology, USA)

The morphology of probiotics was studied by using scanning electron microscope (SEM) (Jeol, model JSM-6460LVl, Tokyo, Japan). The SPI Module™ Sputter Coaters (SPI Supplies/Structure Probe, Inc. West Chester, USA) was utilized for preparing SEM imaging of probiotic cells.

Ultrasonic bath using DT 255 H (Babdelin, Germany) was employed. Refrigerated centrifuge using Centurion Model NF 800R obtained from Nüve (Germany). Water bath using was model TW 12 Julabo, Germany. Freeze drier for drying biomass of probotics was model Freezeone 4.5 obtained from Labconco (USA).

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3.4 Experimental

There are 3 main experiments in this work; development of ED apparatus, development of Se-enriched probiotic bacteria cultivation, and application of the proposed ED to separation of selenium species in Se-enriched probiotic bacteria. The scope of this research is shown in Figure 3.1.

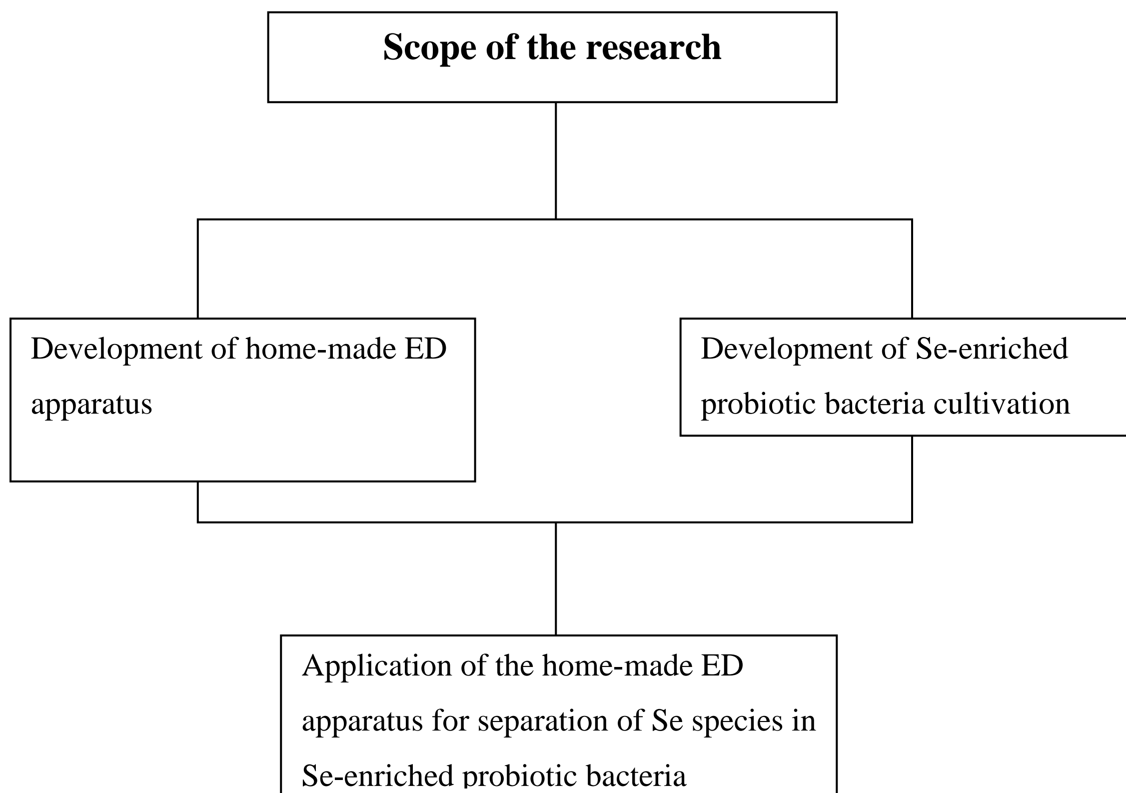

Figure 3.1 Diagram of research scope

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3.4.1 Se-enriched probiotics cultivation

Conditions of Se-enriched probiotics cultivation were optimized and the experimental plan is showed in Figure 3.2.

Development of Se-enriched probiotic bacteria cultivation

↓

Preparation of probiotic inoculums

↓

Studied on growth curve of probiotics

↓

Comparison of Se accumulation and biotransformation of the probiotic strains

↓

Studied on the effect of Se-supplemented concentrations on Se content probiotics

↓

Studied on the effect of initiation time for adding SeO_3^{2-} ion on Se content probiotics

Figure 3.2 Experimental plan of development of Se-enriched probiotic production

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3.4.1.1 Preparation of probiotic inoculums

The strains of *L. casei subsp.rhamnosus* (TISTR No. 372) and *L. bulgaricus* (TISTR No. 451) were individually transferred into 125 mL Erlenmeyer flask containing 50 mL of MRS broth medium (5.15 %w/v). The batch cultures were incubated overnight at 37 °C, and continuously shaken at 150 rpm. The inoculums of probiotics were prepared by loading 0.1 mL of the overnight culture with sterilized micropipette into 1 mL MRS which was contained in screw cap test tube. Afterward, the inoculums of probiotics were cultured at 37 °C and, shaken at150 rpm until the optical density at λ_{max} 660 nm (OD_{660}) was about 1 by sampling to measure with spectrophotometer every 3 h.

3.4.1.2 Growth rate of probiotics

The inoculums of probiotic from section 3.4.1.1 were transferred to 250 mL Erlenmeyer flask (*n* = 3) containing 100 mL of MRS broth medium. The batch cultures were incubated at 37 °C and 150 rpm shaker. Growth rate of the probiotic in the batch cultures was monitored at 0, 3, 6, 12, 18 24, 36, 48, 60, 72 and 96 h of incubation by measuring the OD_{660} value with the spectrophotometer. Harvesting of the culture of each incubation time was performed with aseptic technique by sampling 500 µL of the culture into centrifuge tube using sterilized pipette tip. Then, it was centrifuged at 5000 rpm, 4 °C for 10 min, and removed the supernatant. The cells pellet was washed twice with deionized water after that it was resuspended with deionized water and measured OD_{660} value with the spectrophotometer. The relationship between incubation time and OD_{660} values was plotted as the growth curve for calculating the maximum specific growth rate (μ_{max}) and characterizing the growth phases of probiotic in batch culture.

The maximum specific growth rate (μ_{max}) was calculated from the slope of the log phase using equation (1).

$$\mu_{max} = \frac{\ln X - \ln X_0}{\Delta t} \quad (1)$$

Where: X is OD_{660} value in the end of the log phase, X_0 is OD_{660} value in the beginning of the log phase, and Δt is the time interval between observations (Kask *et al*., 2003). The μ_{max} was calculated using at least three samples taken in the log phase.

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3.4.1.3 Comparison of Se accumulation and biotransformation of the probiotic strains

The inoculums of *L. casei subsp.rhamnosus* and *L. bugaricus* strain were transferred into 100 mL MRS broth medium (n = 3) which was contented SeO_3^{2-} ion 2.5 mg Se L^{-1} from Na_2SeO_3. They were cultured in 250 Erlenmeyer flasks at 37 °C, and shaken at 150 rpm. The cultures of both Se-enriched probiotics were harvested at the end time of cultivation by centrifugation at 5000 rpm for 10 min. The supernatant was removed and the cells pellets were washed three times with deionized water, frozen, lyophilized, and weighed. Se accumulation and biotransformation of *L. casei subsp.rhamnosus* and *L. bugaricus* strain was compared for selecting the strain for the next experiment.

3.4.1.4 The effect of Se-supplemented concentration on Se content in probiotics

The inoculums of probiotic was transferred into 100 mL MRS broth medium (n = 3) for bath culture in 250 Erlenmeyer flask at 37 °C, and shaken at 150 rpm. After initial growth for 3 h, SeO_3^{2-} ion from Na_2SeO_3 stock solution was added into the batch cultures at final concentrations of 0, 1, 2.5, 4.0, 5.5, and 7.0 mg Se L^{-1}, and continuously incubated. At the end of cultivation, the biomass was harvested by centrifugation at 5000 rpm for 10 min. The supernatant was separated and stored in polyethylene bottle. The cells pellets were washed three times with deionized water, frozen, lyophilized, and weighed.

3.4.1.5 The effect of initiation time for adding SeO_3^{2-} ion on Se content in probiotics

The initiation time for adding SeO_3^{2-} ion was studied for comparing the Se accumulation and biotransformation when the SeO_3^{2-} ion was treated into different time of the growth curve of probiotic. The selection of the initiation time for adding SeO_3^{2-} ion was considered from the growth phases of probiotic strains which covered lag phase, log phase and stationary phase. After the probiotic strain was grown with the selected initiation time, the SeO_3^{2-} ion stock solution was added to be the final optimum Se-supplemented concentration. Then, the cultures were continuously incubated, and

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harvested the cells at the end time of cultivation by centrifugation at 5000 rpm for 10 min. The cells pellets were washed three times with deionized water, frozen, lyophilized, and weighed.

3.4.2 Analytical procedures

The experimental plan for analyzing total Se, Se speciation and morphology of Se-enriched probiotic is showed in Figure 3.3.

3.4.2.1 Total selenium determination

The determination of total Se concentrations in dried cells, remained-medium and whole culture from Se-enriched probiotics cultivation was performed by ICP-MS technique. The samples were digested by utilizing the home-made closed digestion system which was developed in our laboratory (Sittipout *et al*., 2011). The 50 mg of Se-enriched dried cells was weighed into the vessel (n=3). Volume of 1 mL nitric acid (68%) and 1 mL H_2O_2 were added. The vessel was closed with its cap and incubated in a hot water bath at 100 °C for 15 min. Then, the vessel was cooled to room temperature. The digest was transfer in to a 25 mL volumetric flask, and made up the volume with deionized water prior to determination of total selenium using the ICP-MS technique.

For the remained-medium and whole culture preparation, a volume of 2 mL sample was loaded into the digestion vessel (n = 3). The digestion procedure was monitored as same as the digestion of dried cells. The conditions of ICP-MS for detection of Se are shown in Table 3.1.

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Table 3.1 ICP-MS operating parameters

Parameters	Setting
Nebulizer gas flow	0.89 L min^{-1}
Auxiliary gas flow	1.10 L min^{-1}
Plasma gas flow	15 L min^{-1}
Lens voltage	7.0 volt
ICP RF power	1200 W
Mass analyzer	Quadrupole
Mass monitoring (m/z)	Se-82
Detection mode	Standard mode

3.4.2.2 Selenium species determination

3.4.2.2.1 Ion paired reversed phase HPLC-ICP-MS for Se speciation analysis

The ion paired reversed phase HPLC-ICP-MS for Se species determination was demonstrated from our previous research (Thosaikham *et al.*, 2014). The conditions of chromatographic system were presented in Table 3.2.

3.4.2.2.2 Preparation of selenium compounds standard solution

The stock standard solutions of selenite ion (SeO_3^{2-}), Se-methyl selenocysteine (SeMC) and selenomethionine (SeM) were prepared from sodium selenite, Semethylseleno-L-cysteine and DL-selenomethionine, respectively. They were prepared by dissolving the solid reagent in deionized water. Selenocysteine (Sec) was specially prepared and adapted from Chery *et al.* (2005). It was prepared by dissolving L-selenocystine into 0.05 M of DTT in 0.1 M HCl solution. The Sec solution was incubated at 50 °C for 30 min until L-selenocystine completely dissolved and then brought up to volume with deionized water. Furthermore, all selenium species standard stock solutions were standardized with the total selenium standard solution (AAS grade) prior to use and stored at -18 °C.

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Table 3.2 Experimental conditions of ion paired reversed phase HPLC-ICP-MS

Parameters	Setting
- Analytical column	C-18 column (250 mm length × 4.6 mm I.D., 5 μm particle size) equipped with C-18 guard column.
- Mobile phase solution	
pH value	4.5
Trifluroacetic acid (TFA)	4 mM
1-butansulfonic acid (BSA)	8 mM
Methanol	0.1 %(v/v)
Injection volume	100 μL
Flow rate	1.0 mL min^{-1} (isocratic system)

3.4.2.2.3 Preparation of Se-enriched probiotics dried cells

The Se species in dried cells of Se-enriched probiotics determination was prepared by enzymatic extraction method. The extraction procedure was performed by weighing 20 mg of dried cells into sterilized polyethylene bottle. The sample was then added 5 mL Tris-HCl buffer (pH = 7.0), 1 mL DTT solution (0.02 M in Tris-HCl buffer pH = 7), 2 mL lysozyme in Tris-HCl (3.5 unit/mL) and 2 mL protease XIV in Tris-HCl (2000 unit/mL). The mixture was sonicated in an ultrasonic bath for 10 min, and moved to incubation at 37℃ and 180 rpm shaking for 24 h. Then, the mixture was centrifuged at 6000 rpm for 30 min, removed the supernatant for determining Se compounds by HPLC-ICP-MS.

3.4.2.2.4 Preparation of remained-medium

The remained-medium was few prepared for determining extracellular Se compounds without hydrolysis procedure. It was directly diluted 5 times with mobile phase solution, and filtered through the nylon syringe filter (0.45 μm) prior to injection into the ion-pairs reversed phased HPLC-ICP-MS. Accordingly, the obtained Se compounds in the remained-medium would be free amino acid or SeO_3^{2-} ion which were not incorporated into protein.

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For checking the position of each Se compounds peaks in the chromatogram, Se compounds standards were individually spiked into the remained-medium to be 100 mg Se L^{-1} at final concentration, and determined with the chromatographic system. Then, the obtained peaks of Se compounds in the non-spiked remain-medium were compared with the peaks of each Se compounds from spiked remain-medium. Moreover, all Se compounds standards were spiked together in the remained-medium for recovery test and calculating their percentage recovery.

3.4.2.2.5 Scanning electron microscopy

The dried Se-enriched probiotics samples from section 3.4.1.4 were directly added onto a filament of graphite, dehydrated at room temperature in a high vacuum coating system and examined by scanning electron microscopy (SEM).

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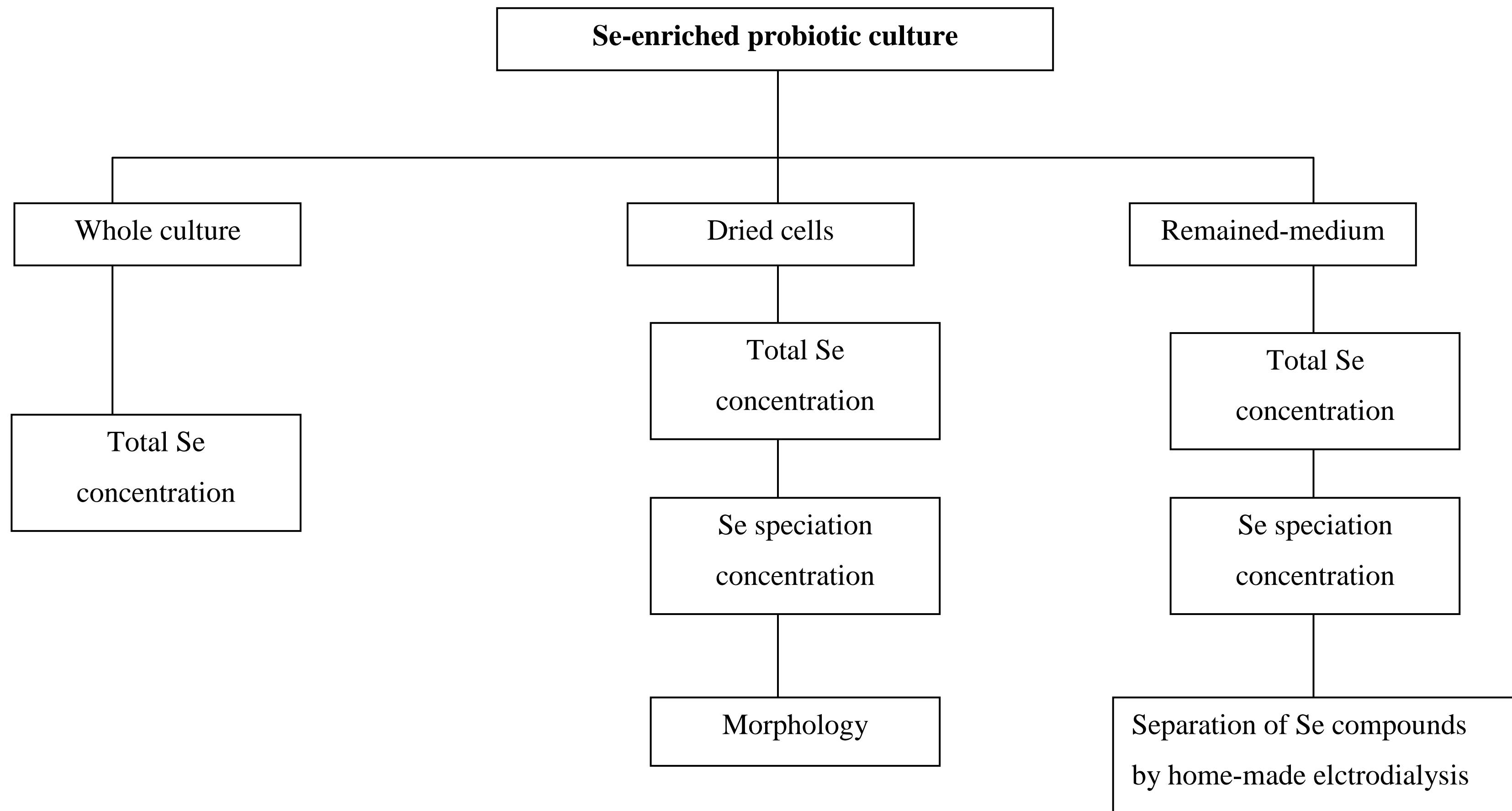

Figure 3.3 Experimental plan for analytical procedure of Se-enriched probiotics

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3.4.3 Development of home-made ED apparatus

The home-made ED apparatus was designed by considering with the separation efficiency of selenium species, invention cost, suitable material, easy operation and available application. In this work, the ED apparatus was designed by using MS-Office PowerPoint 2007 software. It consisted of 2 main parts; ED stack and power supply system. Layout of the designed ED apparatus is displayed in Figure 3.4.

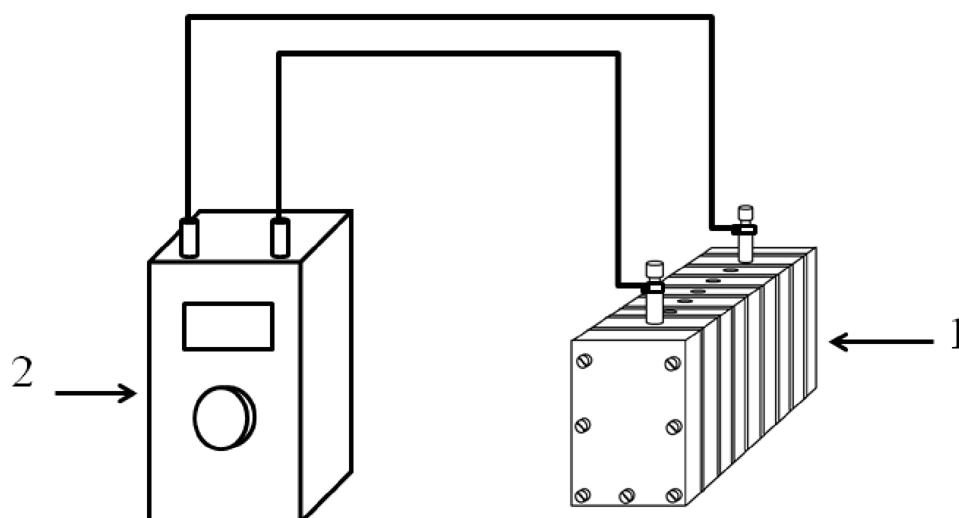

Figure 3.4 The home-made ED apparatus and its main parts; ED stack (1) and power supply system (2).

3.4.3.1 ED stack

ED stack was a unit for containing solution and dialyzing selenium species through a permeable membrane. It consisted of frame cell body, dialysis membrane, sealing system and lock system. The descriptions of the design and function of each device are following as;

3.4.3.1.1 Frame cell body

Frame cell body was a solution containing device which was designed, and shown in Figure 3.5. It was made from acrylic plastic which has the length, width and thickness; 10.7, 4.5 and 1.0 cm, respectively (Figure 3.6 and Figure 3.7). The chamber for containing solution was made by perforating a square space at the

center of frame cell body which the length, width and thickness of this chamber were 7.7, 1.5 and 1.0 cm, respectively. The top side of the frame cell body was countersunk as two holes for solution loading and air passage. Furthermore, seven screwed-holes were countersunk through the cross section side of the frame cell body for wearing screws.

