Enhancement of biofuel production in microalgae by genetic and physiological modification

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นภิศา ภัทรปรัชญากุล : การเพิ่มการผลิตเชื้อเพลิงชีวภาพในจุลสาหร่ายโดยการดัดแปรทางพันธุกรรมและสรีรวิทยา. (Enhancement of biofuel production in microalgae by genetic and physiological modification) อ.ที่ปรึกษาหลัก : ศ. ดร.อรัญ อินเจริญศักดิ์, อ.ที่ปรึกษาร่วม : ศ. ดร.ฮาน มิน อู

จุลสาหร่ายคือจุลินทรีย์ที่สามารถสังเคราะห์แสงได้ ซึ่งมีความสามารถนำมาใช้เป็นโรงงานระดับเซลล์ โดยการเปลี่ยนก๊าซคาร์บอนไดออกไซด์เป็นผลิตภัณฑ์ชีวภาพที่มีมูลค่าสูงและเชื้อเพลิงชีวภาพได้ ในการวิจัยนี้มีความตั้งใจที่จะพัฒนาแหล่งผลิตเชื้อเพลิงชีวภาพจากจุลสาหร่ายสีเขียว *Chlorella* sp. ที่คัดแยกได้จากธรรมชาติ และไซยาโนแบคทีเรียสายพันธุ์ *Synechococcus elongatus* PCC 7942 โดยเพิ่มการผลิตไขมัน (สารตั้งต้นของ FAMEs) และสารเทอร์พีน (α-farnesene) ตามลำดับ โดยในส่วนแรกของงานวิจัยจะเกี่ยวกับการเพิ่มไขมันและการวิเคราะห์ FAMEs และคุณสมบัติของไบโอดีเซลภายใต้การเลี้ยงในสภาวะจำกัดฟอสฟอรัส (0-50%P) ร่วมกับการเติมโลหะหนัก (Fe, Co, Pb) ผลการทดลองพบว่าปริมาณการเพิ่มของไขมันสูงสุดพบในสภาวะขาด P ร่วมกับการเพิ่มโคบอลต์ 17 mM ที่ 19% สูงกว่าคอนโทรล นอกจากนั้นพบว่าการเติมตะกั่วความเข้มข้นต่ำสามารถกระตุ้นการเจริญเติบโตของจุลสาหร่ายได้แม้อยู่ในสภาวะขาด P ถึงแม้ว่าจะมีปริมาณของ MUFAs สูงกว่า PUFAs ในเกือบทุกสภาวะ โดยเฉพาะ palmitoleic acid (C16:1) ภาพรวมของคุณสมบัติของไบโอดีเซลพบว่าอยู่ในคุณภาพที่ยอมรับได้ โดยอ้างอิงจากมาตรฐาน ASTM and EN อีกทั้งยังพบว่าการเปลี่ยนแปลงพลังงานจากแสงเป็นไขมันภายใน 7 วัน มีประสิทธิภาพ 10-16% ซึ่งทัดเทียมกับการเลี้ยงจุลสาหร่ายในสภาวะแบบ mixotrophic condition จากงานวิจัยก่อนหน้า ดังนั้นพบว่าการดัดแปรทางสรีรวิทยาด้วยการใช้ความเครียดในการเลี้ยงสามารถทำให้เกิดการเพิ่มขึ้นของไขมันในจุลสาหร่ายได้แม้จะเป็นสายพันธุ์ที่คัดเลือกจากธรรมชาติและไม่มีฐานข้อมูลของจีโนมก็ตาม