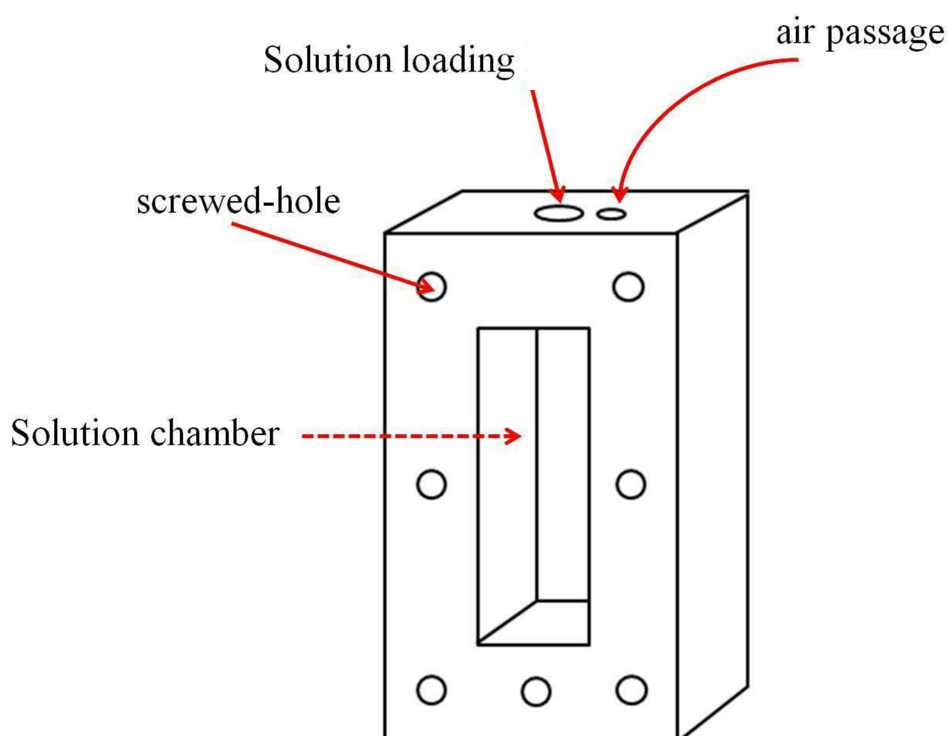

Figure 3.5 The design of frame cell body

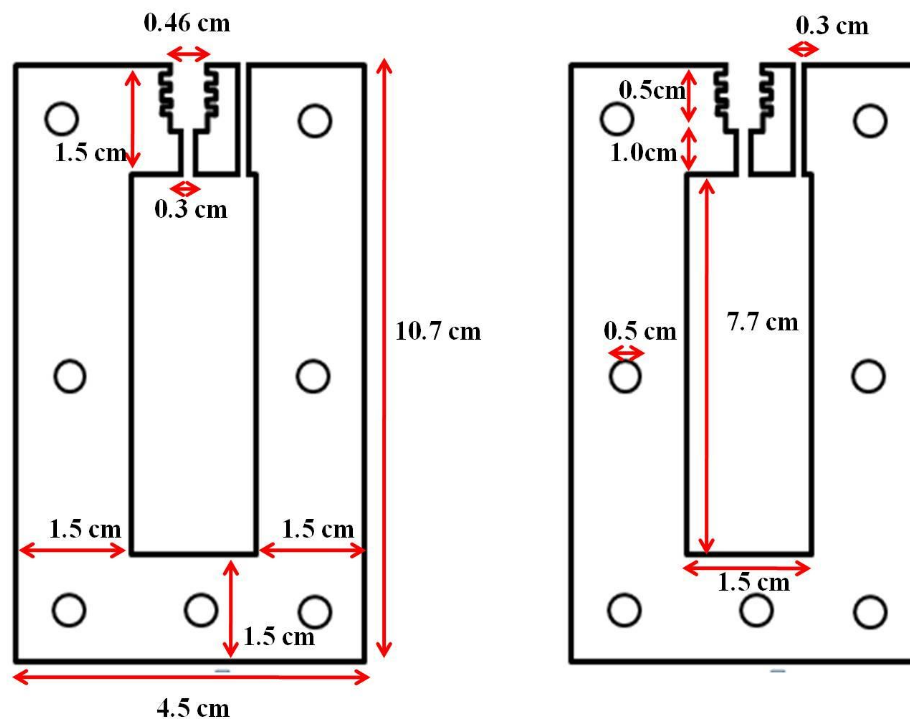

Figure 3.6 Scale of frame cell body

Figure 3.7 The model frame cell body

3.4.3.1.2 Dialysis membrane

Dialysis membrane was used for separating selenium species with their different charge and weight of molecule. The size of dialysis membrane was presented in Figure 3.8. In this work, cellulose membrane was selected for separating small molecules which their molecular weights are lower than 2000 D (Figure 3.9). The dialysis membrane was provided by cutting appropriately for fitting to the frame cell body (Figure 3.10).

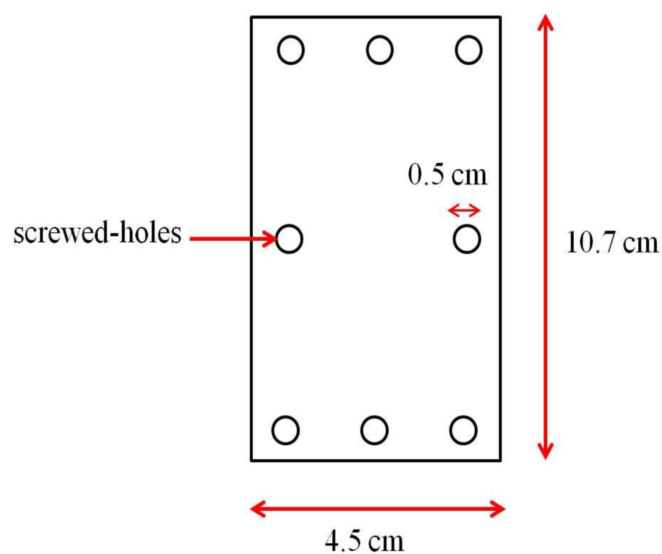

Figure 3.8 Design of dialysis membrane

Figure 3.9 Dialysis membrane used in this work

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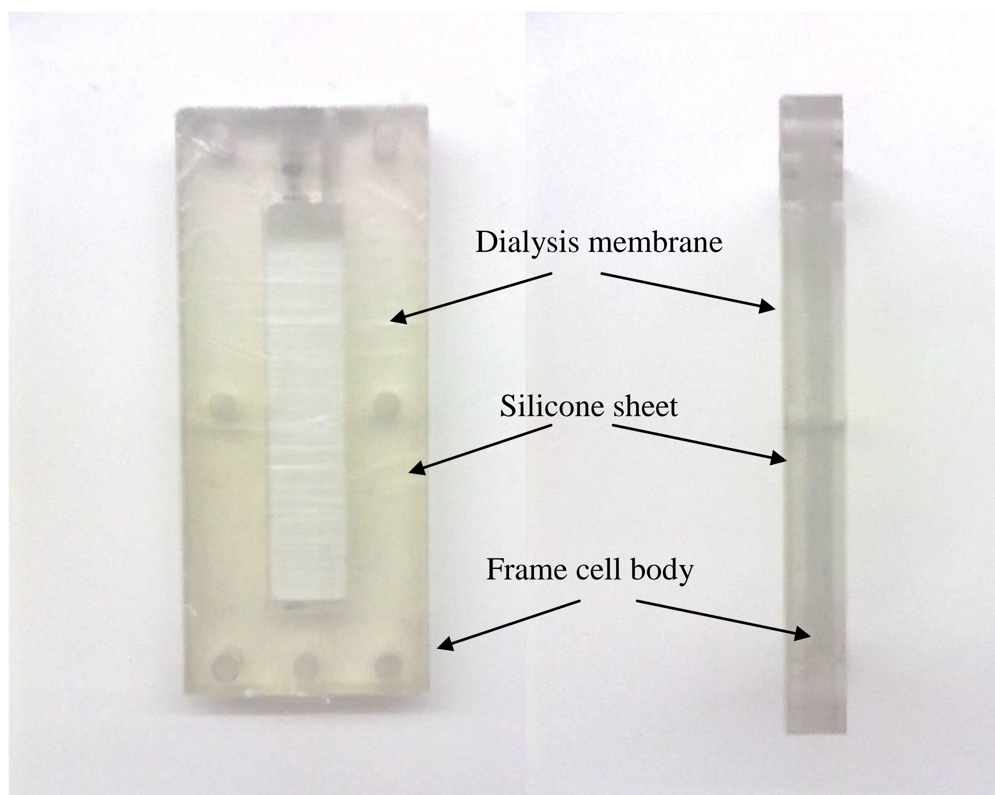

Figure 3.10 Dialysis membrane fitted on silicone sheet and frame cell body

3.4.3.1.3 Sealing system and lock system

The leakage of solution from the ED stack was protected by sealing the frame cell bodies with a silicone sheet. It was appropriately cut and fixed on the frame cell body. Its design and scale are presented in Figure 3.11. In this work, the notebook silicone cover was adapted as the silicone sheet (Figure 3.12 and Figure 3.13). It was exactly attached on the frame cell body prior to installation in the ED stack (Figure 3.14).

The screws and nuts were used for consolidating all devices to be the ED stack. A long screw (Figure 3.15 (A)) was used to fix the frame cell bodies, dialysis membranes, silicone sheets and cover plate together. The ends of screws were locked with nuts. The screws and nuts used in this work were made from stainless steel (Figure 3.15 (B)).

Figure 3.18 presents the arrangement of the devices prior to assembling as ED stack. The end sides of ED stack was enclosed with cover plates, and

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locked with the screws and nuts (Figure 3.16 and Figure 3.17). The complete ED stack was displayed in Figure 3.19.

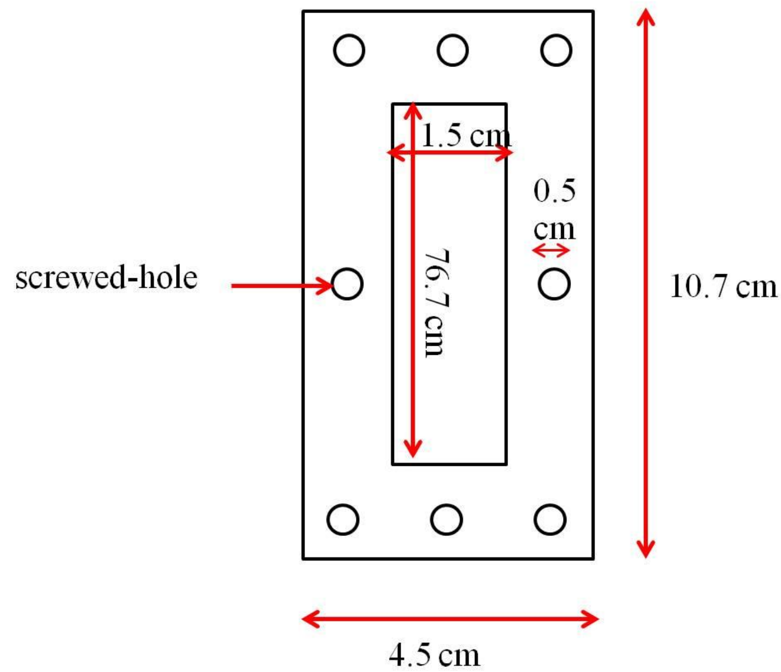

Figure 3.11 The design of silicone sheet

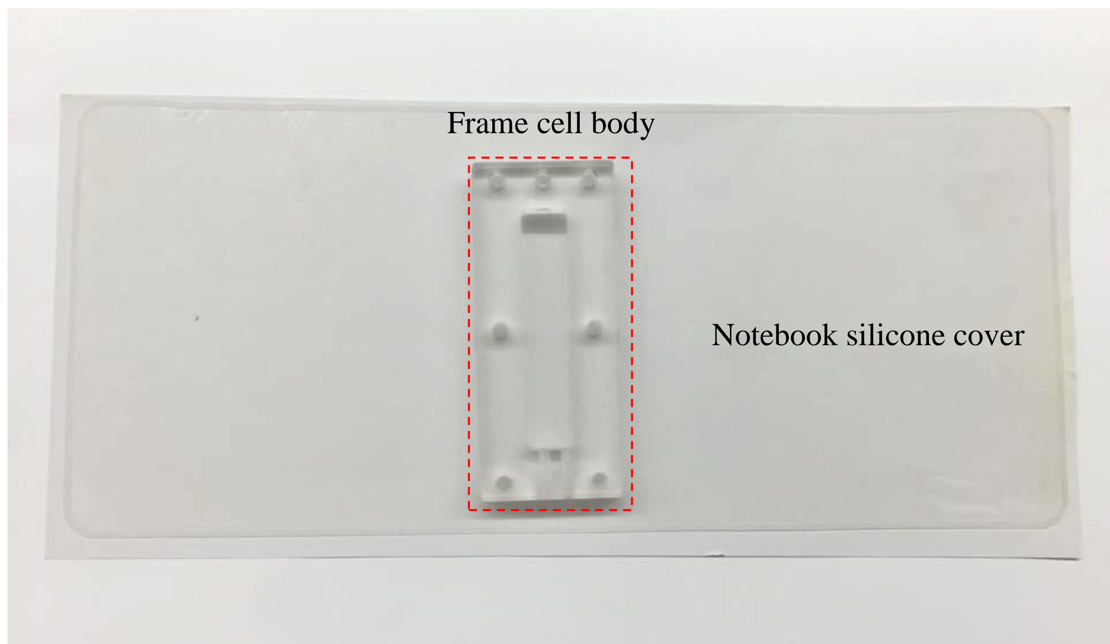

Figure 3.12 Cutting silicone sheet from notebook silicone cover

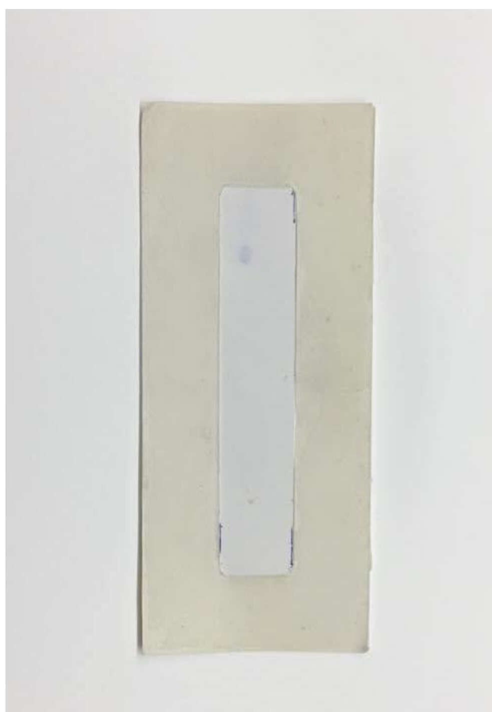

Figure 3.13 The model of silicone sheet

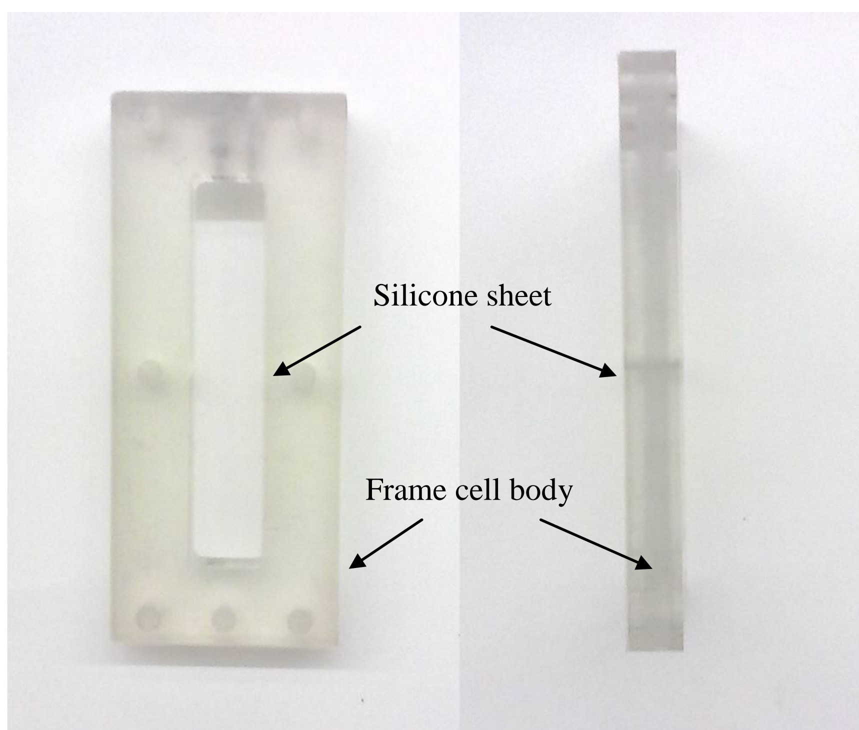

Figure 3.14 Silicone sheet fitted on the frame cell body

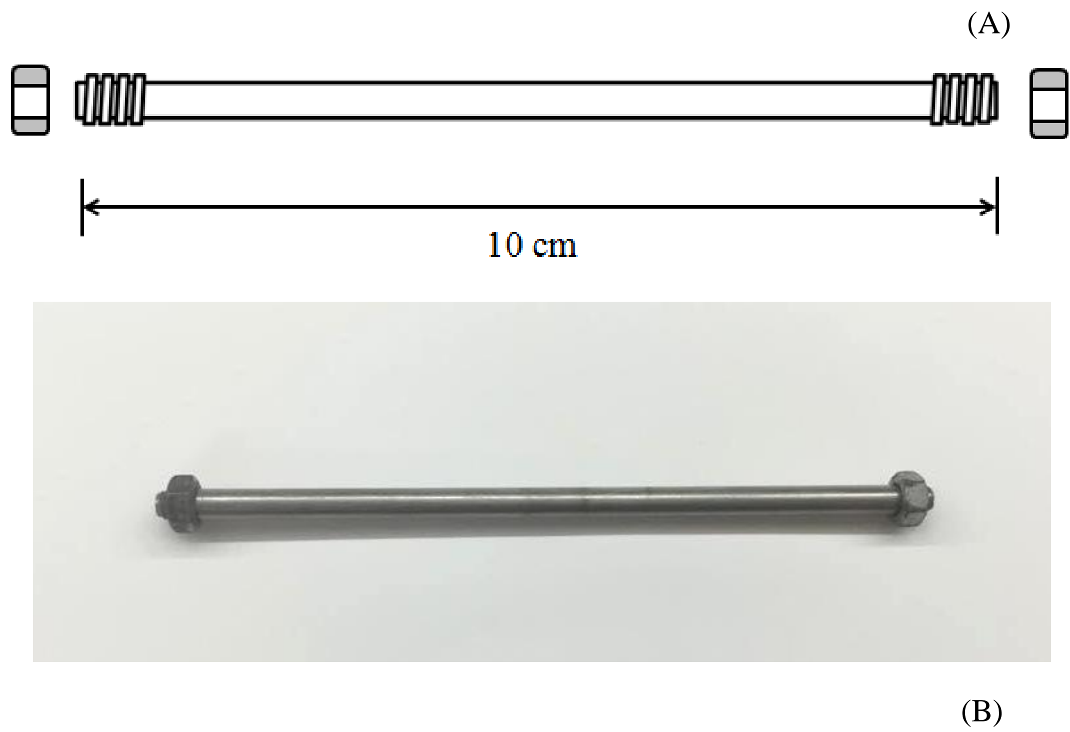

Figure 3.15 The design of screws and nuts (A), the model of screws and nuts (B)

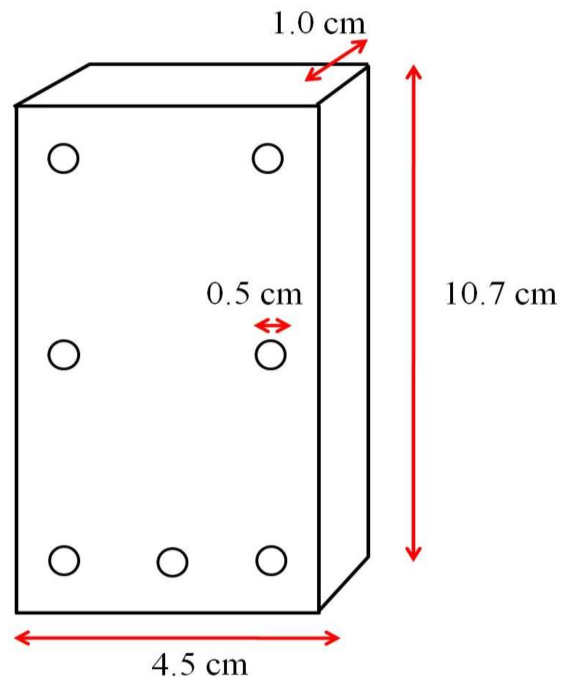

Figure 3.16 The design of cover plate

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Figure 3.17 The model of cover plates

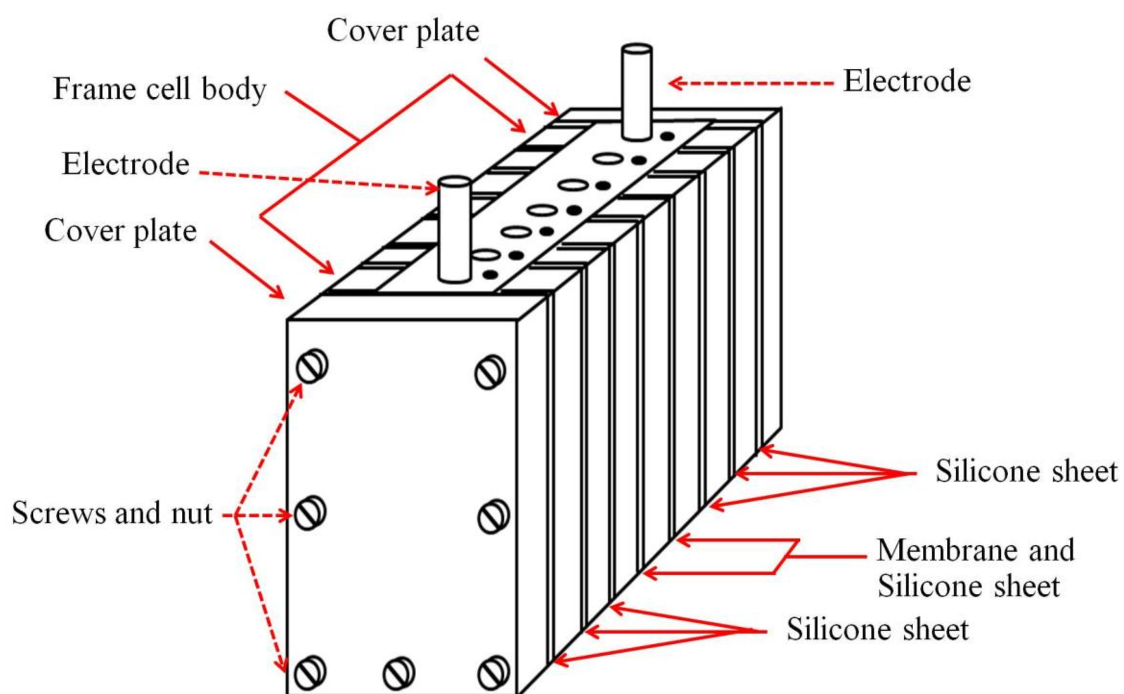

Figure 3.18 Assembly of the ED stack

Figure 3.19 The model of ED stack

3.4.3.2 Power supply system

Applying of electric current to the ED stack was performed by using a power supply system. It composed with electrodes and power supply apparatus. In this work, the graphite electrodes were made from pencil (2B type) that is shown in Figure 3.20. They were installed in the frame cell bodies which were selected as cathode and anode electrode-frame cell bodies. They were fixed in the loading out hole with a nut (HPLC type) that is showed in Figure 3.21. The positive and negative DC voltage from the power supply apparatus was applied to the electrode-frame cell bodies though electric cables to the cathode and anode electrodes, respectively. The picture of ED stack and power supply system is showed in Figure 3.22.

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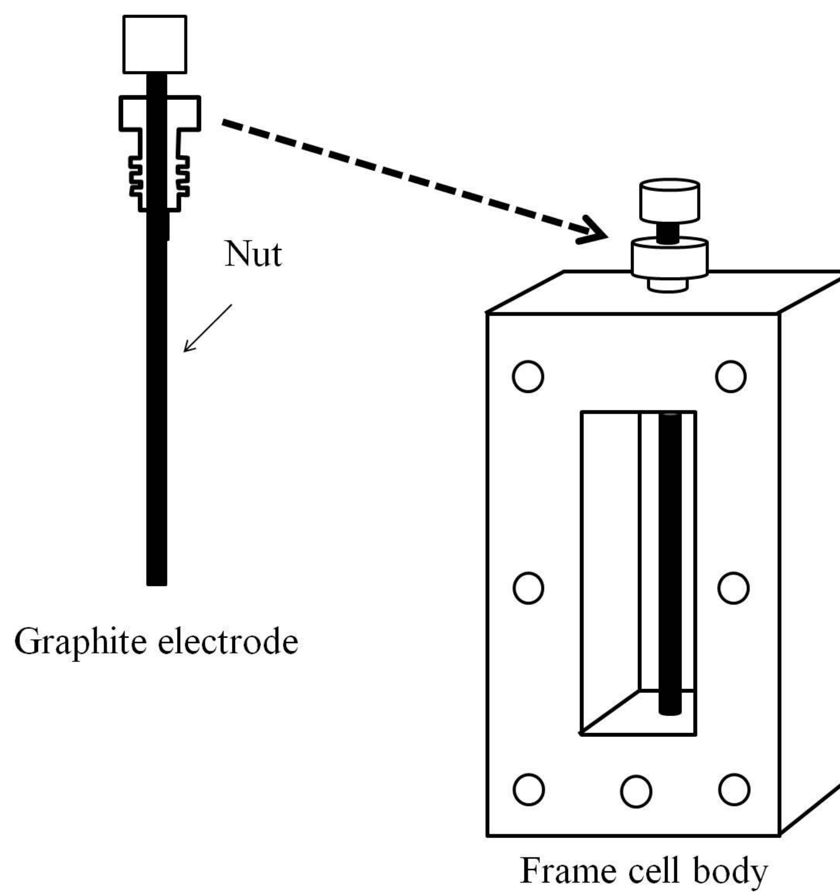

Figure 3.20 The design of graphite electrode and installing in the frame cell body

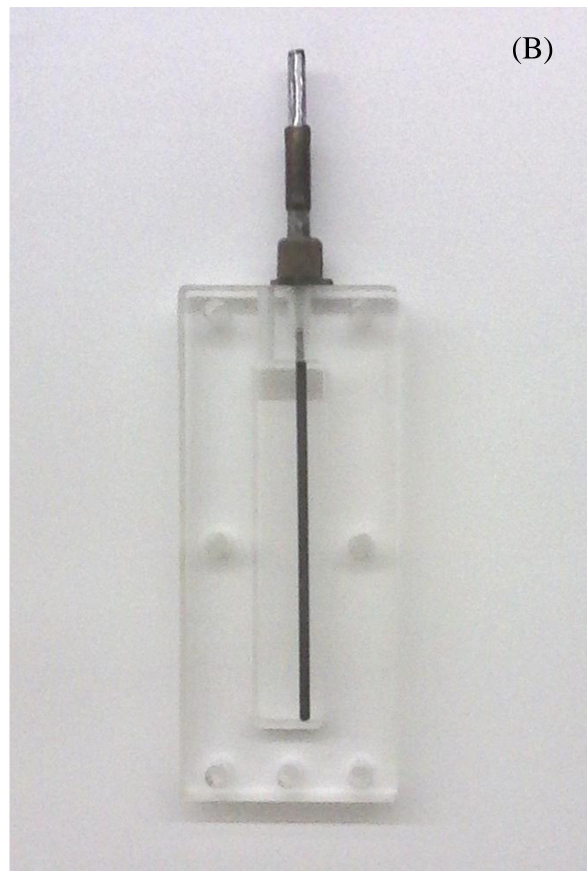

Figure 3.21 The model of graphite electrode (A) and installing in frame cell body (B)

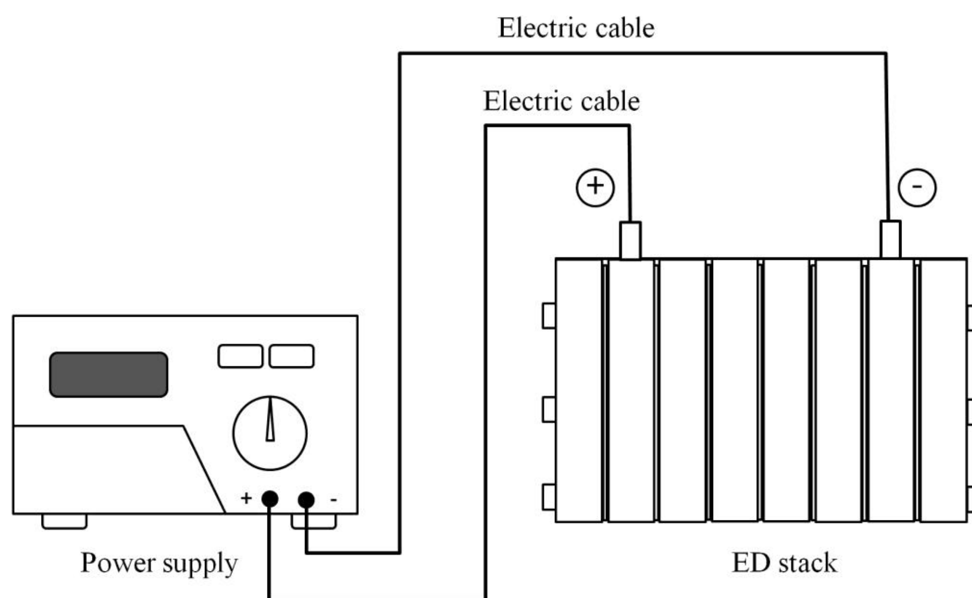

Figure 3.22 The design of home-made ED apparatus

3.4.4 Application of home-made ED apparatus for Se species in Se-enriched probiotics

In Se-enriched probiotic culture, the remained-medium was the largest part that is usually let to be as a by-product after harvesting the biomass. In the previous studies, Se speciation analysis and applications of Se-enriched probiotics have focused only the biomass (Calomme, 1995; Yang *et al.*, 2009; Zhang *et al.*, 2009; Alzate *et al.*, 2007). It is possible that the remained-medium would be also contained organic Se compounds and non-transform SeO_3^{2-} ion. However, the remained-medium should be added value by eliminating the non-transform SeO_3^{2-} ion which is not beneficial for health. Hence, this work interested to apply the home-made ED apparatus for removing the non-transformed SeO_3^{2-} ion from the remained-medium.

3.4.4.1 Determination of Se compounds by HG-AAS

The 4 Se compounds; selenite ion (SeO_3^{2-}), Se-methyl selenocysteine (SeMC) and selenomethionine (SeM) were determined by HG-AAS. The standard solution of each Se compounds was prepared by diluting stock solutions to be final concentration at 20 µg Se L^{-1} with DI water. Afterward, the standard solutions of each Se compounds were individually injected into the hydride generation system. The

method of selenium hydride generation was recommended in user's guide of VGA 77 (Agilent technology, 2014). The conditions of HG-AAS for Se determination are presented in Table 3.3.

Table 3.3 Conditions of HG-AAS for Se determination

Parameters	Setting
VGA 77	
- Reducing solution	0.6% w/v $NaBH_4$ and 0.5% w/v NaOH
- Acid solution	5 M HCl
- Carrier gas	Ar gas
ETC 60	
- Atomization temperature	900℃
- Hollow Cathode Lamp	UltrAA lamp for Selenium detection at λ_{max} 196 nm

3.4.4.2 Optimization of applied voltage

Electric field is an important factor for migrating SeO_3^{2-} ion from the sample frame cell (a) to the electrode-frame cells. Therefore, it was optimized by varying in the range of 25 to 125 volt (n = 3). The optimum applied voltage was selected for the next study.

The setting procedure of the ED apparatus was operated in Figure 3.23. The remained-medium was diluted 10 times with DI water, and loaded 10 mL into the sample unit of ED stack. The 20 mL DI water was loaded into cathode and anode units of the ED stack. Then, the electric field was applied through electric cables to the cathode and anode units for 5 min. For loading out the solution from the ED stack (Figure 3.24), the remained-medium was firstly loaded out by a syringe and collected in PE bottle after that the dialyzed solutions in the cathode and anode units were also loaded out. The collected solutions were determined SeO_3^{2-} concentration by hydride generation - atomic absorption spectrometer (HG-AAS)

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3.4.4.3 Optimization of electrodialysis time

The optimization applied voltage time was studied for evaluating running time of the ED apparatus for selenium species pretreatment. It was optimized in the range of 2.5 to 15 min (*n* = 3) under the optimum applied voltage.

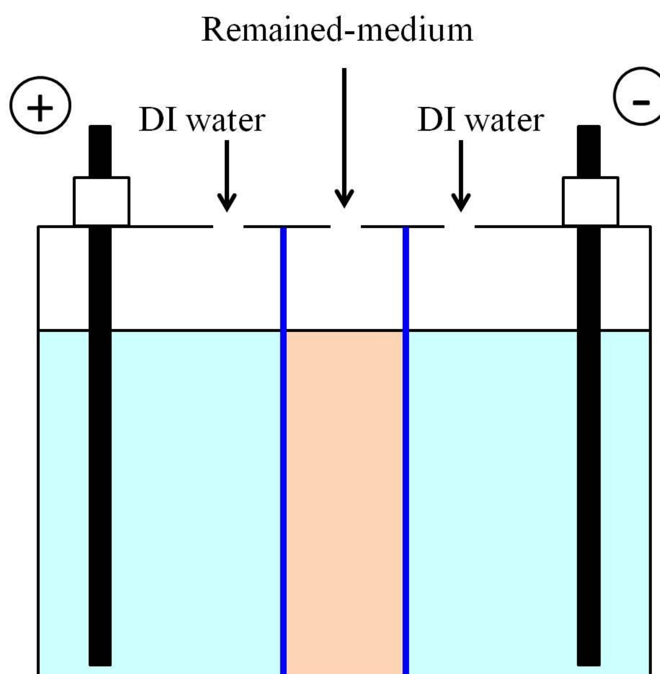

Figure 3.23 The ED stack setting for removing SeO_3^{2-} ion from the remained-medium

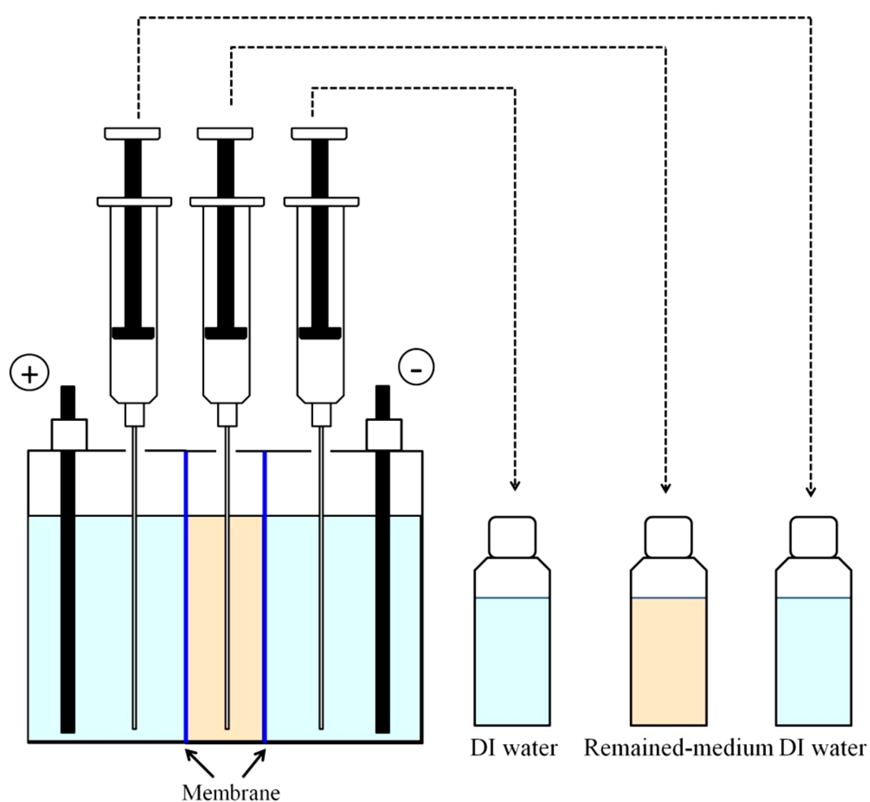

Figure 3.24 Loading out solutions from the ED stack

3.5 Data analysis

All experimental data were expressed as mean ± SD. The data of sample preparation part analyzed for variance (ANOVA) and significant different among the means from triplicate analysis was set at $p < 0.05$. This was determined by General Linear Model multivariate range test using the statistical program for social science (SPSS, Chicago, IL, USA) Version 16.0 for windows

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CHAPTER 4

RESULTS AND DISCUSSION

4.1 Se-enriched probiotics cultivation

4.1.1 Growth rate of probiotic strains

L. casei subp. rhamnosus and *L. bulgaricus* are recognized as beneficial probiotics, and widely used in the dairy industry (Roberfroid, 2000). Hence, they were selected for producing Se-enriched probiotics. The growth rate of the *L. casei subsp. rhamnosus* and *L. bulgaricus* strains in MRS broth medium were studied as the first parameter for further developing the process of Se-enriched probiotics.

The growth of *L. casei subsp.rhamnosus* and *L. bulgaricus* strains were carried out by culturing in MRS broth medium under anaerobic condition at pH 6.88, and incubated at 37℃ and 150 rpm. The growth curves of the both strains were examined by measuring OD_{660} values with spectrophotometer during 0 to 96 h. Figure 4.1 and Figure 4.2 show the growth curves of the both probiotics strains by plotting between the measured OD_{660} values (y) and time (x). It was seen that the length of the lag phase of *L. casei subsp. rhamnosus* was begun at the starting time of culture until the 6th hour. *L. casei subsp. rhamnosus* could mostly grow in the log phase during 6th to 24th hour. The stationary phase of *L. casei subsp. rhamnosus* was started after 24th hour. For the growth curve of *L. bulgaricus*, the length of the lag phase was longer than *L. casei subsp. rhamnosus* which was about 12 hour. Moreover, its log phase was begun from the 12th hour to the 50th hour. After that the growth rate was stable, and entered to the stationary phase. This work demonstrated that the end time for culturing Se-enriched *L. casei subsp. rhamnosus* and *L. bulgaricus* were 48 and 72 hours, respectively.

The specific growth rates (μ_{max}) of *L. casei subsp. rhamnosus* and *L. bulgaricus* were 0.165 ± 0.003 and 0.069 ± 0.002 OD/h, respectively. This result indicated that the growth rate of *L. casei subsp. rhamnosus* strain in the MRS broth medium was better than *L. bulgaricus* strain.

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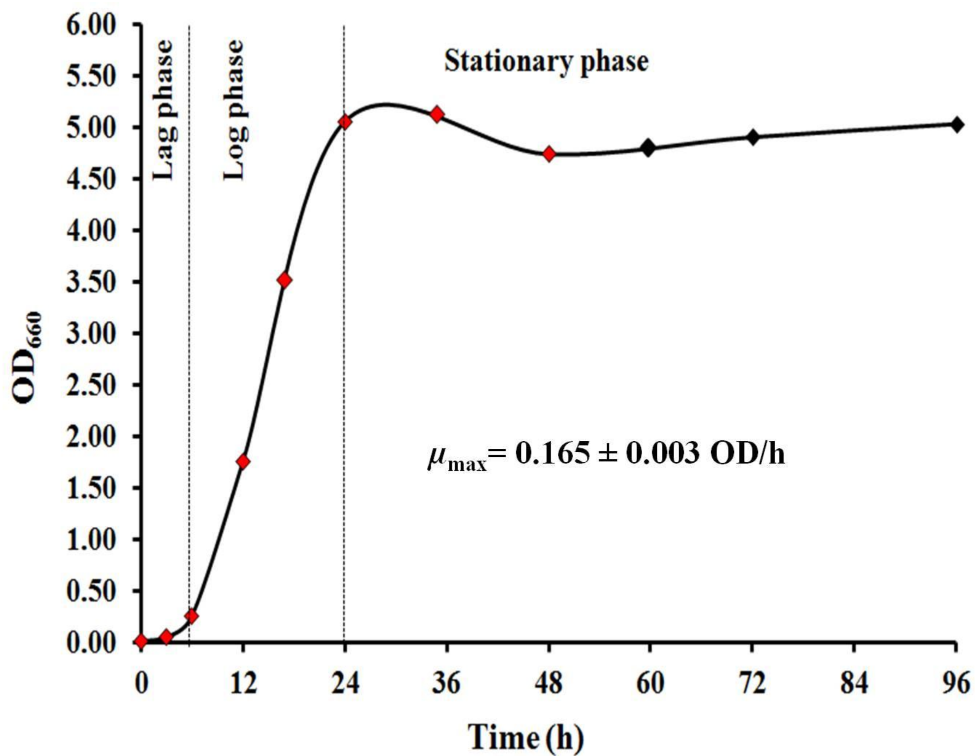

Figure 4.1 Growth curve of *L. casei subsp. rhamnosus*

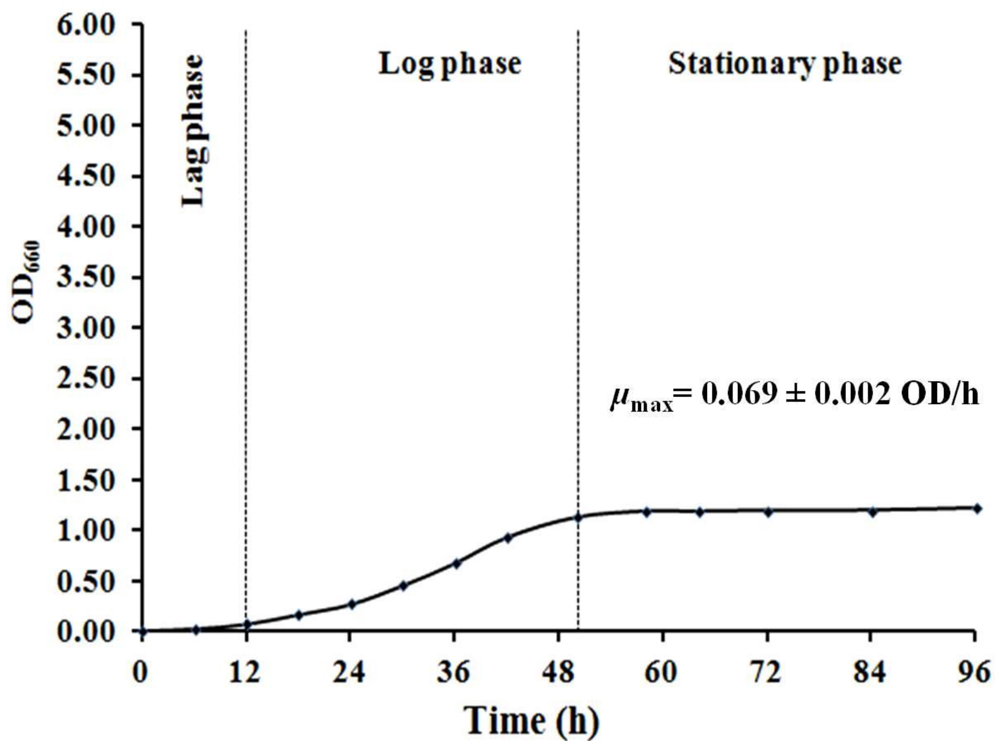

Figure 4.2 Growth curve of *L. bulgaricus*

4.1.2 Comparison of accumulation and biotransformation of selenium in probiotics strains

L. casei subsp. rhamnosus and *L. bulgaricus* were grown in MRS broth medium supplementing with SeO_3^{2-} ion from Na_2SeO_3. The final concentration of Se in the MRS broth medium was 2.5 mg Se L^{-1} which was adapted from the previous reports (Xia *et al*., 2007, Alzate *et al*, 2007). The cultures of *L. casei subsp. rhamnosus* and *L. bulgaricus* were incubated at 37℃ and 150 rpm. The dried weight of *L. casei subsp. rhamnosus* and *L. bulgaricus* were harvested at 48 and 72 h, respectively. Figure 4.3 presents the dried weight from control and Se-supplemented groups of *L. casei subsp. rhamnosus* and *L. bulgaricus*. The results showed that the dried weight of cells pellet from Se-enriched probiotics groups were lower than the control groups. This finding indicated that the growth of the probiotics was inhibited with supplementing Se in the form of SeO_3^{2-} ion (Gerrard *et al*., 1974; Silverberg *et al*., 1976; Turner *et al*., 1998; Xia *et al*., 2007). Furthermore, it was observed that the dried weight of Se-enriched *L. casei subsp. rhamnosus* was higher than *L. bulgaricus*. Therefore, the *L. casei subsp. rhamnosus* strain could resist the toxicity of SeO_3^{2-} ion in the MRS broth medium more than the *L. bulgaricus* strain.

The total Se concentration was utilized for investigating accumulation of Se in the proiotic culture. The results showed that total Se in dried weights of *L. bulcaricus* was higher than *L. casei subsp. rhamnosus* (Figure 4.4). Meanwhile, the obtained total Se content in the remained-medium of *L. casei subsp. rhamnosus* was higher than *L.bulcaricus* (Figure 4.5). Therefore, the *L. bulgaricus* strain could remove SeO_3^{2-} ion from the MRS medium, and accumulate at the cells more than the *L. casei subsp. rhamnosus* strain.

Determination of Se species by ion-pairs reversed phased HPLC-ICP-MS was utilized to study biotransformation of SeO_3^{2-} ion to be organic Se compounds in the probitic strains. Chromatographic profile of Se compound standards; SeO_3^{2-} ion, Sec, SeM and SeMC is shown in Figure 4.6. After preparation of Se-enriched probiotic cells with enzymatic extraction, the obtained Se species in the extracts of the Se-enriched probiotics analyzing by the chromatographic system are presented in Figure 4.7. It was found that Sec and unknown 1 (U1) were determined as the major Se species in the both probiotic strains. Meanwhile, SeO_3^{2-} ion was only found in Se-enriched *L. bulgaricus*.

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Other important organic Se compounds such as SeM and SeMC were only obtained in Se-enriched *L. casei subsp. rhamnosus*. Although the *L. bulgaricus* strain could absorb and accumulate SeO_3^{2-} ion more than the *L. casei subsp. rhamnosus* strain. However, the ability of *L. casei subsp. rhamnosus* strain for transforming SeO_3^{2-} ion to be organic Se compounds was better than the *L. bulgaricus* strain. Thus, the *L. casei subsp. rhamnosus* strain was selected to further study because it could grow and resist the toxicity SeO_3^{2-} ion than the *L. bulgaricus* strain. Moreover, it could highly transform SeO_3^{2-} ion to be Sec with a good yield.