ไซยาโนแบคทีเรีย *S. elongatus* PCC 7942 มีข้อดีที่เหนือกว่าจุลสาหร่ายสีเขียว *Chlorella* ในด้านการจัดการทางพันธุกรรม และมีฐานข้อมูลทางคอมพิวเตอร์ของจีโนมอยู่แล้ว ดังนั้นจึงสามารถนำการดัดแปรทางพันธุกรรมมาใช้ปรับปรุงสายพันธุ์ได้ ในการศึกษานี้การปรับปรุงสายพันธุ์ด้วยวิธีการวิศวกรรมในระบบเมตาบอลิซึมเพื่อเพิ่มการผลิตสาร α-farnesene โดยการปรับแต่ง ribosome-binding site (RBS) ของยีน farnesene synthase ให้แสดงออกอย่างเหมาะสม พบว่าอัตรา translation initiation rate ที่ต่ำกว่า สามารถผลิต α-farnesene ได้มากขึ้น (0.57 mg/L/day) อีกทั้งยังมีการพัฒนาสายพันธุ์ โดยเทคนิค random mutation หลังจากวิเคราะห์ด้วยวิธีการตรวจจับแสงฟลูออเรสเซนซ์ไม่พบสายพันธุ์ที่ดีกว่าสายพันธุ์ RBS-optimized อย่างไรก็ตามพบว่าวิธีทางวิศวกรรมเชิงวิวัฒนาการสามารถเพิ่มการผลิต α-farnesene ถึง 2 เท่า (1.2 mg/L/day) เมื่อเทียบกับงานวิจัยก่อนหน้า ดังนั้นการร่วมกันระหว่างวิศวกรรมในระบบเมตาบอลิซึมและเชิงวิวัฒนาการอาจเป็นประโยชน์ในการทำให้สมรรถนะการทำงานในเซลล์เพื่อการผลิตสารเคมีเป้าหมายของไซยาโนแบคทีเรียดียิ่งขึ้น

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Napisa Pattharaprachayakul : Enhancement of biofuel production in microalgae by genetic and physiological modification. Advisor: Prof. Dr. ARAN INCHAROENSAKDI Co-advisor: Prof. Dr. ĴHan Min Woo

Microalgae are photosynthetic microorganisms, which could be used as potential microbial cell factories by directly converting CO_2 into valuable bioproducts and biofuels. This study aims to improve the target biofuel feedstocks from the isolated green alga *Chlorella* sp. and the engineered cyanobacterium *Synechococcus elongatus* PCC 7942 in terms of the improvement on lipids (as precursors of FAMEs) and terpene (α-farnesene), respectively. The first part is concerned with the enhancement of lipids and the determination of FAMEs and biodiesel properties in *Chlorella* sp. under the phosphorus (P) limitation (0-50%) alone or in combination with heavy metals (Fe, Co, Pb) supplementation. The results showed that the highest yield of lipids was achieved under 0%P with 17 mM Co addition with 19% higher than the control. Moreover, the addition of low Pb concentrations could elevate the cell growth even under P limitation whereas the MUFAs, particularly palmitoleic acid (C16:1), was higher than PUFAs under most conditions. The overall biodiesel properties of the obtained FAMEs were of acceptable quality according to the standards (ASTM and EN). Additionally, the energy conversion from light energy to lipids was shown to be in the range of 10-16% conversion efficiency within 7 days, which corresponded to mixotrophic condition of microalgae cultures from previous studies. Hence, the physiological modification by stress treatments to cultures could offer the improvement of lipid content in microalgae although the genome database was not analyzed.

Cyanobacterium *S. elongatus* PCC 7942 with available genomic database has advantages on genetic manipulation over the isolated *Chlorella* sp., thus the genetic modification was performed on *S. elongatus* PCC 7942 in this study. Herein, *S. elongatus* PCC 7942 was metabolically engineered for an enhanced production of α-farnesene by optimizing the ribosome-binding site (RBS) of the codon-optimized farnesene synthase gene. The production of α-farnesene was found to be enhanced in strains with a low translation initiation rate, resulting in α-farnesene productivity of 0.57 mg/L/day. Using the RBS variants and random mutation, the fluorescence-based analysis was done on cells grown in 96-well culture plates to screen the α-farnesene-producing strains, and the results showed no improvement in the titers by the RBS-optimized strains. However, evolutionary engineering of the RBS-optimized strains resulted in a two-fold increase in α-farnesene productivity (1.2 mg/L/day) compared to the previous study. Therefore, combining metabolic and evolutionary engineering might be helpful for enhancing the cellular fitness of cyanobacteria for the production of target chemicals.