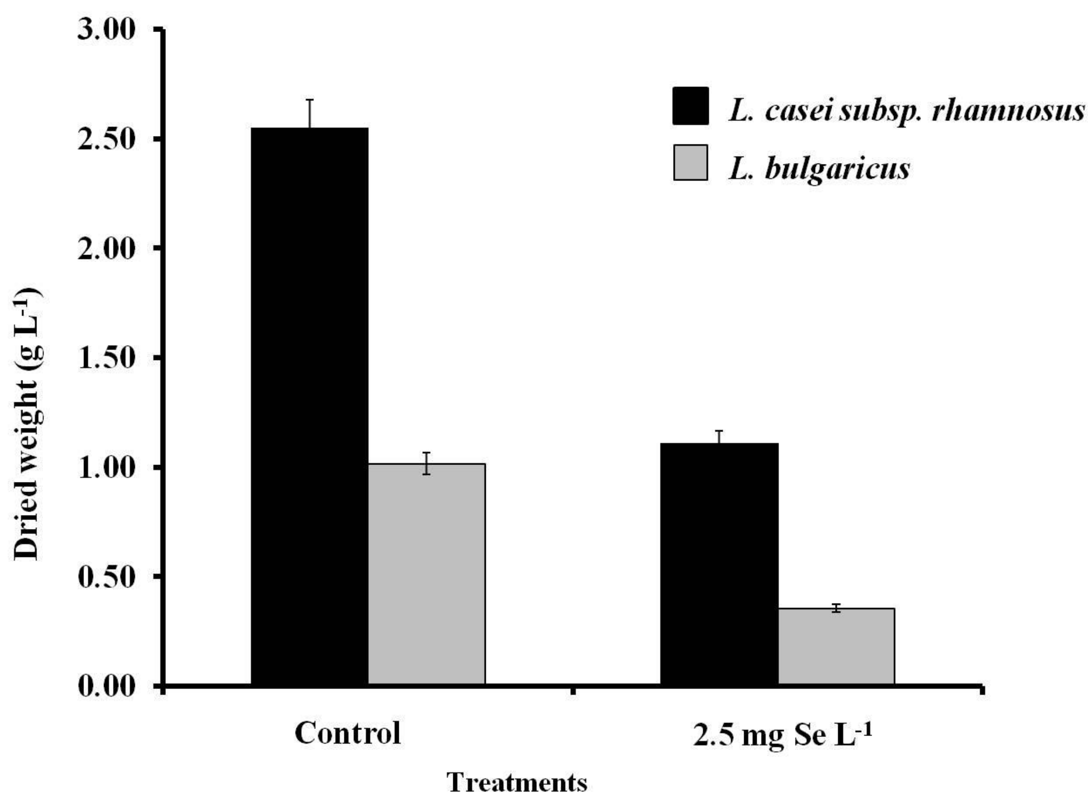

Figure 4.3 The comparison of dried weight harvesting from control and Se-supplemented with 2.5 mg Se L^{-1} groups of *L. casei subsp. rhamnosus* and *L. bulgaricus* cells

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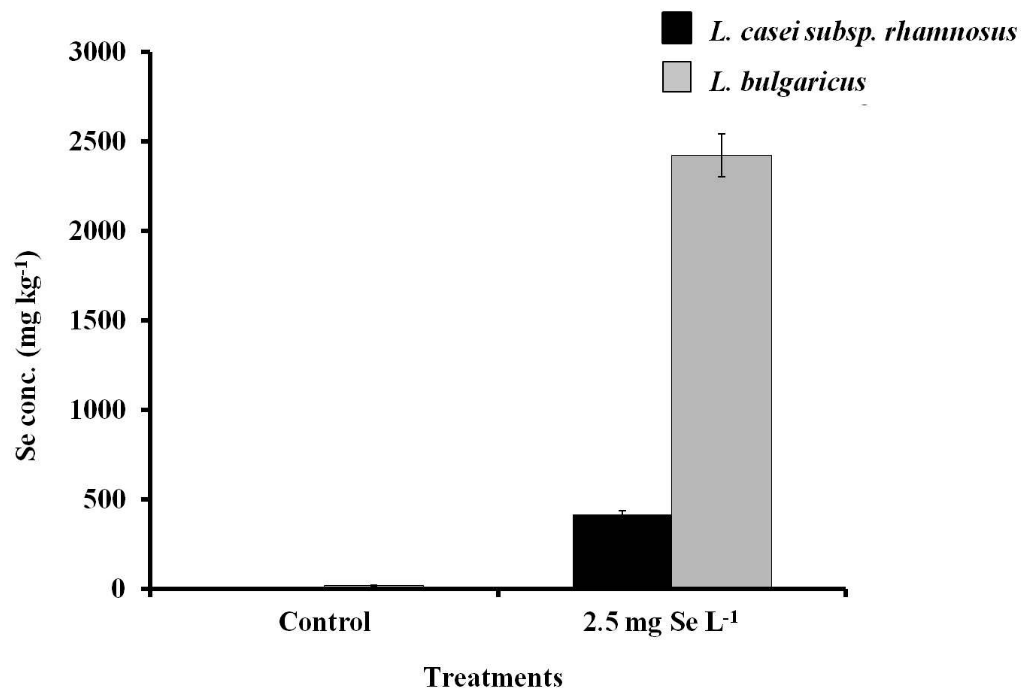

Figure 4.4 The comparison of total Se concentration in dried cells of control and Se-supplemented groups of *L. casei subsp. rhamnosus* and *L. bulgaricus*

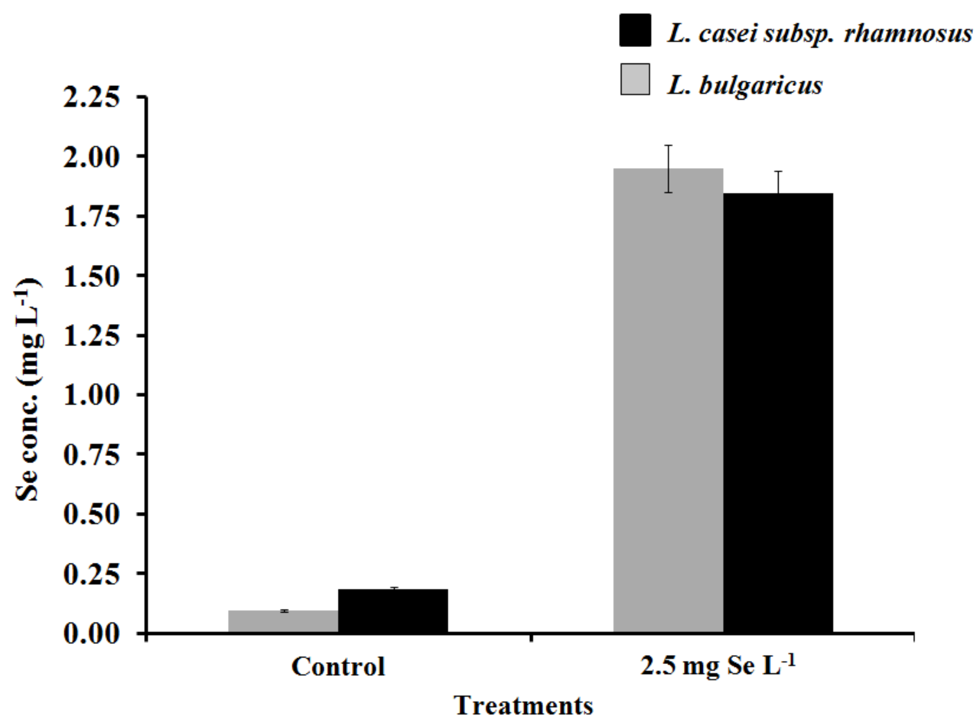

Figure 4.5 The comparison of total Se concentration in remained-medium of control and Se-supplemented groups of *L. casei subsp. rhamnosus* and *L. bulgaricus*

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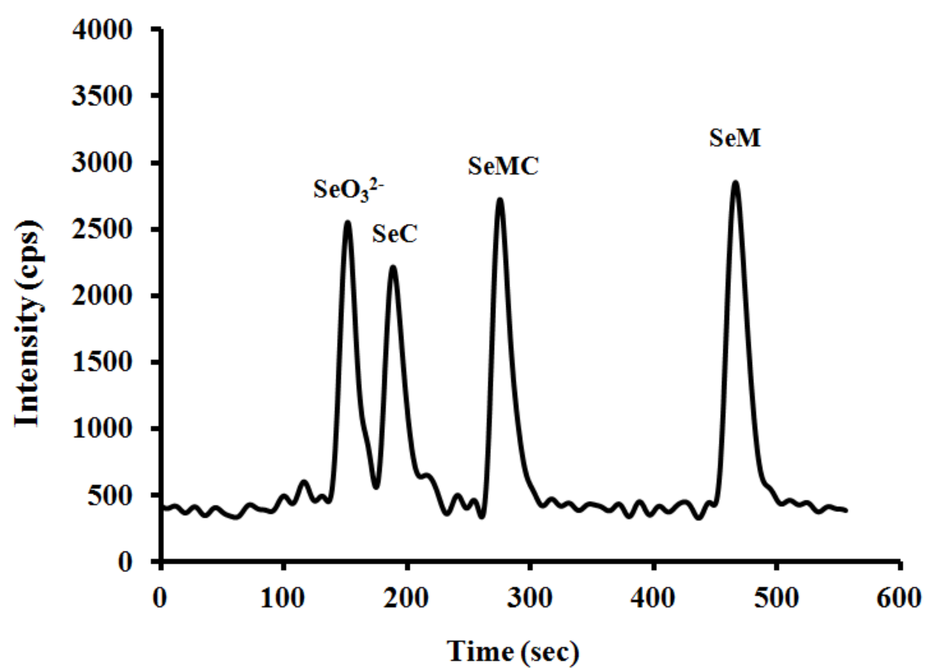

Figure 4.6 Chromatogram of Se species standards determining by ion pairs reversed phase HPLC-ICP-MS

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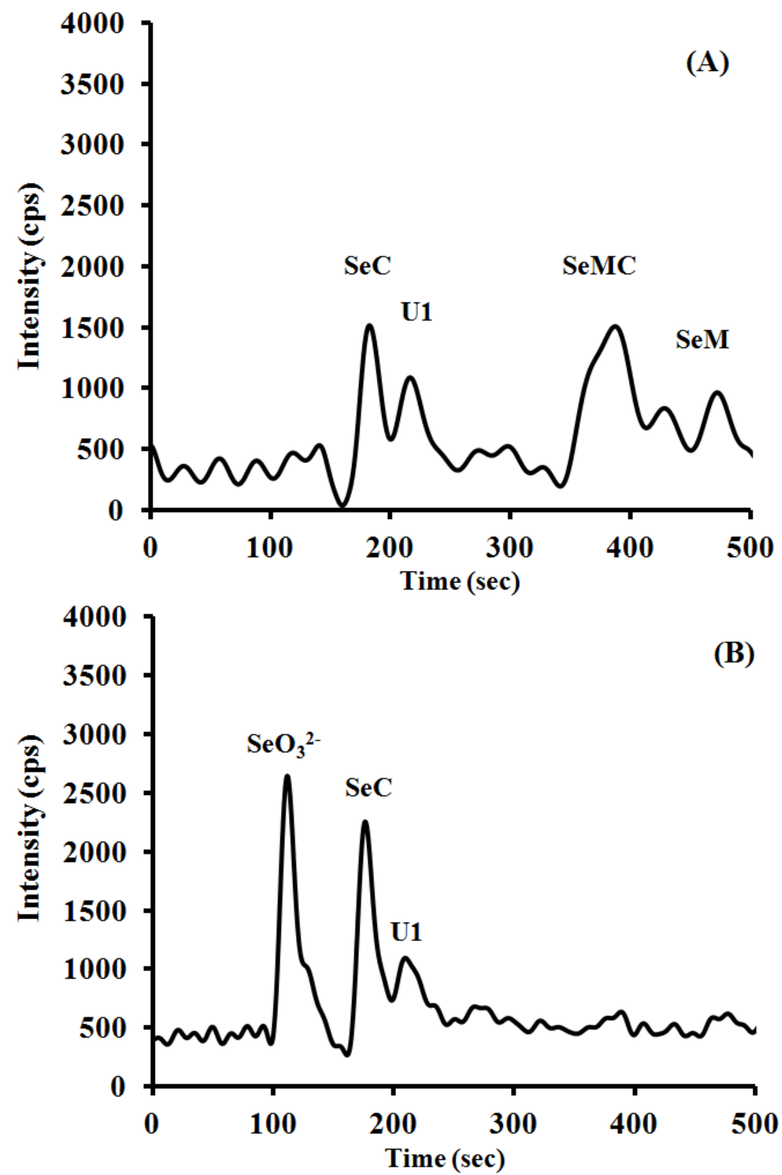

Figure 4.7 Chromatogram of Se species in dried cells of Se-enriched probiotics; *L. casei subsp. rhamnosus* (A) and *L. bulgaricus* (B)

4.2.3 The effect of Se-supplemented concentration on Se content in probiotics

The effect of Se-supplemented concentrations on the growth, and Se accumulation of *L. casei subsp. rhamnosus* was studied in the range of 0 to 7.0 mg Se L^{-1} that shows in Table 4.1. The result found that the dried weight of *L. casei subsp. rhamnosus* was rapidly decreased with an increasing Se-supplemented concentration in the MRS medium (Figure 4.8). Consequently, addition of Se in the form of SeO_3^{2-} ion would inhibit the growth of *L. casei subsp. rhamnosus* when compared with the control group.

This result related to the obtained images from scanning electron microscope that exhibited morphology of *L. casei subsp. rhamnosus* cells treated with or without SeO_3^{2-} ion from Na_2SeO_3 (Figure 4.9). Generally, the morphology of *L. casei subsp. rhamnosus* cells is rod shape, and occurring in short chains that shows in the control group (Salminen *et al*., 2002). In this work, it was found that the abnormal cells were observed when *L. casei subsp. rhamnosus* was treated with SeO_3^{2-} ion above 4 mg Se L^{-1}. Hence, supplementation of high concentration of SeO_3^{2-} ion in the culture medium is a toxic condition which affects to the survival and cell division of *L. casei subsp. rhamnosus.* Therefore, the yield of dried cells was also decreased.

Additionally, the biomass was appeared red color of elemental selenium (Se^0) when Se-supplemented concentration in MRS broth was greater than or equal to 4 mg Se L^{-1}. This is a general pathway for surviving of *Lactobacillus* at high Se-supplemented concentration in culture medium (Xia *et al*., 2007; Yazdi *et al*., 2013; Kheradmand *et al*., 2014). It indicated that *L. casei subsp. rhamnosus* would rapidly detoxify excess SeO_3^{2-} ion concentration in the MRS medium by reducing Se^{4+} to be elemental selenium (Se^0).

An increasing Se-supplemented concentration in the medium culture resulted in an increased Se accumulation in *L. casei subsp. rhamnosus* cells. As demonstrated in Table 4.2, the total Se content in the dry weight of *L. casei subsp. rhamnosus* was rapidly increased from 6.31 ± 1.38 to 2502.40 ± 34.74 mg kg^{-1} with increasing the Se-supplemented concentration from 0 to 7.0 mg L^{-1}. Otherwise, it can be seen that the total Se contents in the remained-mediums were lower than the expected Se-supplemented concentrations in the culture mediums. The finding revealed that the

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SeO_3^{2-} ion in the culture medium was removed to be accumulated at the *L. casei subsp. rhamnosus* cells (Calomme *et al*., 1998).

To understand the distribution of Selenium in the *L. casei subsp. rhamnosus* culture, the accumulation percent of Se in dried weight, remained medium was calculated with equation 4.1 and 4.2.

$$SDP_{bm} = \frac{Se_{bm} \times 100}{Se_w} \quad (4.1)$$

Where SDP_{bm} was selenium distribution percentages of biomass, Se_{bm} was the amount of as Se in biomass, and Se_w was Se concentration in the whole culture.

$$SDP_{rm} = \frac{Se_{rm} \times 100}{Se_w} \quad (4.2)$$

Where SDP_{rm} was selenium distribution percentages of remained-medium, and Se_{rm} was the amount of as Se in remained-medium.

As demonstrated in Table 4.2, the amounts of Se were mostly distributed in the remained-medium (60-85%) while the accumulation of Se in the *L. casei subsp. rhamnosus* cells was only about 12-24%. The percents of Se accumulation in dried weight of each experiment groups were significantly different ($p < 0.05$), and it was mostly obtained in the sample from supplementing with 1.0 mg Se^{-1} group. In the remained-medium, the accumulation percents were not significantly different in the samples from supplementing with 2.5 to 7.0 mg Se L^{-1} group ($p < 0.05$). It was least in the remained-medium from supplementing with 1.0 mg Se^{-1} group. The finding indicated that least amount of SeO_3^{2-} ion in the culture medium was removed to accumulate at *L. casei subsp. rhamnosus* cells. For Se-enriched *L. casei subsp. rhamnosus* growing in high concentration in medium culture, the Se-accumulated at *L. casei subsp. rhamnosus* cells the was not uptake

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Table 4.1 Total Se concentrations in dried weight and remained-medium of *L. casei subsp. rhamnosus* from culturing with different Se-supplemented concentration

Se-supplemented conc. ($mg\ L^{-1}$)	Total Se concentration	
	Dried cells ($mg\ Se\ kg^{-1}$)	Remained-medium ($mg\ Se\ L^{-1}$)
0	6.31 ± 1.38^{a}	0.06 ± 0.001^{a}
1.0	154.67 ± 2.48^{b}	0.82 ± 0.033^{b}
2.5	245.70 ± 19.44^{b}	2.10 ± 0.166^{c}
4.0	512.62 ± 22.96^{c}	3.27 ± 0.209^{d}
5.5	1082.94 ± 92.51^{d}	4.14 ± 0.071^{e}
7.0	2502.40 ± 34.74^{e}	4.38 ± 0.209^{e}

[a, b, c, d and e] The mean difference for significance of total Se concentrations in column at $p < 0.05$ (n = 3).

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Table 4.2 Distribution of Se *L. casei subsp. rhamnosus* culture

Se conc. addition ($mg\ L^{-1}$)	Amount of Se in distribution in whole sample 1000 mL (mg)			Accumulation percentage of Se (% ± SD)	
	whole cells	RM	Cells + RM	Dried weight	RM
1.0	0.24 ± 0.018	0.61 ± 0.028	1.0 ± 0.16	24.25 ± 1.84	62.11 ± 2.74
2.5	0.31 ± 0.015	2.04 ± 0.017	2.5 ± 0.19	12.51 ± 0.61	85.72 ± 8.76
4.0	0.63 ± 0.011	3.54 ± 0.29	4.1 ± 0.19	15.71 ± 0.28	83.31 ± 2.23
5.5	0.98 ± 0.097	4.65 ± 0.37	5.3 ± 0.14	17.28 ± 1.77	83.42 ± 6.66
7.0	1.26 ± 0.019	6.06 ± 0.36	7.0 ± 0.014	18.24 ± 0.28	85.23 ± 5.07

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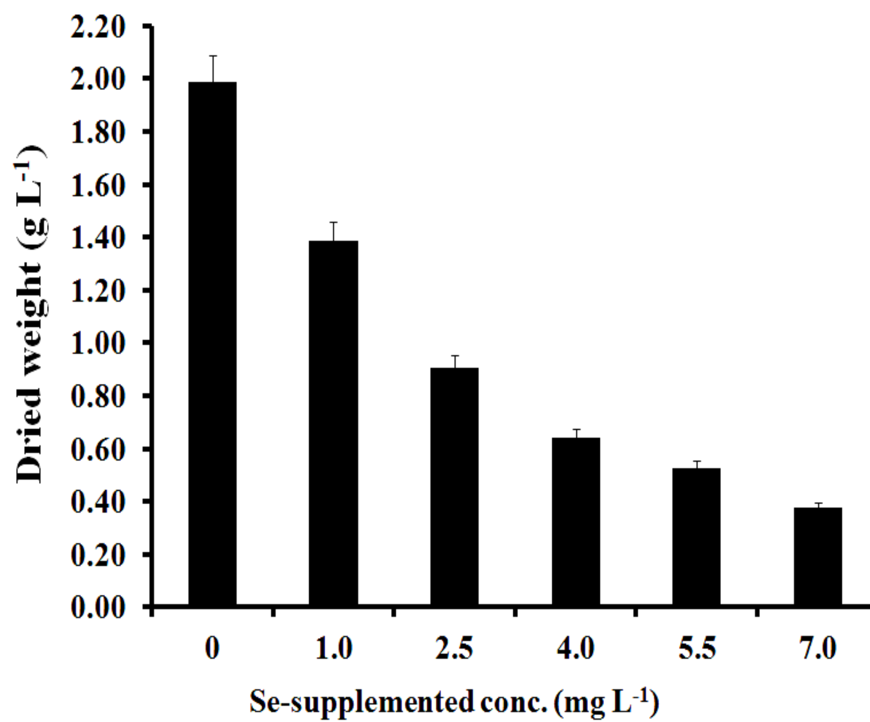

Figure 4.8 The effect of Se-supplemented concentration on the dried weight of Se-enriched *L. casei subsp. rhamnosus* cells

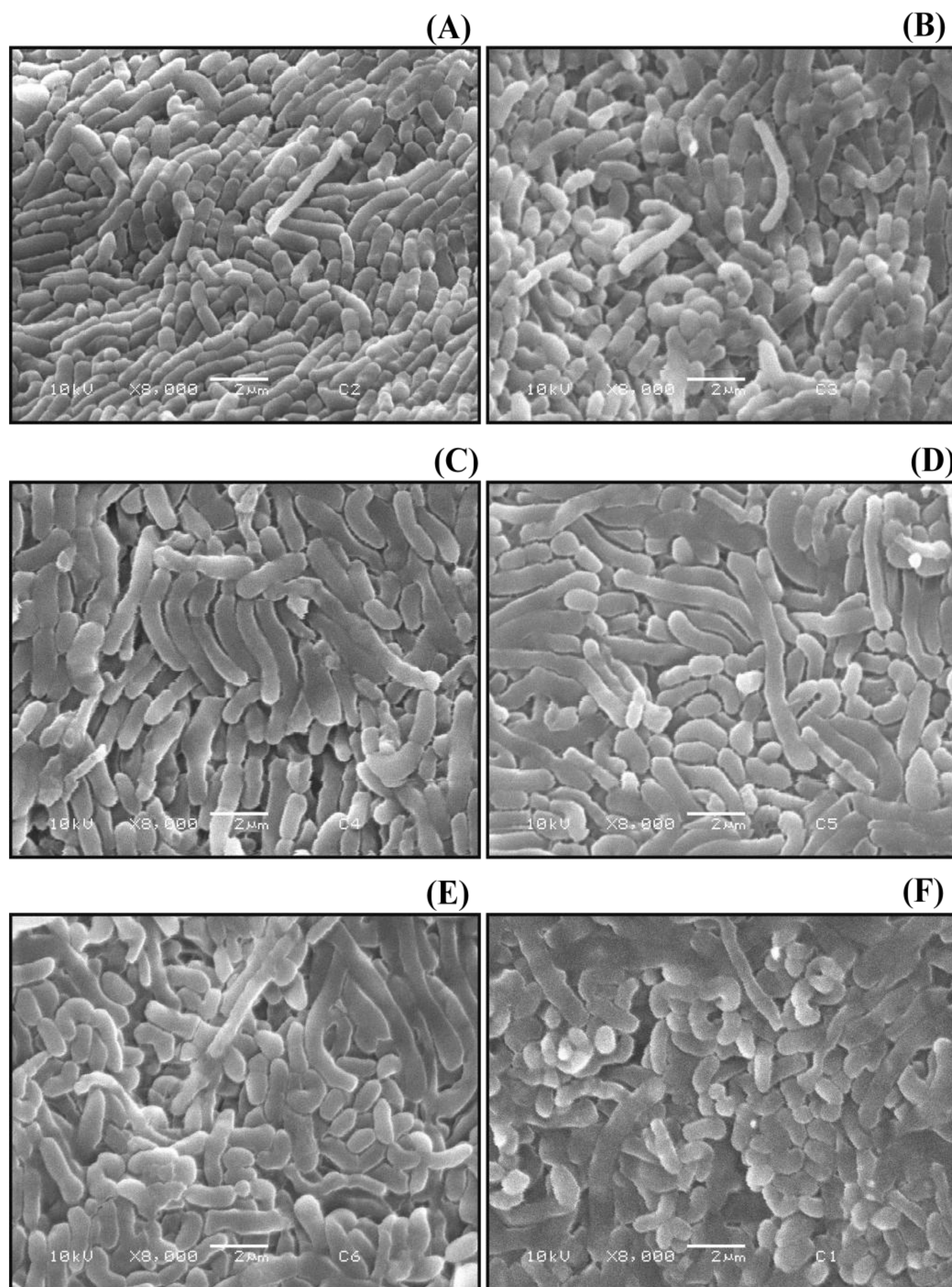

Figure 4.9 SEM photographs of *L. casei subsp. rhamnosus* treated with or without SeO_3^{2-} ion from Na_2SeO_3; (A) = 0, (B) = 1.0, (C) = 2.5, (D) = 4.0, (E) = 5.5 and (F) = 7.0 mg Se L^{-1}

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4.2.4 Determination of extracellular Se species in the remained-medium of Se-enriched *L. casei subsp. rhamnosus*

Remained-medium is the largest part of the culture of Se-enriched probiotics. However, it is few studied, and usually let to be as a by-product after harvesting the biomass of Se-enriched probiotics. Previously, the accumulation and biotransformation of Se is mainly investigated in the intracellular part of Se-enriched probiotics (Andreoni *et al*., 2000; Alzate *et al*., 2007; Galano *et al*., 2013). Meanwhile, determination of Se species in the extracellular environment has not been mentioned. Therefore, the understanding about accumulation and biotransformation of Se have not been covered all part of Se-enriched probiotic culture.

The analysis of extracellular Se species in the remained-medium of Se-enriched *L. casei subsp. rhamnosus* was carried out by ion-pairs reversed phased HPLC-ICP-MS. The remained-medium was few prepared by diluting with mobile phase solution and filtered with nylon membrane (0.45 µm). The chromatogram of Se species standards are shown in Figure 4.10. After determining Se species in the remained-medium, SeO_3^{2-} ion and Sec were only found as the major Se species (Figure 4.11). The obtained SeO_3^{2-} ion was the access amount in the remained-medium that was not absorbed and transformed to be organic Se by *L. casei subsp. rhamnosus*. Moreover, the detected Sec could be defined as free amino acid form of Sec in extracellular environment of the *L. casei subsp. rhamnosus* culture because the remained-medium was not pretreated with hydrolysis procedure.

To verify the presence peak of SeO_3^{2-} ion and Sec, the standard solution of the both Se species were spiked into the remained-medium prior to injecting into the chromatographic system. Figure 4.12 shows chromatographic profile of recovery test of both Se species. The results presented that intensity of SeO_3^{2-} peak was increased with spiking only SeO_3^{2-} standard while the peak of Sec was constant (Figure 4.12 (B)). In the other hand, the peak of Sec was only increased when it was only spiked (Figure 4.12 (C)). The peaks of SeO_3^{2-} and Sec were already increased with spiking the both Se species standards together (Figure 4.12 (D)). Additionally, the percentage recovery of SeO_3^{2-} and Sec were 98 and 95 %, respectively. Accordingly, the presence Se species peaks of the remained-medium determining by the HPLC-ICP-MS could be

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corroborated that they were non-transformed SeO_3^{2-} and free amino acid form of Sec in remained-medium from Se-enriched *L. casei subsp. rhamnosus* culture.

The concentrations of SeO_3^{2-} ion and Sec in the remained-medium are shown in Figure 4.13. It was found that an increasing of Se-supplemented concentration in MRS broth medium affected on increment of the obtained concentrations of SeO_3^{2-} ion and Sec in the remained-medium. However, the tendency of Sec concentration was decreased when the Se-supplemented concentration in MRS broth was above 4 mg Se L^{-1}. This observation could be discussed that supplementation of Se in MRS medium above 4 mg Se L^{-1} was toxic condition to the growth of *L. casei subsp. rhamnosus*which was related to the previous results. Hence, it also affected on biosynthesis of Sec in *L. casei subsp. rhamnosus*. Moreover, SeO_3^{2-} ion and Sec were also found in the remained-medium of Se-enriched *L. bulgaricus* (Figure 4.14) which confirmed the results of Se-enriched *L. casei subsp. rhamnosus*.

In discussion of the finding, SeO_3^{2-} ion was transported into *Lactobacillus* cells, and synthesized to be Sec as a primary selenoamino acid through the biosynthetic pathway (Lamberti *et al*., 2011; Galano *et al*., 2013). Normally, Sec is incorporated in to peptide chain as selenoproteins in response to UGA codons and Sec insertion sequence (SECIS) (Zhang and Gladyshev, 2005; Zhang *et al*., 2009; Galano *et al*., 2013). From the observation, it indicated that partial of Sec was not incorporated into selenoproteins and accumulated in each part of *Lactobacillus* cells, while it was also exported to the extracellular environment in the form of free amino acid. However, this was only the primary finding of extracellular Se speciation in *Lactobacillus.* The other Se species in extracellular environment especially selenoprotein should be further studied for extending the understanding about biotransformation and distribution of Se in *Lactobacillus*.

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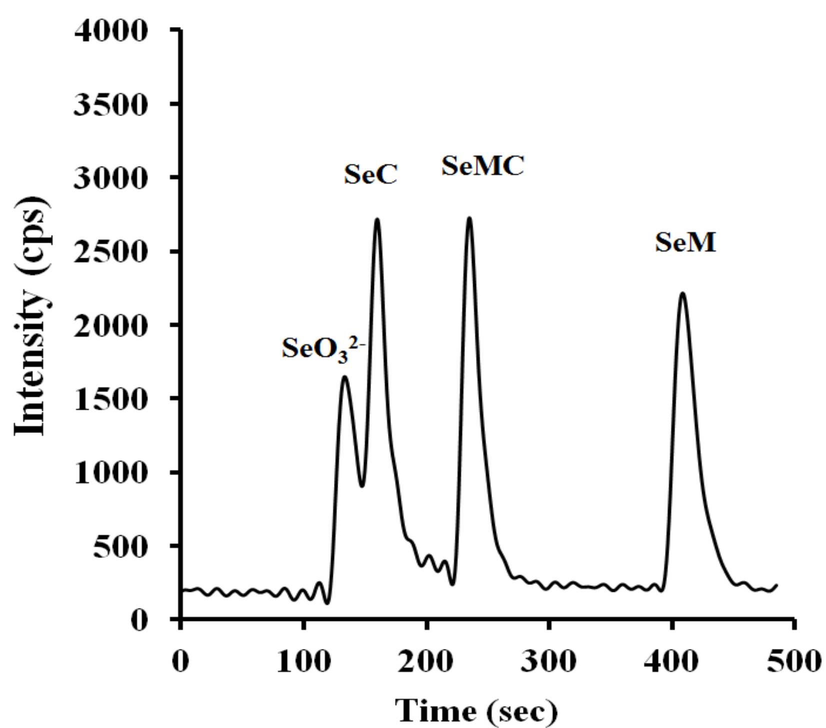

Figure 4.10 Chromatogram of mixed standard of selenium species; selenite ion (SeO_3^{2-}), selenocysteine (SeC), Se-methylselenocysteine (SeMC) and selenimethionine (SeM)

Figure 4.11 Chromatograms of selenium species in remained-medium from the cultures of differences Se supplemented concentrations; (A) = 0, (B) = 1.0, (C) = 2.5, (D) = 4.0, (E) = 5.5 and (F) = 7.0 mg Se L^{-1}

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Figure 4.12 Chromatograms for recovery test; (A) RM from the culture of supplementing 2.5 mg Se L^{-1}, (B) spiked SeO_3^{2-} ion, (C) spiked SeC, and (D) spiked both of SeO_3^{2-} ion and SeC.

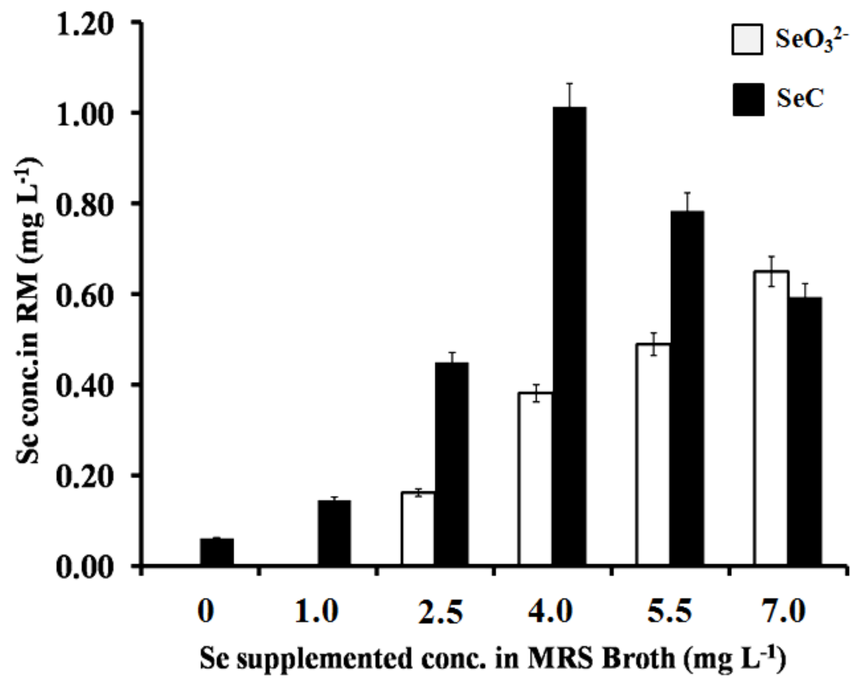

Figure 4.13 Concentrations of SeO_3^{2-} ion and SeC in the remained-medium (RM) from the cultures of different Se-supplemented concentrations in MRS Broth medium.

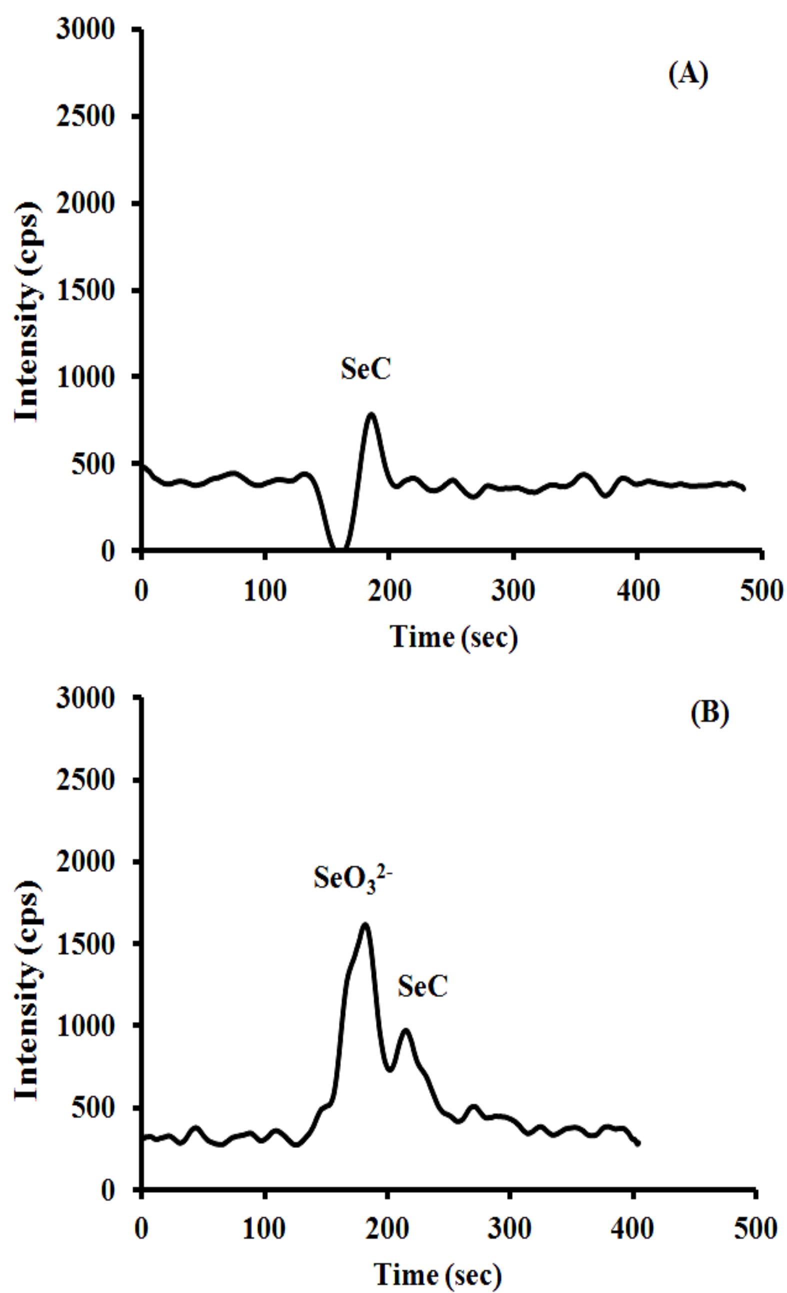

Figure 4.14 Chromatogram of Se speciation in remained-medium of Se-enriched *L. bulgaricus*; control group (A) and Se-supplemented group (B)

4.2.5 The effect of initiation time for adding SeO_3^{2-} ion on Se content probiotics

The initiation time for adding SeO_3^{2-} ion was studied for comparing the Se accumulation and biotransformation when the SeO_3^{2-} ion was treated into different time of the growth curve of *L. casei subsp. rhamnosus*. Figure 4.15 presents the criteria for selecting the time in the growth curve for adding SeO_3^{2-} ion into *L. casei subsp. rhamnosus* culture to be 2.5 mg Se L^{-1} at the final concentration. The selected initiation times were 0, 3, 6, 12, 18 and 24 h that covered the starting point of culture, lag phase, starting point of log phase, log phase and end point of log phase, respectively.

The effect of the initiation time for adding SeO_3^{2-} ion on dried weight of *L. casei subsp. rhamnosus* is showed in Table 4.3. The result exhibited that the dried weights of *L. casei subsp. rhamnosus* from all Se-enriched groups were lower than the control group. The highest and lowest dried weights of *L. casei subsp. rhamnosus* were obtained in adding SeO_3^{2-} ion in the end point of log phase and lag phase, respectively. Moreover, it was observed that the dried weights of adding SeO_3^{2-} ion at the lag phase and starting point of log phase were not significantly different ($p < 0.05$).

Table 4.4 presents the total Se concentrations in dried cells and remained-medium of each Se addition times. As can be seen that the total Se concentrations in dried weight was mostly obtained with adding SeO_3^{2-} ion at the starting point of culture and the end point of log phase. The lowest total Se concentrations in dried weight were obtained with adding SeO_3^{2-} ion at the log phase groups (12 and 18h). In the other hands, the total Se concentrations in dried weight were not significantly different with addition of SeO_3^{2-} ion at the lag phase and the starting point of log phase.

Moreover, the result showed that adding SeO_3^{2-} ion at lag phase, starting point of log phase, log phase and end point of log phase did not significantly affect the total Se concentrations in remained-mediums ($p < 0.05$). Meanwhile, the *L. casei subsp. rhamnosus* adding SeO_3^{2-} ion at the starting point of culture and the end point of log phase gave lower total Se concentration of Se in the remained-medium. It could be discussed that Se was mostly accumulated at *L. casei subsp. rhamnosus* cells when it was added at the starting point of culture and the end point of log phase.

Table 4.5 expresses the distribution of Se in *L. casei subsp. rhamnosus* culturing with different inanition times of adding SeO_3^{2-} ion. It can be observed total Se

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concentrations in the whole cultures were closed to the expected Se-supplemented concentration (2.5 mg Se L^{-1}) except at the end point of log phase. Moreover, the result confirmed that Se was usually accumulated in the remained-medium more than in dried cells.

The Se distribution percentage in dried cells and remained-medium of *L. casei subsp. rhamnosus* culturing at the lag phase, starting point of log phase, and log phase were not significantly different ($p< 0.05$). The total Se was enormously accumulated in the *L. casei subsp. rhamnosus* growing by adding SeO_3^{2-} ion at the starting point of culture and the end point of log phase. Furthermore, the results were observed that the occurring of the red color of elemental Se (Se^0) at the cell pellets of *L. casei subsp. rhamnosus* were observed in the addition times of SeO_3^{2-} ion at the starting point of culture, log phase and the end point of log phase. It indicated that adding SeO_3^{2-} ion at the both time of the growth curve was toxic conditions for *L. casei subsp. rhamnosus*. However, these results were not observed in the addition time of SeO_3^{2-} ion at the lag phase and the starting point of log phase of *L. casei subsp. rhamnosus* growth curve.

The results of Se speciation in the dried cells are presented in Figure 4.16. It can be seen that Sec obtained as the major Se species in all experimental groups. For the other minor Se species such as the unknown Se species (U1) and SeMC were found in some experimental groups. Moreover, SeM was highly found in the dried cells of *L. casei subsp. rhamnosus* growing by adding SeO_3^{2-} ion at the lag phase of *L. casei subsp. rhamnosus* growth curve. Figure 4.17 expresses the chromatograms of Se speciation in the remained-mediums. It was found that SeO_3^{2-} ion and Sec was obtained as the major Se species in the remained-mediums.

These observations indicated that addition of SeO_3^{2-} ion at the lag phase of *L. casei subsp. rhamnosus* was mostly suitable because the *L. casei subsp. rhamnosus* strain was started to assimilate for the new environment of the MRS broth medium (Cooper, 1991). Furthermore, synthesis of RNA, enzymes and other molecules are occurred during the lag phase of bacteria (Khan, 2001; Rolfe *et al.*, 2012). Hence, the biosynthesis pathway of Sec was mostly operated when SeO_3^{2-} was added during the lag phase of *L. casei subsp. rhamnosus*.

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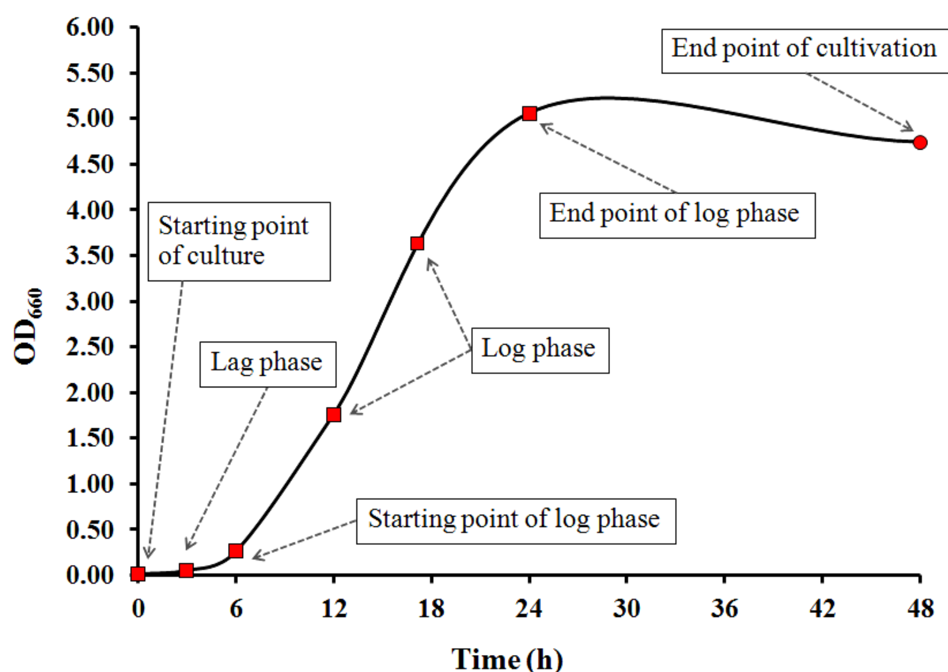

Figure 4.15 The selection criteria of initiation time for adding Se(IV) ion into *L. casei subsp. rhamnosus* culture

Table 4.3 Dried weights of *L. casei subsp. rhamnosus* cultured with different initiation times of adding SeO_3^{2-} ion

Initiation time for adding Se (h)	Dried weight (g L^{-1})
Control	2.55 ± 0.20^{d}
0	1.42 ± 0.10^{b}
3	0.95 ± 0.04^{a}
6	0.97 ± 0.12^{a}
12	1.61 ± 0.01^{b}
18	1.99 ± 0.04^{c}
24	2.10 ± 0.12^{c}

[a, b, c, d] The mean difference for significance of dried weight of *L. casei subsp. rhamnosus* in column at $p < 0.05$ (n = 3)

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Table 4.4 Comparison of the total Se concentrations in dried cells and remained-medium of *L. casei subsp. rhamnosus* cultured with different initiation times of adding SeO_3^{2-} ion

Initiation time for adding Se (h)	Total Se concentration	
	Dried cells (mg Se kg^{-1})	Remained-medium (mg Se L^{-1})
Control	8.06 ± 0.10[a]	0.18 ± 0.002[a]
0	414.99 ± 20.72[d]	1.85 ± 0.02[c]
3	244.94 ± 33.92[c]	2.25 ± 0.10[d]
6	238.38 ± 5.40[c]	2.32 ± 0.03[d]
12	167.94 ± 12.38[b]	2.22 ± 0.15[d]
18	153.76 ± 11.35[b]	2.10 ± 0.08[d]
24	386.49 ± 36.31[d]	1.08 ± 0.02[b]

[a, b, c, d] The mean difference for significance of total Se concentrations in column at $p < 0.05$ (n = 3)

Table 4.5 Distribution of Se in *L. casei subsp. rhamnosus* cultured with different initiation times of adding SeO_3^{2-} ion

Initiation time for adding Se (h)	Amount of Se in each part of the culture 1000 mL (mg)			Se distribution percentage (% ± SD)	
	Dried cells	RM	Whole culture	Dried cells	RM
0	0.49 ± 0.041	1.85 ± 0.02	2.34 ± 0.038	21.11 ± 1.47[b]	78.89 ± 1.42[a]
3	0.28 ± 0.014	2.25 ± 0.10	2.50 ± 0.136	10.84 ± 0.041[a]	89.92 ± 1.30[c]
6	0.21 ± 0.035	2.32 ± 0.03	2.59 ± 0.096	7.63 ± 0.67[a]	89.63 ± 4.55[c]
12	0.29 ± 0.035	2.22 ± 0.15	2.51 ± 0.19	11.47 ± 0.97[a]	88.53 ± 0.91[c]
18	0.31 ± 0.029	2.10 ± 0.08	2.46 ± 0.029	12.72 ± 0.83[a]	87.28 ± 0.80[c]
24	0.83 ± 0.12	1.08 ± 0.02	1.89 ± 0.089	43.27 ± 4.24[c]	53.73 ± 6.33[b]

[a, b, c,] The mean difference for significance of Se distribution percentage in column at $p < 0.05$ (n = 3).