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LIST OF ABBREVIATIONS

P	Phosphorus
N	Nitrogen
Fe	Iron
Co	Cobalt
Pb	Lead
SFAs	Saturated fatty acids
MUFAs	Monounsaturated fatty acids
PUFAs	Polyunsaturated fatty acids
FAMEs	Fatty acid methyl esters
CO_2	Carbon dioxide
Dxs	1-deoxy-D-xylulose-5-phosphate synthase of *E. coli*
Idi	Isopentenyl diphosphate isomerase of *E. coli*
IspA (Ec)	Farnesyl diphosphate synthase (IspA) of *E. coli*
FS (Apple fruit)	Farnesene synthase of *Malus* X *domestica* Borkh
G3P	Glyceraldehyde 3-phosphate
DXP	1-Deoxy-D- xylulose-5-phosphate
MEP	2-C-Methyl-D-erythritol-4-phosphate
ME	4-Diphosphocytidyl-2-C-methyl-D-erythritol
CDP-MEP	4-Diphosphocytidyl-2C-methyl-D-erythritol-2-phosphate
MEcPP	2C-Methyl-D-erythritol-2,4-cyclodiphosphate
HMBPP	(E)-4-Hydroxy-3-methylbut-2-enyl-diphosphate
IPP	Isopentenyl diphosphate
DMAPP	Dimethylallyl diphosphate
FPP	Farnesyl diphosphate
LacI	Lac repressor
RBS	Ribosome binding site
ALE	Adaptive laboratory evolution

CHAPTER I: INTRODUCTION

To date, the use of biofuels instead of fossil fuel is a worldwide issue, which includes the consideration of both economic and environmental aspects [1]. Rising costs of petroleum oil is general economic issue in many countries for decades and fossil fuel combustion leads to increase in greenhouse gases, especially carbon dioxide [2]. The demand of diesel is higher in markets compared with gasoline and also no need of modified engine [3, 4]. Then biodiesel research and development is a major plan of the policies starting from 2008 and ending at 2022, aiming to change the use of diesel from crude palm oil to biodiesel [5]. Biodiesel is biodegradable fuel that is mainly made from oleaginous crops in the form of fatty acid methyl esters (FAMEs) or ethyl esters (FAEEs), where the major source of biodiesel in Thailand is crude palm oil [6]. However, palm oil is generally used for food production as cooking oil, which causes then the competition between uses for food and biodiesel is a challenge [7]. Moreover, the palm yield has slightly decreased since 2014 due to rising drought condition whereas the mandate is stable [8]. Furthermore, the limited arable land and additional fertilization requires more budgets for improvement. Hence, the new alternative biodiesel feedstocks need to be considered.

In terms of biofuel production, microalgae obtain several advantages over oleaginous crops, including higher growth rate, CO_2 neutral and no needs of arable lands [9]. Additionally, the direct conversion of solar energy into target biofuel is also a potential benefit as similar to plants, in which lipids and fatty acids profiles are varied depending on species or conditions [10]. Some microalgae mainly produce high unsaturated fatty acids (UFAs), which have lower gel point than saturated fatty acids (SFAs) biodiesel hence it is suitable to use in cold climate countries. On the other hand, higher saturated-fats could provide greater properties on oxidative stability and cetane number [11]. Furthermore, biodiesel produced from microalgae is the nontoxic [12]. Thus, using microalgae as the third generation of biodiesel could desirably facilitate the way on bioenergy applications.