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Figure 4.16 Chromatograms of Se compounds contents in the dried cells of *L. casei subsp. rhamnosus* cultured with different initiation times of adding SeO_3^{2-} ion; (A) = 0 h, (B) = 3 h, (C) = 6 h, (D) = 12 h, (E) = 18 h and (F) = 24 h

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Figure 4.17 Chromatograms of Se compounds contents in the remained-medium of *L. casei subsp. rhamnosus* cultured with different initiation times of adding SeO_3^{2-} ion; (A) = 0 h, (B) = 3 h, (C) = 6 h, (D) = 12 h, (E) = 18 h and (F) = 24 h

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4.2 Development of home-made ED apparatus

Development of home-made ED apparatus was completely designed and constructed that is displayed in Figure 4.18. It consisted of 2 main parts; ED stack and power supply system. The ED stack was a unit for containing solution and dialyzing selenium species through a permeable membrane. The electric current was applied from the power supplier through to the ED graphite electrodes which were installed in the ED stack.

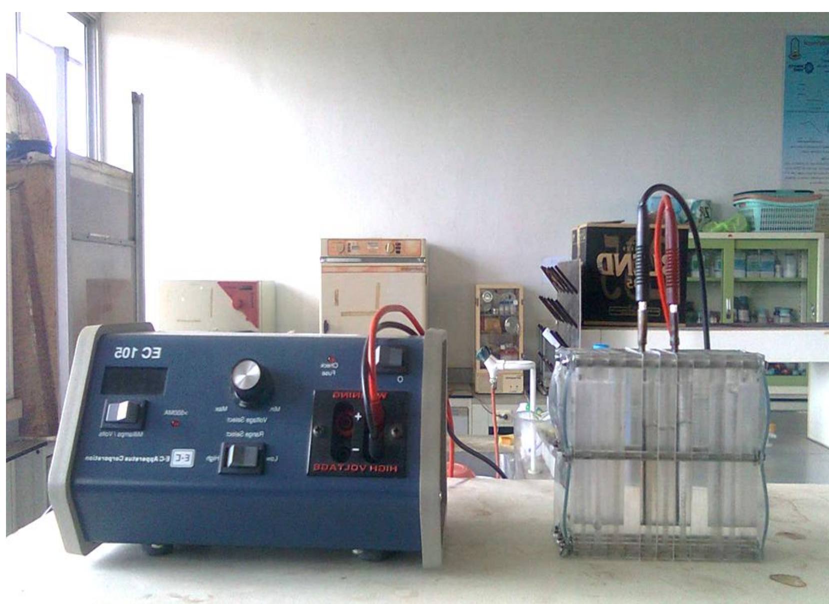

Figure 4.18 The home-made ED apparatus

4.3 Application of home-made ED apparatus for removing SeO_3^{2-} ion in the remained-medium in Se-enriched probiotics

4.3.1 Determination of Se compounds by HG-AAS

Hydride generation - atomic absorption spectrometry (HG-AAS) was utilized for determining Se concentration in electrodialyzed solutions of the remained-medium. The 4 kinds of selenium compounds; SeO_3^{2-} ion, SeC, SeM and SeMC were individually prepared at 20 µg Se L^{-1} and analyzed by HG-AAS. The result showed that the HG-AAS could specifically detect only SeO_3^{2-} ion. Meanwhile, the analytical signal

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of the other selenium compounds were not obtained (Table 4.6). It indicated that the hydride generation is very specific to react with Se^{4+} of SeO_3^{2-} ion (Foster and Sumar, 1996). Hence, the HG-AAS was available to apply for determination of SeO_3^{2-} ion in the remained-medium.

Table 4.6 Comparison of concentration of Se compounds determining by HG-AAS

Se compounds	Se concentration (μg L^{-1}) (n=5)
SeO_3^{2-}	21.51 ± 0.169
SeC	n.d.
SeM	n.d.
SeMC	n.d.
Expected concentration	20

4.3.2 The effect of applied voltage on SeO_3^{2-} ion migration

The effect of applied voltage on SeO_3^{2-} ion migration was studied by using the remained-medium which was supplemented with 2.5 mg Se L^{-1} as the sample. The concentration of SeO_3^{2-} ion in this remained-medium was 160 ± 0.008 μg Se L^{-1}. The results showed that SeO_3^{2-} ion was only migrated from the sample unit to the anode unit. The concentrations of Se in the anode unite were increased with increasing the applied voltage from 20 to 100 volt. Moreover, the concentration of Se was decreased when the applied voltage was over 100 volt (Figure 4.19). Table 4.7 presented the migration percent of SeO_3^{2-} ion to the anode unit by calculating with equation 4.3.

$$\text{Percent migration of } SeO_3^{2-} \text{ ion} = \frac{Se_{an} \times N_{fb} \times 100}{Se_{rm}} \qquad 14.3$$

Where Se_{an} was selenium concentration in the anode unit, N_{fb} was number of frame cell bodies for using as anode unit and Se_{rm} was the Se concentration in diluted remained-medium.

The result was expressed that the percent migration of SeO_3^{2-} ion was mostly observed with applying voltage at 100 volt to the ED stack. Thus, it was selected as the optimum applied voltage for the next experiment.

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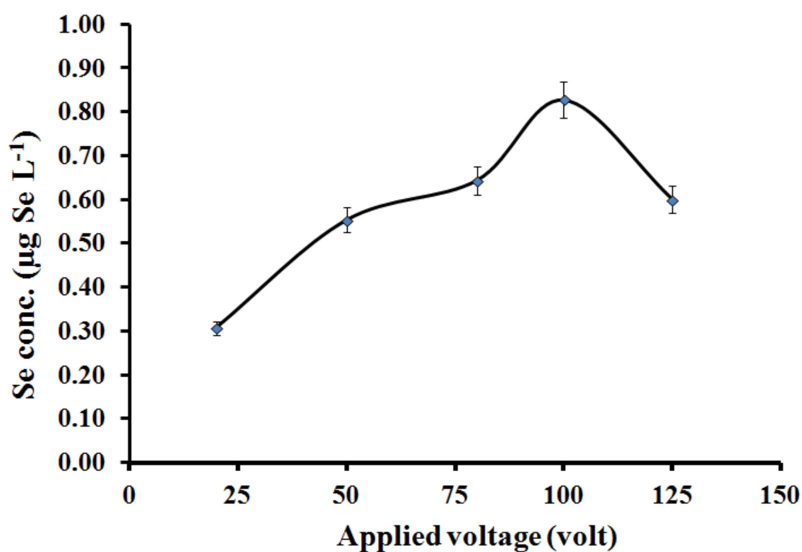

Figure 4.19 The effect of applied voltage on SeO_3^{2-} ion migration to the anode unit of ED stack

Table 4.7 Percent migration of SeO_3^{2-} ion in the anode unit of the ED stack with applying different voltages

Applied voltage (volt)	Migration percent of SeO_3^{2-} ion (%)
20	3.84 ± 0.16^a
50	7.22 ± 0.52^b
80	8.05 ± 0.51^b
100	10.95 ± 1.29^c
125	7.50 ± 0.71^b

[a, b and c] The mean difference for significance of migration percent of SeO_3^{2-} ion in column at $p < 0.05$ (n = 3)

4.3.3 The effect of electrodialysis time on SeO_3^{2-} ion migration

The effect of electrodialysis time on SeO_3^{2-} ion migration to the anode unit of ED stack is shown in Figure 4.20. The result exhibited that SeO_3^{2-} ion concentrations in the anode unit were increased with increasing electrodialysis time. Table 4.8 presents migration percent of SeO_3^{2-} ion in the anode unit of ED stack. The result exhibited that the ED apparatus could remove 50% SeO_3^{2-} ion in the remained-medium to the anode

unit within 15 min. Furthermore, it was observed that the temperature of solution in the ED stack was also increased when increased the electrodialysis time. This observation was affected from the Joule heating effect when the ions in solution are moved in electric field (Evenhuis and Haddad, 2009). Thus, some organic Se compound could be degraded when the temperature of solution was increased (Zhang et al. 2000). However, this result is a data for further developing the ED apparatus.

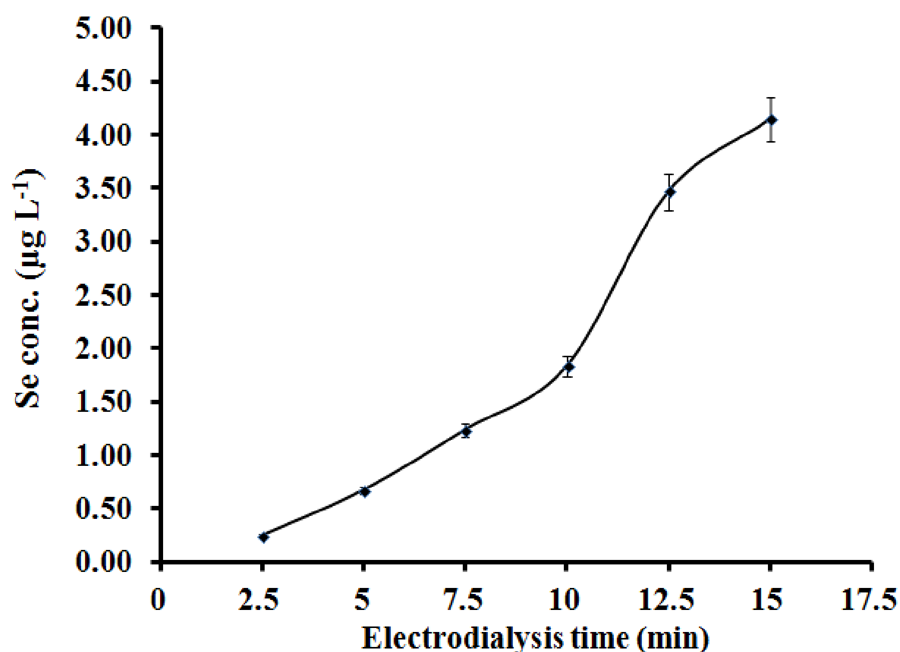

Figure 4.20 The effect of different electrodialysis time on SeO_3^{2-} ion migration to the anode unit of ED stack

Table 4.8 Percent migration of SeO_3^{2-} ion in the anode unit of the ED stack with different electrodialysis time

Electrodialysis time (min)	Migration percent of SeO_3^{2-} ion (%)
2.5	3.07 ± 0.10
5.0	8.39 ± 1.10
7.5	15.46 ± 3.59
10.0	22.91 ± 0.98
12.5	43.36 ± 2.04
15.0	51.86 ± 3.44

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CHAPTER 5

CONCLUSIONS

The development of Se-enriched probiotics process was begun by comparing the accumulation and biotransformation of Se in the *L. casei subsp. rhamnosus* (TISTR No. 372) and *L. bulgaricus* (TISTR No. 451) strains. It could be seen that the *L. bulgaricus* strain could accumulate SeO_3^{2-} ion at the biomass greater than the *L. casei subsp. rhamnosus*is strain. However, the *L. casei subsp. rhamnosus* strain could transform SeO_3^{2-} ion to be organic Se compounds better than the *L.bulgaricus* strain. In this work, the *L. rhamnosus* strain was selected to further study because it could better grow and resist the toxicity SeO_3^{2-} ion than the *L.bulgaricus* strain. Moreover, it could highly transform SeO_3^{2-} ion to be Sec and other organic Se compounds with a good yield.

The conditions of Se-enriched *L. casei subsp. rhamnosus* cultivation, Se-supplemented concentration and initiation time for adding SeO_3^{2-} ion into the culture were optimized. The production of Se-enriched *L. casei subsp. rhamnosus* was achieved by uptake SeO_3^{2-} ion with 2.5 mg Se L^{-1} into the culture medium at the lag phase of growth curve. The accumulation of Se *L. casei subsp. rhamnosus* culture was mostly obtained in the remained-medium than the dried weight. For Se speciation analysis in Se-enriched *L. casei subsp. rhamnosus* culture, Sec was the major Se compound that could be determined in both the remained-medium and the dried cells. Therefore, SeO_3^{2-} ion was transported into *Lactobacillus* cells, and synthesized to be Sec as a primary selenoamino acid though the biosynthetic pathway. From the previous reports, Sec is normally incorporated in to peptide chain as selenoproteins. However, the finding of this work indicated that the partial of Sec was not incorporated into selenoproteins and accumulated in each part of *Lactobacillus* cells, while it was also exported to the extracellular environment in the form of free amino acid.

The growing of the *L. casei subsp. rhamnosus* with high SeO_3^{2-} ion concentration in the culture medium would rapidly detoxify excess SeO_3^{2-} ion by reducing oxidation state of Se^{4+} to be elemental selenium (Se^0) particle that was adhered at the cell membrane. Moreover, this phenomenon of Se detoxification was also

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observed when SeO_3^{2-} ion was added at the starting point of culture, during the range of log phase and the end point of log phase.

Development of home-made ED apparatus was carried out by designing and constructing based on separation efficiency of selenium species, invention cost, suitable material, easy operation and versatile application. The structure of home-made ED apparatus consisted of 2 main parts; ED stack and power supply system. The ED stack was a unit for containing solution and dialyzing selenium species through a permeable membrane. It consisted of frame cell body, dialysis membrane and sealing system and lock system. Applying of electric current to the ED stack was performed by using a power supply system that composed with graphite electrodes and power supply apparatus.

The home-made ED apparatus was achieved for using to remove SeO_3^{2-} ion from the remained-medium. The optimum applied voltage for driving the SeO_3^{2-} ion from the sample unit to the anode unit was 100 volt. It could remove 50 % SeO_3^{2-} ion from the remained-medium within 15 min. Thus, the home-made ED apparatus could be used to eliminate SeO_3^{2-} ion from the remained medium.

From all of the results, this work achieved to develop a new design and construct the home-made apparatus. It could be applied to eliminate SeO_3^{2-} ion from the remained-medium of Se-enriched probiotics. However, it should be improved some part of the design, material and application. Moreover, the result of this work provided the primary data for further developing the ED apparatus.

For Se-enriched probiotics, this work carried out to produce Se-enriched *L. casei subsp. rhamnosus*. It could be used as a guideline for supplementing SeO_3^{2-} ion into other lactobacillus species. Nevertheless, Se-enriched *L. casei subsp. rhamnosus* should be intensively study about biotransformation of Se and also its applications.

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REFERENCES

Mahasarakham University

REFERENCES

Agilent Technologies (2014) User's Guide of Agilent Vapor Generation Accessory VGA 77 and VGA 77P, Agilent Technologies Inc., USA, pp 66-67.

Aguilar F, Autrup H, Barlow S, Castle L, Crebelli R, Dekant W, Engel KH,… Toldrá F (2008) Selenium-enriched yeast as source for selenium added for nutritional purposes in foods for particular nutritional uses and foods (including food supplements) for the general population. *EFSA Journal*, 30 (766), 1-42.

Aguilar F, Charrondiere UR, Dusemund B, Galtier P, Gilbert J, Gott DM,…Woutersen R (2009) L-selenomethionine as a source of selenium added for nutritional purposes to food supplements. *EFSA Journal*, 1082, 1-39.

Allmang C and Krol A (2006) Selenoprotein synthesis: UGA does not end the story. *Biochimie*, 88(11)1561-1571.

Alzate A, Cañas B, Pérez-Munguía S, Hernández-Mendoza H, Pérez-Conde C, Gutiérrez AM and Cámara C (2007) Evaluation of the Inorganic Selenium Biotransformation in Selenium-Enriched Yogurt by HPLC-ICP-MS. *Journal of Agricultural and Food Chemistry*, *55*, 9776–9783.

Alzate A, Fernandez-Fernandez A, Perez-Conde MC, Gutierrez A M and Camara C (2008) Comparison of Biotransformation of Inorganic Selenium by *Lactobacillus* and *Saccharomyces* in Lactic Fermentation Process of Yogurt and Kefir. *Journal of Agricultural and Food Chemistry*, 56, 8728-8736.

Andoh A, Hirashima M, Maeda H, Hata K, Inatomi O, Tsujikawa T,…Fujiyama Y (2005) Serum selenoprotein-P levels in patients with inflammatory bowel disease. *Nutrition,* 21, 574-579.

Angstwurm MWA and Gaertner R (2006) Practicalities of selenium supplementation in critically ill patients. *Current Opinion in Clinical Nutrition & Metabolic Care*, 9, 233-238.

Andreoni V, Luischi MM, Cavalca L, Erba D, Ciappellano S (2000) Selenite tolerance and accumulation in the Lactobacillus species. *Annual Microbiology*, 50(1), 77-88.

Arnér E and Holmgren A (2000) Physiological functions of thioredoxin and thioredoxin reductase. *European Journal of Biochemistry* 267, 6102-9.

Mahasarakham University

Behne D, Hammel C, Pfeifer H, Röthlein D, Gessner H and Kyriakopoulos A (1998) Speciation of selenium in the mammalian organism. *Analyst*, 123, 871–873.

Batley GE (1990) *Trace Element Speciation Analytical Methods and Problems,* Boca Raton, CRC press.

Behne D and Kyriakopoulos A (2001) Mammalian selenium-containing proteins. *Annual Review of Nutrition*, 21, 453-473.

B'Hymer C and Caruso JA (2006) Selenium speciation analysis using inductively coupled plasma-mass spectrometry. *Journal of Chromatography A*, 1114, 1-20.

Böck A (2000) Biosynthesis of selenoproteins - an overview. *BioFactors*, 1, 77-78.

Böck A (2001) *"Selenium metabolism in bacteria" in Selenim*, Springer US.

Boyle RJ, Robins-Browne RM and Tang ML (2006)Probiotic use in clinical practice: what are the risks?. *The American Journal of Clinical Nutrition*, 83(6), 1256-64.

Brown KM and Arthur JR (2001) Selenium, selenoproteins and human health: a review. *Public Health Nutrition*, (2B), 593-9.

Burk R and Hill K (2005) Selenoprotein P: an extracellular protein with unique physical characteristics and a role in selenium homeostasis. *Annual Review of Nutrition*, 25, 215-235.

Burk RF (1991) Molecular biology of selenium with implications for its metabolism. *FASEB*, 5, 2274-2279.

Burk RF and Levander OA (2006) *Seleium in Modern Nutrition in Health and Disease.* Philadelphia, Lippincott Williams & Wilkins.

Calomme MR, Hu J, Van DBK and Vanden BD (1995) Seleno-lactobacillus. *Biological Trace Element Research*, 47, 379-383.

Calomme MR Van DBK and Vanden BDA (1995) Selenium and *Lactobacillus* species. *Journal of Applied Microbiology*, 79, 331-340.

Cankur O, Yathavakilla SKV and Caruso JA (2006) Selenium speciation in dill (Anethum graveolens L.) by ion pairing reversed phase and cation exchange HPLC with ICP-MS detection. *Talanta*, 70, 784-790.

Capelo JL, Ximenez-Embun P, Madrid-Albarran Y and Camara C (2003) Enzymatic Probe Sonication: Enhancement of Protease-Catalyzed Hydrolysis of Selenium Bound to Proteins in Yeast. *Analytical Chemistry*, 76, 233-237.

Mahasarakham University

Casiot C, Szpunar J, Łobinski R and Potin-Gautier M (1999) Sample preparation and HPLC separation approaches to speciation analysis of selenium in yeast by ICP-MS. *Journal of Analytical Atomic Spectrometry*, 14, 645–650.

Charalampopoulos D, Pandiella SS and Webb C (2003) Evaluation of the effect of malt, wheat and barley extracts on the viability of potentially probiotic lactic acid bacteria under acidic conditions. *International Journal of Food Microbiology*, 82(2), 133-41.

Chantiratikul A, Aengwanich W, Chinrasri O and Chantiratikul P (2008) Plasma Selenium Concentration and Glutathione Peroxidase Activity in Red Blood Cells of Laying Hens Fed Sodium Selenite or Zinc-L-Selenomethionine. *International Journal of Poultry Science*, 7, 692-695.

Chantiratikul A, Chinrasri O, Pakmaruek P, Chantiratikul P, Thosaikham W and Aengwanich W (2011) Responses of Growing Japanese Quails that Received Selenium from Selenium Enriched Kale Sprout (*Brassica oleracea var. alboglabra* L.). *Biological Trace Element Research*, 144, 760-768.

Chen L, Pan DD, Zhou J and Jiang Y Z (2005) Protective effect of selenium-enriched *Lactobacillus* on CCl_4-induced liver injury in mice and its possible mechanisms. *World Journal of Gastroenterology*, 11, 5795-5800.

Chassaigne H, Chry CC, Bordin G and Rodriguez AR (2002) Development of new analytical methods for selenium speciation in selenium-enriched yeast material. *Journal of Chromatography A,* 976, 409-422.

Clement I, Birringer M, Block E, Kotrebai M, Tyson JF, Uden PC and Donal J (2000) Chemical speciation influences comparative activity of selenium-enriched garlic and yeast in mammary cancer prevention. *Journal of Agricultural and Food Chemistry*, 48, 2062–2070.

Combs GF, Clark LC and Turnbull BW (1997) Reduction of cancer risk with an oral supplement of selenium. *Biomedical and environmental sciences : BES*, 10, 227-234.

Combs GF (2005) Current Evidence and Research Needs to Support a Health Claim for Selenium and Cancer Prevention. *The Journal of nutrition*, 135, 343-347.

Mahasarakham University

Corcoran BM, Stanton C, Fitzgerald GF, and Ross RP (2005) Survival of Probiotic Lactobacilli in Acidic Environments Is Enhanced in the Presence of Metabolizable Sugars. *Applied and Environmental Microbiology*, 7(6), 3060–3067.

Ding WK and Shah NP (2007) Acid, Bile, and Heat Tolerance of Free and Microencapsulated Probiotic Bacteria. *Journal of Food Science*, 72, M446-M450.

Drummond KE and Brefere LM (2004) *Nutrition for food service and Culinary Professionals,* Hoboken, John Wiley and Son.

Dungan R S, Yates SR and Frankenberger WT (2003) Transformations of selenate and selenite by *Stenotrophomonas maltophilia* isolated from a seleniferous agricultural drainage pond sediment. *Environmental Microbiology*, 5, 287-295.

Dumont E, Vanhaecke F and Cornelis R (2006) Selenium speciation from food source to metabolites: a critical review. *Analytical and Bioanalytical Chemistry*, 385, 1304-1323.

El-Ghonemy AMK.(2006) Water desalination systems powered by renewable energy sources: Review. *Renewable and Sustainable Energy Reviews*, 16, 1537-1556.

Ellis DR, Sors TG, Brunk DG, Albrecht C, Orser C, Lahner B, Wood KV, Harris HH, Pickering IJ and Salt DE (2004) Production of Se-methylselenocysteine in transgenic plants expressing selenocysteine methyltransferase. *BMC Plant Biology,* 4, 1-11.

Evenhuis CJ and Haddad PR (2009) Joule heating effects and the experimental determination of temperature during CE. *Electrophoresis*, 30, 897–909.

Food and Nutrition Board Institute of Medicine (2000) *Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium and Carotenoides.,* Washington DC, National Academics Press.

Foster LH and Sumar S (1996) Hydride generation atomic absorption spectrometric (HGAAS) determination of selenium in term and preterm infant formulae available in the United Kingdom. *Food Chemistry*, 55, 293–298.

Frankenberger WT and Benson S (1994) *Selenium in the Environmental*, New York, Marcel Dekker.

Mahasarakham University

Gan F, Ren F, Chen XX, Lv C, Pan C, Ye G, ShiJ, Shi X, Zhou H, Shituleni SA, and Huang K (2013) Effects of Selenium-Enriched Probiotics on Heat Shock Protein mRNA Levels in Piglet under Heat Stress Conditions. *Journal of Agricultural Food Chemistry*, 61(10), 2385–2391.

Gerrard TL, Telford JN and Williams HH (1974,) Detection of Selenium Deposits in *Escherichia coli* by Electron Microscopy. *Journal of Bacteriology*, 119, 1057-1060.

Goldstein J and Mass W. *Electrodialysis Apparatus and Process*. United State Patent. USA. 4614576.

Gromer S, Eubel J, Lee B and Jacob J (2005) Human selenoproteins at a glance. *Cellular and Molecular Life Sciences*, 62, 2414-243.

Guo Y, Pan D, Li H, Sun Y, Zeng X and Yan B (2013) Antioxidant and immunomodulatory activity of selenium exopolysaccharide produced by *Lactococcus lactis subsp. lactis*. *Food Chemistry*, 138, 84–89

Guthrie HA and Picciano MF (1995) *Human Nutrition.*, St. Louis, Mary Frances Picciano.

Hatfield DL, Berry MJ and Gladyshev VN (2006) *Selenium: Its Molecular Biology and Role in Human Health,* New York, Springer-Verlag New York Inc.

Hartikainen H (2005) Biogeochemistry of selenium and its impact on food chain quality and human health. *Journal of Trace Elements in Medicine and Biology*, 18, 309-318.

Heller KJ (2001) Probiotic bacteria in fermented foods: product characteristics and starter organisms. *American Journal of Clinical Nutrition*, 73, 374S-379S.

Hérold A (2006) An arrangement of the chemical elements in several classes inside the periodic table according to their common properties. *Comptes Rendus Chimie*, 9, 148-153.

Hinsenkamp MH (2001) Kashin-Beck disease. *International Orthopaedics*, 25, 133-133.

Holzapfel WH, Haberer P, Geisen R, Björkroth J and Schillinger U (2001) Taxonomy and important features of probiotic microorganisms in food and nutrition. *American Journal Clinical Nutrition*, 73, 365S-373S.

Mahasarakham University

Jeong D, Kim TS, Chung YW, Lee BJ and Kim IY (2002) Selenoprotein W is a glutathione-dependent antioxidant in vivo. *FEBS Letters*, 517, 225-228.

Jin M, Wang Y, Xu C, Lu Z, Huang M and Wang Y (2010) Preparation and biological activities of an exopolysaccharide produced by *Enterobacter cloacae* Z0206. *Carbohydrate Polymers*, 81, 607–611.

Johanson L, Gafvelin G and Arnèr ESJ (2005) Selenocysteine in proteins - properties and biotechnological use. *Biochimica et Biophysica Acta*, 1726, 1-13.

Kápolna E, Shaha M, Carusoa JA and Fodorb P (2007) Selenium speciation studies in Se-enriched chives (*Allium schoenop*rasum) by HPLC-ICP-MS. *Food Chemistry*, 101, 1398-1406.

Kariduraganavar MY, Nagarale RK, Kittur AA and Kulkarni SS (2006) Ion-exchange membranes: preparative methods for electrodialysis and fuel cell applications. *Desalination*, 197, 225-246.

Kask S, Adamberg K, Orłowski A, Vogensen FK, Møller PL, Ardö Y, Paalme T (2003) Physiological properties *of Lactobacillus paracasei*, *L. Danicus* and *L-curvatus* strains isolated from Estonian semi-hard cheese. *Food Research International*, 36, 1037—1046.

Kiffney P and Knight A (1990) The toxicity and bioaccumulation of selenate, selenite and seleno-L-methionine in the cyanobacterium *Anabaena flosaquae*. *Archives of Environmental Contamination and Toxicology*, 19, 488-494.

Kim H-Y and Gladyshev V (2005) Different Catalytic Mechanisms in Mammalian Selenocysteine- and Cysteine-Containing Methionine-R-Sulfoxide Reductases. *PLoS Biology*, 3, 2080-2088.

Klein G, Pack A, Bonaparte C and Reuter G (1998) Taxonomy and physiology of probiotic lactic acid bacteria. *International Journal of Food Microbiology*, 41, 103-125.

Klein EA (2004) Selenium: Epidemiology and Basic Science. *The Journal of Urology,* 171, S50-S53.

Korngold E, Kock K and Strathmann H (1978) Electrodialysis in Advanced Waste Water Treatment. *Desalination*, 24, 129-139.

Kotrebai M, Birringer M, Tyson JF, Block E and Uden PC (2000) Selenium speciation in enriched and natural samples by HPLC-ICP-MS and HPLC-ESI-MS with perfluorinated carboxylic acid ion-pairing agents. *Analyst*, 125(1), 71-8.

Krebs B, Bonmann S and Eidenschink I (1994) *Encyclopedia of Inorganic Chemistry,* New York, John Willey and Son, Chichester. England. pp 1668-1678.

Larsen EH, Lobinski R, Burger-Meÿer K, Hansen M, Ruzik R, Mazurowska L, Rasmussen PH, Sloth JJ, Scholten O and Kik C (2006) Uptake and speciation of selenium in garlic cultivated in soil amended with symbiotic fungi (mycorrhiza) and selenate. *Analtical Bioanalytical Chemistry*, 385, 1098-1108.

Li HF, McGrath SP and Zhao FJ (2008) Selenium uptake, translocation and speciation in wheat supplied with selenate or selenite. *New Phytologist*, 178, 92-102.

Li J, Liang D, Qin S, Feng P and Wu X (2015) Effects of selenite and selenate application on growth and shoot selenium accumulation of pak choi (*Brassica chinensis L.*) during successive planting conditions. *Environmental Science and Pollution Research*, 22(14), 11076-11086.

Li S, Li W, Hu X, Yang L and Xirao R (2009) Soil selenium concentration and Kashin-Beck disease prevalence in Tibet, China. *Earth and Environmental Science*, 3, 62-68.

Lieberman HR, Kanarek R and Prasad C (2005) *Nutritional neuroscience*, CRC Press. Lobinski, R. & Szpunar, J. (1999) Biochemical speciation analysis by hyphenated techniques. *Analytica Chimica Acta*, 400, 321-332.

Loflin J, Lopez N, Whanger PD and Kioussi C (2006) Selenoprotein W during development and oxidative stress. *Journal of Inorganic Biochemistry*, 100, 1679-1684.

Liu Y, Chiba M, Inaba Y and Kondo M (2002) Keshan disease-a review from the aspect of history and etiology. *Nihon Eiseigaku Zasshi*, 56, 641-648.

Makarova K, Slesarev A, Wolfa Y, Sorokin A, Mirkin B, Koonin E,…Mills D (2006) Comparative genomics of the lactic acid bacteria. *PNAS*, 17, 15611-15616.

Maneetong S, Chookhampaeng S, Chantiratikul A, Chinrasri O, Thosaikham W, Sittipout R and Chantiratikul P (2013) Hydroponic cultivation of selenium-enriched kale (*Brassica oleracea var. alboglabra L.*) seedling and speciation of selenium with HPLC–ICP-MS. *Microchemical Journal*, 108, 87-91.

Mahasarakham University

Mangiapane E, Lamberti C, Pessione A, Galano E, Amoresano A and Pessione E (2014) Selenium effects on the metabolism of a Se-metabolizing *Lactobacillus reuteri*: analysis of envelope-enriched and extracellular proteomes. *Molecular BioSystems*, 10(6), 1272–1280.

Marteau PR, de Vrese M, Cellier CJ and Schrezenmeir J (2001) Protection from gastrointestinal diseases with the use of probiotics. *American Journal of Clinical Nutrition*, 73, 430S-436S.

Mazan S, Gilon N, Crètier G, Rocca JL and Mermet JM (2002) Inorganicselenium speciation using HPLC-ICP-hexapole collision/reaction cell-MS. *Journal of Analytical Atomic Spectrometry*, 17, 366-370.

Montes-Bayón M, Yanes EG, León CP, Jayasimhulu K and Stalcup A (2002) Initial Studies of Selenium Speciation in *Brassica juncea* by LC with ICP-MS and ES-MS Detection: an Approach for Phytoremediation Studies. *Analytical Chemistry*, 74, 107-113.

Moreno-Reyes R, Suetens C, Mathieu F, Begaux F, Zhu D, Rivera MT,…Vanderpas J (1998) Kashin–Beck Osteoarthropathy in Rural Tibet in Relation to Selenium and Iodine Status. *New England Journal of Medicine*, 339, 1112-1120.

Montes-Bayón M, Molet MJD, González EB and Sanz-Medel A (2006) Evaluation of different sample extraction strategies for selenium determination in selenium-enriched plants (*Allium sativum* and *Brassica juncea*) and Se speciation by HPLC-ICP-MS. *Talanta*, 68, 1287-1293.

Mostert V (2000) Selenoprotein P: Properties, Functions, and Regulation. *Archives of Biochemistry and Biophysics*, 376, 433-438.

Moxon AL and Rhian M (1943) Selenium Poisoning. *Physiological Reviews*, 23(4), 305-337.

Mueller EG (2009) Seeing into selenocysteine biosynthesis. *Nature Chemical Biology*, 5(9), 611-2.

Murphy AP and Colo L (1990) *Chemical Process for Removing Selenium from Water*. United State Patent. USA. 5089141.

Mustacich D and Powis G (2000) Thioredoxin reductase. *Biochemical Journal*, 346, 1-8.

Mahasarakham University

Narasingarao P and Häggblom MM (2007) Identification of Anaerobic Selenate-Respiring Bacteria from Aquatic Sediments. *Applied and Environmental Microbiology*, 73, 3519-3527.

National Center for Complementary and Integrative Health [2015] *Probiotics.* [Online] Available from: https://nccih.nih.gov/health/probiotics [Cited 3 September 2015].

Nauser T, Steinmann D and Koppenol WH (2012) Why do proteins use selenocysteine instead of cysteine?. *Amino Acids*, 42(1), 39-44.

Nève J (1999) Combined Selenium and Iodine Deficiency in Kashin-Beck Osteoarthropathy. *New England Journal of Medicine,* 54, 1-4.

O'Toole D and Raisbeck MF (1995) Pathology of experimentally induced chronic selenosis (alkali disease) in yearling cattle. *Journal of Veterinary Diagnostic Investigation*, 7, 364-373.

Ogra Y, Kitaguchi T, Ishiwata K, Suzuki N, Toida T and Suzuki KT (2009) Speciation of selenomethionine metabolites in wheat germ extract. *Metallomics*, 1, 78-86.

Oremland RS, Hollibaugh JT, Maest AS, Presser TS, Miller LG and Culbertson CW (1989) Selenate Reduction to Elemental Selenium by Anaerobic Bacteria in Sediments and Culture: Biogeochemical Significance of a Novel, Sulfate-Independent Respiration. *Applied Environmental Microbiology*, 55, 2333-2343.

Orero Iserte L, Roig-Navarro AF and Hernández F (2004) Simultaneous determination of arsenic and selenium species in phosphoric acid extracts of sediment samples by HPLC-ICP-MS. *Analytica Chimica Acta*, 527, 97–10.

Palacios Ò and Lobinskia R (2007) Investigation of the stability of selenoproteins during storage of human serum by size-exclusion LC-ICP-MS. *Talanta*, 71, 1813-1816.

Pan C, Zhao Y, Liao SF, Chen F, Qin S, Wu X, Zhou H and Huang K (2011) Effect of Selenium-Enriched Probiotics on Laying Performance, Egg Quality, Egg Selenium Content, and Egg Glutathione Peroxidase Activity. *Journal of Agricultural and Food Chemistry*, 59, 11424-11431.

Papp LV, Lu J, Holmgren A and Khanna KK (2007) From selenium to selenoproteins: synthesis, identity, and their role in human health. *Antioxidants & Redox Signaling*, 9(7):775-806.

Pessi T, Sütas Y, Hurme M and Isolauri E (2000) Interleukin-10 generation in atopic children following oral Lactobacillus rhamnosus GG. *Clinical & Experimental Allergy*, 30, 1804-1808.

Pilon-Smits EAH and LeDuc DL (2009) Phytoremediation of selenium using transgenic plants. *Current Opinion in Biotechnology*, 20, 207-212.

Pieniz S, Okeke BC, Andreazza R and Brandelli A (2011) Evaluation of selenite bioremoval from liquid culture by Enterococcus species. *Microbiology Research*, 166(3), 176-85.

Pophalya SD, Poonama, Singh P, Kumar H, Tomar SK and Singha R (2014) Selenium enrichment of lactic acid bacteria and bifidobacteria: A functional food perspective. *Trends in Food Science & Technology*, 39(2), 135–145.

Pyrzynska K (2009) Selenium speciation in enriched vegetables. *Food Chemistry,* 114, 1183-1191.

Rayman MP (2004) The use of high-selenium yeast to raise selenium status: how does it measure up?. *British Journal of Nutrition*, 92, 557–573.

Reilly CS (1996) S*elenium in food and health,* London, Chapman & Hall.

Reyes HL, Sanz MF, Espilez HP, Marchante-Gayon JM, Alonso GJI and Sanz-Medel A (2004) Biosynthesis of isotopically enriched selenomethionine: application to its accurate determination in selenium-enriched yeast by isotope dilution analysis-HPLC-ICP-MS. *Journal of Analytical Atomic Spectrometry*, 19, 1230-1235.

Roberge M, Borgerdin A and Finley J (2003) Speciation of Selenium Compounds from High Selenium Broccoli is Affected by the Extracting Solution. *Journal of Agricultural and Food Chemistry*, 51, 4191-4197.

Roberfroid MB (2000) Prebiotics and probiotics: are they functional foods?. *American Journal of Clinical Nutrition*, 71, 1682S-1687S.

Robles LC, Feo JC, de Celis B, Lumbreras JM, Garcoa-Olalla C and Aller AJ (1999) Speciation of selenite and selenate using living bacteria. *Talanta*, 50, 307-325.

Rolfe RD (2000) The role of probiotic cultures in the control of gastrointestinal health. *The Journal of Nutrition*, 130, 396S-402S.

Mahasarakham University

Salminen MK, Tynkkynen S, Rautelin H, Saxelin M, Vaara M, Ruutu P, Sarna S, Valtonen V and Järvinen A (2002) *Lactobacillus* Bacteremia during a Rapid Increase in Probiotic Use of *Lactobacillus rhamnosus* GG in Finland. *Clinical Infectious Diseases*, 35, 1155-1160.

Sanders ME (2009) How Do We Know When Something Called "Probiotic" Is Really a Probiotic? A Guideline for Consumers and Health Care Professionals. *Functional Food Reviews*, 1, 3-12.

Schlosser PM, Riedy G and Bailey JE (1994) Ethanol Production in Baker's Yeast: Application of Experimental Perturbation Techniques for Model Development and Resultant Changes in Flux Control Analysis. *Biotechnology Progress*, 10, 141-154.

Schrauzer GN (2000) Selenomethionine: A Review of Its Nutritional Significance, Metabolism and Toxicity. *Journal of Nutrition*, 130(7), 1653-1656.

Schrezenmeir and de Vrese M (2001) Probiotics, prebiotics, and synbiotics - approaching a definition. *The American Journal of Clinical Nutrition*, 73(2), 361S-364S.

Segers ME and Lebeer S (2014) Towards a better understanding of *Lactobacillus rhamnosus* GG--host interactions. *Microbial Cell Factories*, 13, 1-16.

Shao S and Zheng B (2008) The biogeochemistry of selenium in Sunan grassland, Gansu, Northwest China, casts doubt on the belief that Marco Polo reported selenosis for the first time in history. *Environmental Geochemistry Health*, 30, 307-314.

Shen Q, Zhang B, Xu R, Wang Y, Ding X and Li P (2010) Antioxidant activity in vitro of the selenium-contained protein from the Se-enriched *Bifidobacterium animalis* 01. *Anaerobe*, 16, 380-386.

Shia L,Yuea W, Zhanga C, Rena Y, Zhua X, Wanga Q, Shia L and Lei F (2010) Effects of maternal and dietary selenium (Se-enriched yeast) on oxidative status in testis and apoptosis of germ cells during spermatogenesis of their offspring in goats. *Animal Reproduction Science*, 119, 212-218.

Shiobara YY, Ogra & Suzuki K (1999) Speciation of metabolites of selenate in rats by HPLC-ICP-MS. *Analyst,* 124, 1237–1241.

Mahasarakham University

Siddique T, Zhang Y, Okeke BC and Frankenberger WT (2006) Characterization of sediment bacteria involved in selenium reduction. *Bioresource Technology*, 97, 1041-1049.

Sittipout R, Thosaikham W, Chantiratikul, P (2011). *Thai Patent No. 6432*. Bangkok: Department of Intellectual Property.

Silverberg BA, Wong P TS and Chau YK (1976) Localization of selenium in bacterial cells using TEM and energy dispersive X-ray analysis. *Archives of Microbiology*, 107, 1-6.

Stadtman T (1974) Proteins containing selenium are essential components of certain bacterial and mammalian enzyme systems. *Science*, 183, 915 - 922.

Stadtman T (1996) Selenocysteine. *Annual Review of Biochemistry*, 63, 83-100.

Strathmann H (1992) *Membrane Handbook*. Van Nostrand Reinhold, New York, 255-262.

Subcommittee on Selenium, National Research Council (U.S.) (1983) *Selenium in nutrition*, Washington D.C.: National Academic Press.

Sugihara S, Kondo M, Chihara Y, Yuji M, Hattori H and Yoshida M (2004) Preparation of Selenium-enriched Sprouts and Identification of Their Selenium Species by High-performance Liquid Chromatography-Inductively Coupled Plasma Mass Spectrometry. *Bioscience Biotechnology and Biochemistry*, 68, 193-199.

Szpunar J (2005) Metabolomics of Selenium: Se Metabolites Based on Speciation Studies. *Journal of Health Science*, 51, 107-114.

Tang X, Zhou Z-K, Shen B, Kang P-D, Yang J, Li J, and Pei F-X (2014) Total knee arthroplasty in elderly patients with severe Kashin-Beck disease of the knee. *International Orthopaedics*, 38(4), 753–759.

Terry N, Zayed AM, De Souza MP and Tarun AS (2000) Selenium in Higher Plants. *Annual Review of Plant Physiology and Plant Molecular Biology*, 52, 401-432.

Templeton DM, Ariese F, Cornelis R, Lars-Goran D, Herbert M, Herman PV and Ryszard L (2000) Guidelines for terms related to chemical speciation and fractionation of elements. Definitions, structural aspects, and methodological approaches. *Pure Applied Chemistry*, 72, 1453-1470.

Mahasarakham University

Thosaikham W, Jitmanee K, Sittipout R, Maneetong S, Chantiratikul A and Chantiratikul P (2014) Evaluation of selenium species in selenium-enriched pakchoi (*Brassica chinensis Jusl var parachinensis (Bailey) Tsen & Lee*) using mixed ion-pair reversed phase HPLC-ICP-MS. *Food Chemistry*, 145, 736-42.

Tomei FA, Barton LL, Lemanski CL, Zocco TG, Fink NH. and Sillerud LO (1995) Transformation of selenate and selenite to elemental selenium by *Desulfovibrio desulfuricans*. *Journal of Industrial Microbiology & Biotechnology*, 14, 329-336.

Turner RJ, Weiner JH and Taylor DE (1998) Selenium metabolism in *Escherichia coli*. *BioMetals*, 11, 223-227.

Turanov AA, Xu X-M, Carlson BA, Yoo MH, Gladyshev VN and Hatfield DL (2011) Biosynthesis of Selenocysteine, the 21st Amino Acid in the Genetic Code, and a Novel Pathway for Cysteine Biosynthesis. *Advance Nutrition*, 2(2), 122–128.

Umesh C, Gupta and Guptab SC (1998) Trace element toxicity relationships to crop production and livestock and human health: implications for management. *Communications in Soil Science and Plant Analysis*, 29, 491-1522.

Wapnir RA (1990) *Protein Nutrition and Mineral Absorption.* Baca Raton: CRC Press.

Wardlaw GM, Disilvestro RA and Hampl JS (2004) *Perspective Nutrition,* New York: Mc Graw-Hill.

Watanabe C (2005) *Nutritional Neuroscience,* Boca Raton, CRC press.

Whanger PD (2005) Selenium and its relationship to cancer: an update. *British Journal of Nutrition*, 91, 11-28.

WHO/WFO (2004) *Vitamin and Mineral Requirements in Human Nutrition*, Rome: WHO/WFO.

Wróbel K, Wróbel K, Kannamkumarath SS, Caruso JA, Wysocka IA, Bulska E, Swiatek J and Wierzbicka M (2004) HPLC-ICP-MS speciation of selenium in enriched onion leaves - a potential dietary source of Se-methylselenocysteine. *Food Chemistry*, 86, 617-623.

Xia SK, Chen L and Liang JQ (2007) Enriched Selenium and Its Effects on Growth and Biochemical Composition in *Lactobacillus bulgaricus*. *Journal of Agricultural and Food Chemistry*, 55, 2413-2417.

Mahasarakham University

Xu T (2005) Ion exchange membranes: State of their development and perspective. *Journal of Membrane Science*, 263, 1-29.

Yang F (2006) Keshan disease and mitochondrial cardiomyopathy. *Science in China Series C: Life Sciences*, 49, 513-518.

Yang X, Hill KE, Maguire MJ and Burk RF (2000) Synthesis and secretion of selenoprotein P by cultured rat astrocytes. *Biochimica et Biophysica Acta*, 1474, 390-396.