Microalgae could live under harsh environments, which could trigger some metabolites production under those undesirable environments [10], corresponding to aquatic ecosystems that are regulated by ecological conditions with numerous factors [13]. Moreover, there is continuous degradation of nutrients and toxic substances in water, especially heavy metals from industry as wastewater discharges [14]. Therefore, the physiochemical approaches are necessary in industrial wastewater treatment process because of the high costs of chemicals [15].

The regulations in wastewater treatment have restrictions and rules on effluent discharge into the environment resources by the multiple steps of treatment [16, 17]. For instance, a phytoremediation of heavy metal ions in wastewater using algae instead of plants – e.g. water hyacinths – could provide ecologically safer, cheaper, and more flexible processes to remove metal ions from industrial wastewater [17, 18]. Microalgae have attracted a significant amount of attention due to their ability to raise their lipid accumulation under various concentration of nitrogen and phosphorus [15, 19]. However, to reach the desired levels of tolerance, each algal physiology is essentially required to be observed with individual conditions. In addition, different levels of metal dissolved in wastewater can also induce their great potential in lipid production [20]. Hence, we can use this opportunity to cultivate and trigger the microalgae for biodiesel production simultaneously as the biological process for further industrial applications [14, 21], which can consequently resolve Thailand's situation in terms of the reduction of petroleum-based fuel uses.

In general, microalgae are more suitable to be studied as biodiesel feedstocks over crops and cyanobacteria due to their higher lipid productivities, especially green algae – e.g. *Botryococcus braunii*, *Chlorella* sp., *Isochrysis galbana*, *Nannochloropsis* sp. and

Shizochytrium sp. [22-24]. Therefore, most of researchers have been interested in investigating the lipid content and fatty acid profiles of microalgae under severe conditions including the desirable methods for cell pretreatment and lipid extraction [10, 12, 25]. For instances, S Chakravarty, et al. [26] have recently studied the optimization of lipid accumulation in green microalga *Selenastrum* sp. under different levels of NaCl, nitrate and phosphate concentrations. The results showed that the highest lipid yield (33.72%) has been achieved under nitrogen starvation condition at day 9, then dramatically dropped after that. Another study has been done on *Chlorella pyrenoidosa* cultivation under oxidative stress induced by nitrate, phosphate and sulphate regimes, which resulted in the maximal lipid content at 48.90% under nitrogen starvation [27]. However, the both results demonstrated the strong growth inhibition because nitrogen is an essential nutrient for protein synthesis in organisms. Interestingly, nutrients limitation conditions could differently affect lipid accumulation in individual species [10]. Hence, the optimization of stress conditions is necessary for lipid accumulation and biodiesel quality of microalgae.

On the other hand, cyanobacteria also obtain several advantages as microalgae compared with higher plants. Notably, the advancement of synthetic biology and genetic manipulation has permitted engineering of

cyanobacteria to produce non-natural chemicals, whereby microalgae studies have more obstacle. Several synthesized biofuel precursors have been successfully produced by engineered cyanobacteria achieved from both conventional and synthetic engineering approaches such as alcohols, fatty acids and isoprenoids [9]. For biodiesel production, terpenes also attain good properties as precursors besides fatty acids after saturation by hydrogenation, including a lower freezing point, higher energy content and good fluidity at low temperatures due to their special carbon skeletons [28-30]. Terpenes are volatile hydrocarbon compounds that are naturally emitted by several higher plants, thus they could be extracted or derived with difficulty [9]. Similar to other hydrocarbons, terpenes have obtained structures in forms of short-, middle- and long-chain, and been classified by their number of carbon in terpenoid molecules, including hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), triterpenes (C30) and tetraterpenes (C40) [31]. Generally, terpenes could be naturally derived from either the methylerythritol 4-phosphate (MEP) pathway or the mevalonate (MVA) pathway, where MEP is often found in bacteria and plants plastid and MVA pathway is mostly found in eukaryotic organisms [29, 32]. The process of terpenes synthesis in cyanobacteria is involved with MEP pathway. However, cyanobacteria are devoid of key enzymes for terpenes synthesis, especially the last step

enzymes e.g. isoprene synthase (IspS), limonene synthase (LS), farnesene synthase (FS) or squalene synthase (SQS) [31]. Therefore, the metabolic engineering approach is required for terpenes-producing cyanobacteria.