Yang J, Huang K, Qin S, Wu X, Zhao Z and Chen F (2009) Antibacterial Action of Selenium-Enriched Probiotics Against Pathogenic *Escherichia coli*. *Digestive Diseases and Sciences*, 54, 246-254.

Yathavakilla SVK., Shah M, Mounicou S and Caruso JA (2005) Speciation of cationic selenium compounds in *Brassica juncea* leaves by strong cation-exchange chromatography with inductively coupled plasma mass spectrometry. *Journal of Chromatography A*, 1100, 153-159.

Yazdi MH, Mahdavi M, Setayesh N, Esfandyar M and Shahverdi AR (2013) Selenium nanoparticle-enriched *Lactobacillus brevis* causes more efficient immune responses in vivo and reduces the liver metastasis in metastatic form of mouse breast cancer. DARU *Journal of Pharmaceutical Sciences,* 21(33) 2–9.

Yoshida M, Sugihara S, Inoue Y, Chihara Y, Kondo M, Miyamoto S and Sukcharoen, B (2005) Composition of chemical species of selenium contained in selenium-enriched shiitake mushroom and vegetables determined by high performance liquid chromatography with inductively coupled plasma mass spectrometry. *Journal of nutritional science and vitaminology*, 51, 194-199.

Yunbao L, Momoko C, Yutaka I and Masao K (2002) Keshan disease-a review from the aspect of history and etiology. *Japanese Journal of Hygiene*, 56, 641-648.

Zhang B, Zhou K, Zhang J, Chen Q, Liu G, Shang N, Qin W, Li P and Lin F (2009) Accumulation and species distribution of selenium in Se-enriched bacterial cells of the *Bifidobacterium animalis* 01. *Food Chemistry*, 115, 727-734.

Zayed A, Lytle CM and Terry N (1998) Accumulation and volatilization of different chemical species of selenium by plants. *Planta,* 2006, 248-292.

Mahasarakham University

Zheng J, Ohata M, Furuta N and Kosmus W (2000) Speciation of selenium compounds with ion-pair reversed-phase liquid chromatography using inductively coupled plasma mass spectrometry as element-specific detection. *Journal of Chromatography A,* 874, 55-64.

Zhong L and Holmgren A (2000) Essential Role of Selenium in the Catalytic Activities of Mammalian Thioredoxin Reductase Revealed by Characterization of Recombinant Enzymes with Selenocysteine Mutations. *Journal of Biological Chemistry,* 275, 18121–18128.

Zhang Y and Gladyshev VN (2005) An algorithm for identification of bacterial selenocysteine insertion sequence elements and selenoprotein genes. *Bioinformatics*, 21, 2580-2589.

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APPENDICES

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Appendix A

Preparation of solutions

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1. List of chemicals and reagents

Name	Formula	Molar mass (g/mol)	Density	Grade	Purity (%)
Ammonia solution	NH_4OH	35.05	0.893	HPLC	30.0
1-butanesulfonic acid	$C_4H_9O_3SNa$	160.17	-	HPLC	98.0
DL-1,4-dithiothreitol	$C_4H_{10}O_2S_2$	152.24	-	AR	99.0
Hydrochloric acid	HCl	36.46	1.180	AR	35.4
Methanol	CH_3OH	32.04	0.791	HPLC	99.0
Nitric acid	HNO_3	63.01	1.400	AR	65.0
Selenium standard	Se	78.96	-	AAS	-
Sodium selenite	Na_2SeO_3	172.94	-	AR	95.0
Se-methyl seleno-L-cysteine	$C_4H_9NO_2Se$	192.08		AR	98.0
DL-selenomethionine	$C_5H_{11}NO_2Se$	196.11	-	AR	99.0
L-selenocystine	$C_6H_{12}N_2O_4Se_2$	334.09	-	AR	98.0
Tetramethylammo-nium hydroxide pentahydrate	$C_4H_{13}NO._5H_2O$	181.23	-	AR	99.0
Trifluoroacetic acid	CF_3CO_2H	114.02	1.535	AR	99.0
Tris-hydrochloride	$NH_2C(CH_2OH)_3 \cdot HCl$	157.60	-	BioPerformance Certified	99.0

2. Selenium standards stock solution preparation

1.1 Preparation selenite standard solution

Stock standard solution (500 mg Se/L) of selenite was prepared by dissolving 54.76 mg of sodium selenite in 50 mL of deionized water.

1.2 Preparation Se-methylselenocysteine standard solution

Stock standard solution (500 mg Se/L) of Se-methylselenocysteine was prepared by dissolving 60.82 mg of sodium Se-methylseleno-L-cysteine in 50 mL of deionized water.

1.3 Preparation selenomethionine standard solution

Stock standard solution (500 mg Se/L) of selenomethionine was prepared by dissolving 62.09 mg of DL-selenomethionine in 50 mL of deionized water.

1.4 Preparation selenocysteine standard solution

Stock standard solution (500 mg Se/L) of selenocysteine was prepared by dissolving 62.09 mg of L-selenocystine in 5 mL of 0.05 M of DTT in 0.1 M HCl

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solution. The mixture was incubated at 50 °C for 30 min, made up to volume with deionized water in 50 mL volumetric flask.

All selenium compounds standard stock solutions were standardized with selenium standard solution (AAS grade) and determinedby ICP-MS.

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Appendix B
The photograph of probiotics

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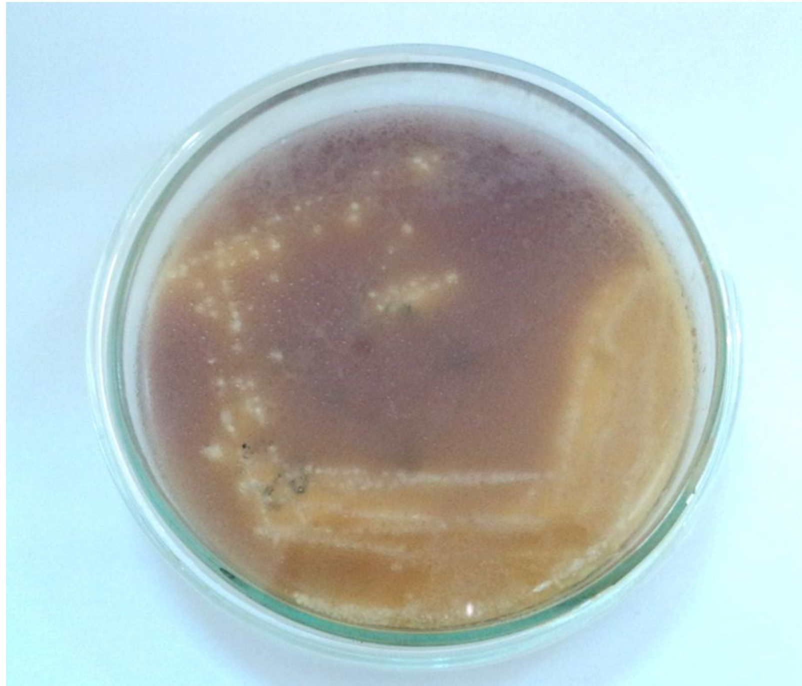

Figure B1 Colony morphology of *L. casei subsp. rhamnosus*is in MRS agar

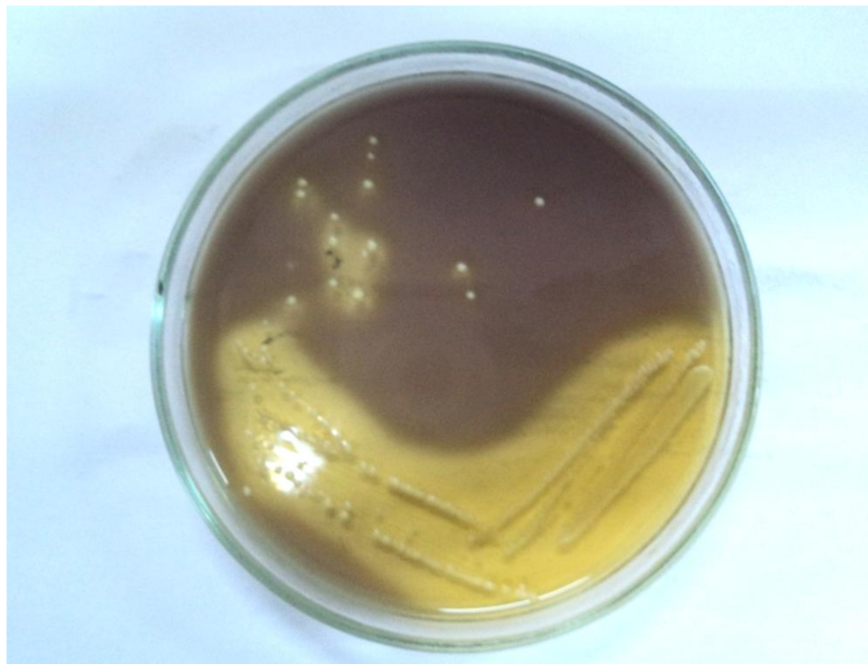

Figure B2 Colony morphology of *L. bulgaricus* in MRS agar

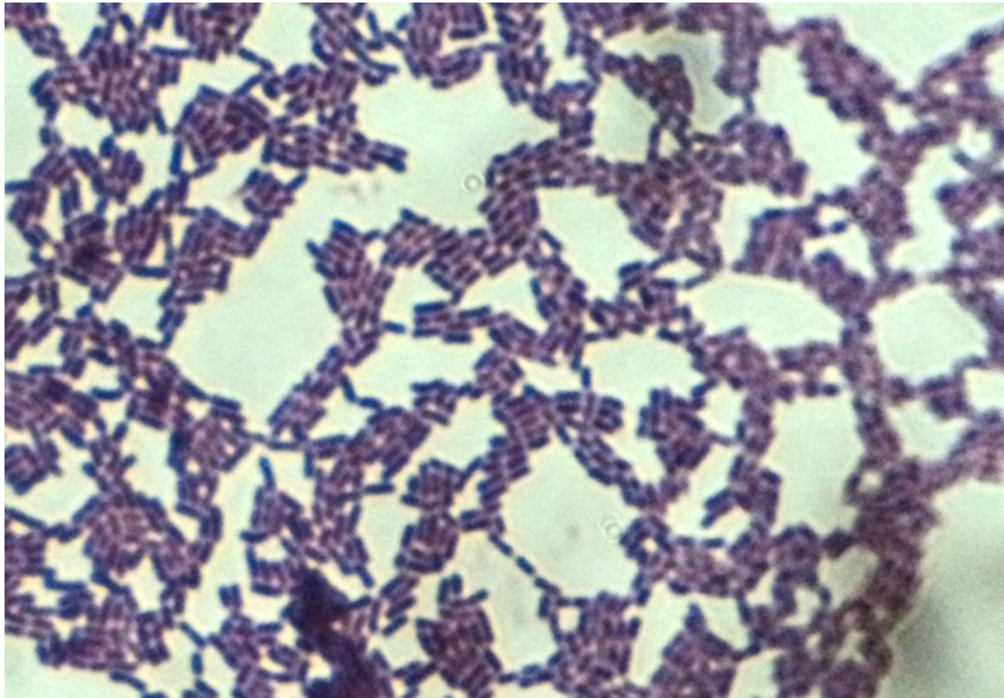

Figure B3 Gram strain of *L. casei subsp. rhamnosus*is

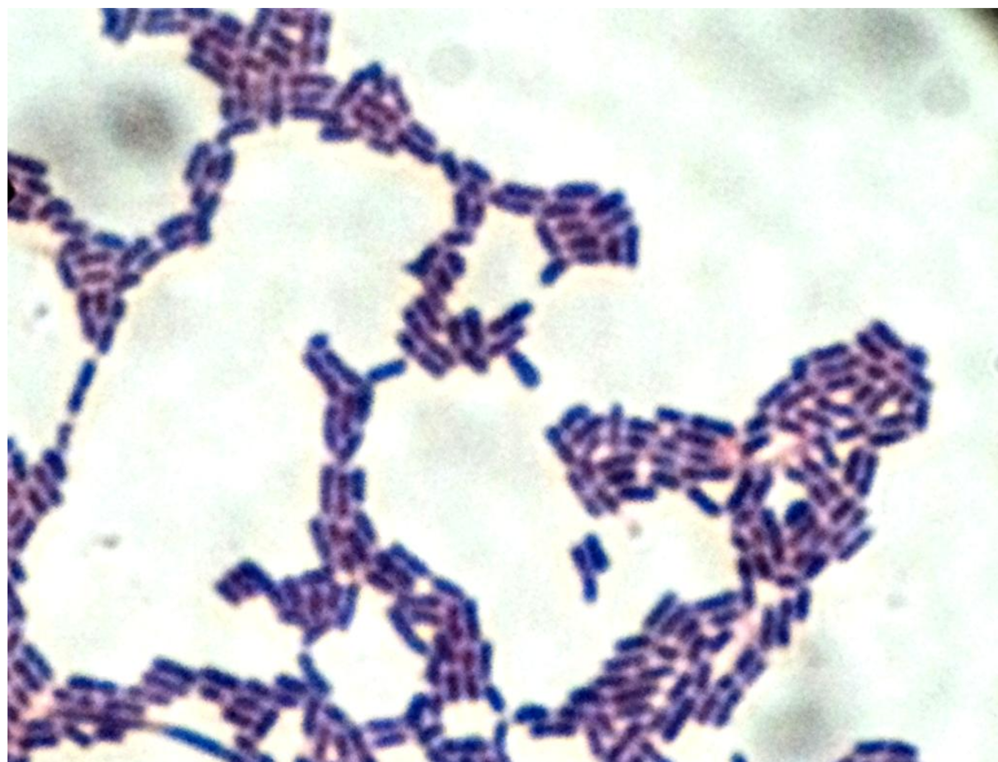

Figure B4 Gram strain of *L. bulgaricus*

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BIOGRAPHY

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Biography

Name	Mr. Witchapol Thosaikham
Date of birth	March 24, 1984
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2010	Master of Science degree in Chemistry at Mahasarakham University, Thailand
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Research grants & awards

2006	Research grant for senior project of B.Sc. in Chemistry supported by Mahasarakham University
2007 - 2010	Scholarship for M.Sc. in Chemistry from Center of Excellence for Innovation in Chemistry (PERCH-CIC)
2009	Research grant for thesis topic of M.Sc. in Chemistry supported by Mahasarakham University
2012	Research grant for dissertation topic of Ph.D in Chemistry supported by Mahasarakham University
2013	King Bhumibol's scholarship

Mahasarakham University

Research output

Publications

1. **Thosaikham W** and Chantiratikul P (2008) Application of HPLC-ICP-MS technique on elemental speciation analysis. *Naresuan Phayou Journal*, 3(1), 154-163.

2. Chinrasri O, Chantiratikul P, **Thosaikham W**, Atiwetin P, Chumpawadee S, Saenthaweesuk S and Chantiratikul A (2009) Effect of Selenium-enriched Bean Sprout and Other Selenium Sources on Productivity and Selenium Concentration in Eggs of Laying Hens. *Asian-Australasian Journal of Animal Sciences*, 22(12), 1661-1666.

3. Chantiratikul A, Chinrasri O, Pakmaruek P, Chantiratikul P, **Thosaikham W** and Sittipout R (2012) Responses of Growing Japanese Quails that Received Selenium from Selenium-Enriched Kale Sprout (*Brassica oleracea var. alboglabra* L.). *Biological Trace Element Research*, 144, 760-768.

4. Chantiratikul A, Chinrasri O, Chantiratikul P, **Thosaikham W**, Maneethong S and Aengwanich W (2011) Effect of Selenium from Selenium-Enriched Kale Sprout (*Brassica oleracea var. alboglabra* L.) on Selenium Concentration in Tissue of Spent Hens. *Khon Kean Agricultural Journal*, 40(2), 295-298.

5. Maneetong S, Chookhampaeng S, Chantiratikul A, Chinrasri O, **Thosaikham W**, Sittipout R and Chantiratikul P (2013) Hydroponic cultivation of selenium-enriched kale (*Brassica oleracea var. alboglabra L.*) seedling and speciation of selenium with HPLC–ICP-MS. *Microchemical Journal*, 108:87-91.

6. **Thosaikham W**, Jitmanee K, Sittipout R, Maneetong S, Chantiratikul A and Chantiratikul P (2014) Evaluation of selenium species in selenium-enriched pakchoi (*Brassica chinensis Jusl var parachinensis (Bailey) Tsen & Lee*) using mixed ion-pair reversed phase HPLC-ICP-MS. *Food Chemistry*, 145, 736-42.

Patents

Sittipout R, **Thosaikham W** and Chantiratikul, P (2011). Thai Patent No. 6432. Bangkok: Department of Intellectual Property.

Thosaikham W and Chantiratikul, P (2016). Application No.1303001389. Bangkok: Department of Intellectual Property.

Mahasarakham University

Proceeding

1. **Thosaikham W**, Sittipout R and Chantiratikul A (2009) Sample preparation methods for selenium determination with ICP-MS technique. *Mahasarakham University Science and Technology Journal*. Special volume, 71-78.

2. Sittipout R, **Thosaikham W**, Chantiratikul P and Burakham R (2011) Development of Home-made Digestion Apparatus for Determination of Iron and Zinc with Flame Atomic Spectrometry Technique in Biological Samples. *Mahasarakham University Science and Technology Journal*, Special volume, 74-79.

3. **Thosaikham W**, Maneethong S, Sittipout R and Chantiratikul P (2012) Extraction Method for extracting Selenium from Se-enriched Plants. *Mahasarakham University Science and Technology Journal*, Special volume, 74-79. 146-151.

4. Borisuth L, Saenthaweesuk N, Chinrasri O, **Thosaikham W**, Chantiratikul P, Sriart N, and Chantiratikul A (2015) Toxicity of selenium from selenium-enriched kale sprout (*Brassica oleracea var.albogiabla L.*) on performance and egg quality in laying hens. Mahasarakham University Science and Technology Journal, Special volume, 270-275.

Conferences

Oral presentation

1. **Thosaikham W**, Chantiratikul P and Chantiratikul A (2007) Determination of plasma selenium content in laying hens fed supplemental sodium selenite or Zinc-L-selenomethionine using ICP-MS Technique. *The 3th Mahasarakham University Research Conference*, 6th - 7th September 2007, Maha Sarakham, Thailand. P.74.

2. **Thosaikham W**, Sittipout R and Chantiratikul A (2009) Sample preparation methods for selenium determination with ICP-MS technique. *The 5th Mahasarakham University Research Conference*, 10th - 11th September 2009, Maha Sarakham, Thailand.

3. **Thosaikham W**, Jitmanee K**,** Chantiratikul A, Sittipout R, Maneethong S and Chantiratikul P (2010) Selenium extraction using mixture of pepsin and cellulase for selenium-enriched Chinese kale (*Brassica alboglabra* L.H. Bailey). *Pure and Applied Chemistry International Conference (PACCON 2010)*. 21st - 23rd January 2010, Ubon Rachathani, Thailand. P.70.

Mahasarakham University

4. **Thosaikham W**, Jitmanee K**,** Chantiratikul A, Sittipout R, Maneethong S and Chantiratikul P (2012) Ion-Paired Reversed Phase HPLC-ICP-MS for Selenium Speciation Analysis in Se-enriched Yeast. *Pure and Applied Chemistry International Conference (PACCON 2012).* 11st - 13rd January 2012, Chiang Mai, Thailand. P.70.

5. **Thosaikham W**, Maneethong S, Sittipout R and Chantiratikul P (2012) Extraction Method for extracting Selenium from Se-enriched Plants. *The 8th Mahasarakham University Research Conference*, 8th - 9th November 2012, Maha Sarakham, Thailand.

6. **Thosaikham W**, Sittipout R and Chantiratikul P (2012) Modification of Water Bath as a Newly Digestion Apparatus and Application for Preparation of Mushroom Samples Prior to Determine Fe Concentration by AAS. *Pure and Applied Chemistry International Conference (PACCON 2014).* 8th - 10th January 2014, Khon Kean, Thailand. P.98.

7. **Thosaikham W**, Maneerat S, Chantiratikul P (2016) Application of Ion-paired reversed phase HPLC-ICP-MS for Determination of Extracellular Selenium Speciation in Selenium-enriched *L. rhamnosus* culture. European Symposium on Atomic Spectrometry 2016, Eger, Hungary, 31 March – 2 April 2016.

Poster presentation

1. **Thosaikham W**, Chantiratikul P and Chantiratikul A (2007) Quantitative determination of selenium contents in plasma of laying hen using ICP-MS Technique. *The Colloquium Spectroscopicum Internationale XXXV*, 23rd - 27th September 2007, Xiamen, China. P.54.

2. **Thosaikham W**, Chantiratikul P and Chantiratikul A (2008) Determination of plasma selenium content in laying hens fed supplemental sodium selenite or Zinc-L-selenomethionine using ICP-MS Technique. *The 34th Congress on Science and Technology of Thailan*d. 31st October - 2nd November 2008, Bangkok, Thailand. P.117.

3. **Thosaikham W**, Phansi P, Chantiratikul P, Maneechote U and Chantiratikul A (2009) Determination of selenium content in Water meal (*Wolffia globosa*) supplemental sodium selenate using ICP-MS Technique. *Pure and Applied Chemistry International Conference (PACCON 2009)*. 14th – 16th January 2009, Pitsanulok, Thailand. P.151-152.

4. **Thosaikham W**, Chantiratikul P, Chantiratikul A, Atiwetin P and Jitmanee K (2009) Development of extraction method for Se-enriched plant preparation. *The 6th PERCH-CIC congress*. 3rd - 6th May 2009, Pattaya, Thailand. P.149.

5. Sittipout R, **Thosaikham W,** Maneethong S and Chantiratikul P (2010) Sample preparation methods for selenium determination with ICP-MS technique. *Pure and Applied Chemistry International Conference (PACCON 2010)*. 21st - 23rd January 2010, Ubon Rachathani, Thailand. P.93.

6. Maneethong S, **Thosaikham W,** Sittipout R and Chantiratikul P (2010) Speciation and determination of selenium in selenium-enriched duckweed (*Lemna minor*). *Pure and Applied Chemistry International Conference (PACCON 2010)*. 21st - 23rd January 2010, Ubon Rachathani, Thailand. P.162.

7. Sittipout R, **Thosaikham W**, Maneethong S and Chantiratikul P (2011) Development of Home-made Digestion Apparatus for Trace Elements Analysis in Biological Samples. *The 7th PERCH-CIC congress*. 4rd – 7th May 2011, Pattaya, Thailand. P.134.

8. **Thosaikham W**, Maneerat S, Chantiratikul P (2016) Quantitative of Total Selenium in Selenium-enriched Yeast Products with ICP-MS Using Home-made Closed-digestion System form Sample Preparation. European Symposium on Atomic Spectrometry 2016, Eger, Hungary, 31 March – 2 April 2016.

Mahasarakham University