To develop cyanobacteria-based bio-solar cell factories, certain key genes coding for chemicals that are absent in the native strains can be derived from the original source or made synthetically for heterologous expression of these target genes. Moreover, the factors involved in gene expression also need to be optimized in each species [33], such as promoters, ribosome binding sites (RBSs) [34], replicons and terminators. As mentioned above, cyanobacteria gain the advantage on genomic database over microalgae and also the genetic manipulation efficiency. *Synechococcus elongatus* PCC 7942 (Syn7942) is one of novel cyanobacteria, which has been studied as a host for terpenes production few years ago such as isoprene [35], limonene [36], farnesene [37, 38], amorpha-4,11-diene [39] and squalene [39, 40]. Unlike fatty acids-based biodiesel production, the number of carbons in terpenes can be controlled by each specific terpene synthetic enzyme, especially monoterpene (C10) and sesquiterpene synthases (C15) [30]. Most of terpene synthetic genes are derived from plants or yeasts due to their high activity such as LS from *Mentha spicata* [36, 41], FS from *Malus x domestica* [38], (E)-α-bisabolene synthase (BLS) from *Abies grandis* [42], SQS from

Saccharomyces cerevisiae and amorpha-4,11-diene [39, 40] and β-caryophyllene synthases from *Artemisia annua* [43]. In addition, the MEP pathway key genes have been mostly derived from native genes or bacteria i.e. *Escherichia coli* [38, 39, 44]. For example, SY Choi, et al. [40] had studied squalene production in Syn7942, which could achieve 11.98 mg/L/OD_{730} of squalene as the maximum production. The strategies used in this study were gene optimization (SQS derived from *S. cerevisiae*), gene overexpression (the *ispA*, *dxs*, *idi* gene) and fusion protein (CpcB·SQS) to initiate and improve the squalene production in Syn7942. Thus, the conventional engineering and synthetic biology could be cooperated as powerful tools for obligating and developing the terpene-derived biofuel production of engineered cyanobacteria.

Though similar in hydrocarbon composition, fatty acids and terpenoids are desirably used as biodiesel precursors, which should have major components as C9-C23 hydrocarbons [45]. To transform biofuels from an idea into a real industrial alternative to petroleum-based fuels, it is essential to understand how one can best design efficient biomaterials [46]. In contrast, a major challenge is the low amount of biofuel production in microalgae and cyanobacteria compared with other heterotrophic microorganisms [47]. Hence, the improvement of lipids and terpenoids by physiological and genetic modification approaches could

provide the promising biodiesel feedstocks – i.e. microalgae and cyanobacteria, which can be used as the feasible strategies for further industrial aspects.

CHAPTER II: ENHANCEMENT OF LIPID CONTENT IN *Chlorella sp.* ISOLATE FOR BIODIESEL PRODUCTION

2.1 Literature reviews

Microalgae obtain several advantages compared with crops, which including higher growth rate, zero CO_2 producing and no need of arable lands for cell growing. Notably, microalgae is attractive to because of their ability to raise their lipid accumulation under various concentration of nitrogen and phosphorus [15, 19]. Focus on phosphate limitation, higher lipid contents – i.e. neutral lipids and glycolipids – and unsaturated fatty acids (UFAs) at lower concentrations of phosphate are widely reported in some microalgae, including *Chlorella* sp. [10, 48]. The phosphorus found in biodiesel comes from the phospholipids contained in the oil used as feedstock, which could interrupt the engine systems by damage the catalytic converters [49]. Moreover, a maximum level of 10 mg/kg of phosphorus in biodiesel samples has been established by the ASTM 6751 standard. Furthermore, micronutrients or heavy metals also essentially participate in microalgae metabolic functions in form of co-enzymes or energy carriers [50]. In general, heavy metals could be toxic to microalgae. Although the addition of heavy metal concentrations has

been found that could enhance lipid content of microalgae (even in the same species) with different trends and patterns [51]. For instances, *Chlorella vulgaris* demonstrated that could enhance the absorption of Cu^{2+} by addition of HCl for approximately 70% [52]. Additionally, JPK Wong, et al. [53] had studied on nickel absorption by two *Chlorella* species – i.e. *C. vulgaris* (commercial) and *C. miniata* (a local isolate), which then found that the *C. miniata* isolate could absorb the maximal Ni(II) 2-times greater than *C. vulgaris*. PC Gorain, et al. [54] also had taken the opportunity from this ability of microalgae to adapt in biodiesel production by treating heavy metals (Ca, Mg and NaCl) to two green microalgae – i.e. *C. vulgaris* and *Scenedesmus obliquus*. Interestingly, increased concentration of Mg could enhance the lipids and biomass production in both microalgae, even though the higher NaCl could also improve lipid content approximately 40% but strongly reduce the biomass yields. Moreover, the major advantage from using microalgae as biofuel generators is their capability on CO_2 capture and could convert it to a more stable form for long term storage, which obtain the capture efficiency as high as 99% under optimal conditions [55]. In contrast, the conversion efficiency from solar energy to biomass is also significantly higher than higher plants, which is 100-fold faster on biomass productivity [56, 57]. Thus, the phosphorus limitation and metal stress

conditions need to be studied to investigate the trends and potential as a biodiesel feedstock, since some microalgae revealed the outstanding high lipid accumulation potential under various stresses. And the energy conversion efficiency could also represent the capability on biodiesel production in terms of economic aspects for further large-scale studies.

In this study, isolated microalga *Chlorella* sp. has been observed for the biomass, lipid content and lipid production for biodiesel production under the heavy stress and phosphorus limitation conditions. The heavy metals used in this study are iron (Fe), cobalt (Co) and lead (Pb). Fe and Co are general metal ions used in BG11 medium whereas Pb is the heavy metal found in contaminated area or wastewater. Several literatures have reported on their oxidative stress to microalgae leading to the desirably increase of lipid content, especially Fe and Co [10, 58-60]. Owing to Pb as non-BG11 based metals, the studies on their effects combined with P limitation are still unavailable so far. Additionally, the fatty acids compositions and biodiesel properties affected by each Pb condition had been also determined. In this background, the present study was purposed at improving the isolated green microalga, *Chlorella* sp. as the candidate for biodiesel feedstock by the heavy metal stress and P limitation.

2.2 Materials and Methods

2.2.1 Microalgal cultivation and biomass collection

Chlorella sp. was one of collection that isolated from natural brackish water in Thailand. The cultures of microalga were separated into 2 phases: Growth phase and Production phase. Growth phase was aimed to increase biomass production. The start optical density of microalgae is 0.5 at 680 nm in 800 mL BG11 medium [61]. All samples were then cultured under constant cool white light 40 µmol/m^2/s, 150 rpm at 30ºC for 14 days.

The production phase was anticipated to induce lipid accumulation in microalgae for 7 days with triplicates. The BG11-based metals had been observed under P deprivation and Fe^{3+} and Co^{2+} stress combinations, which Fe ($FeCl_3{\cdot}6H_2O$; UNILAB®, Australia) and Co ($Co(NO_3)_2{\cdot}6H_2O$; Sigma-Aldrich, USA; 98%) concentrations were 3, 6 µM and 1.7, 17 mM, respectively. Noted that the basic Fe^{3+} source in BG11 is Ammonium ferric citrate ($(NH_4)_5[Fe(C_6H_4O_7)_2]$; UNILAB®, Australia) with 21 µM as a final concentration and also 0.17 mM for Cobaltous nitrate. In non-BG11-based metal experiments, Pb^{2+} with concentration of 0.1, 1, 10 µM ($Pb(NO_3)_2$; Ajax Finechem, AR grade) was subjected into P limitation; 0%P, 50%P (0.115 mM) and 100%P or

normal BG11 (0.230 mM). According to A Piotrowska-Niczyporuk, et al. [62], exceeding 10 µM of Pb cab cause the toxic effect to microalgal growth. The biomass production was achieved by collecting 45 mL samples were collected in pre-weighted Eppendorf tubes at 0, 1, 3 and 7 days. Then centrifuged cell samples were kept in -80ºC until used and disrupted by -50ºC freeze dryer (Labconco®, USA) overnight. The lyophilized cells were weighted by gravimetric method.

2.2.2 Total lipid extraction and determination

Several solvent systems can be used for lipid extraction in microalgae. Using *n*-hexane is one of the suitable systems, which a simple method that revealed high efficiency in lipid extraction from green algae [63]. Microalgal total lipids were extracted by *n*-hexane system, which adapted from R Halim, et al. [64]. Briefly, 5-10 mg lyophilized biomass of each condition were suspended in 800 µL *n*-hexane. Total lipids were subsequently extracted in hexane phase after incubated shaking at ambient temperature overnight. Centrifuge at 8000 rpm (6000 × *g*) for 2 min and remove an upper layer to a new pre-weighted glass vial. Then evaporate residual hexane for 2-3 h or until it is constant weight. Each dried total lipid sample was determined by gravimetric analysis (**Appendix C**).

2.2.3 Transesterification and biodiesel properties analysis

Transesterification and fatty acid profiles analysis were partly adapted from M Limsuwatthanathamrong, et al. [65]. Briefly, each dried total lipid was firstly esterified into methyl esters by saponification with 0.5 N methanolic NaOH and transesterified with 14% BF_3 in methanol (v/v) [66]. Finally, cooling down the solution and adding NaCl for precipitation and remove the derivatized solution as form as fatty acid methyl esters (FAMEs) into a new vial. After transesterification, the FAMEs were analyzed by gas chromatography (GC 6890N/FID HP-INNOWax, Agilent, USA). Individual FAMEs were identified by comparison with C14-C20 FAMEs standard (Sigma-Aldrich, USA). The GC chromatographic temperature program was set as follow: initial temperature of 150ºC and the oven temperature was setting to gradient increasing to 180ºC at 10ºC/min, to 200ºC at 5ºC/min, to 205ºC at 0.5ºC/min and held at 205ºC for 2 min. Finally, the temperature was rising to 250ºC at 5ºC/min and then held for 5 min (30 min as a total running time).

According to R Sivaramakrishnan, et al. [67], the determination of biodiesel characteristics had been characterized by equations published earlier. Saponification value (SV) and iodine value (IV) were determined

by the equations from ÉC Francisco, et al. [47]. Cetane number (CN) was determined by equations published by MJ Ramos, et al. [11]. Cloud point (CP) was calculated using the equation from A Sarin, et al. [68]. KV, density, and higher heating value (HHV) were determined by equations published by LF Ramírez-Verduzco, et al. [69].

2.2.4 Oxidative stress from Pb treatment determination

The $Pb(NO_3)_2$ treated cells of H_2O_2 content was determined by treating the cells with 1 M potassium iodide (KI). The experiments were performed according to the V Alexieva, et al. [70] and the absorbance was read at 390 nm using UV spectrophotometer. A fresh known H_2O_2 concentration solution is used for standard curve [70]. The supernatant was mixed with 1 mL 0.5% thiobarbituric acid and 0.5 mL of 20% trichloroacetic acid to determine the MDA content. The reaction mixture then incubated in a boiling water bath for 15 min and centrifuged at 2790 $\times\ g$ for 10 min. The samples were analyzed using spectrophotometer at 450, 532 and 600 nm and the different values obtained from different nm were used to calculate the MDA content by using following equation [71].

$$MDA = \frac{[6.45 \times (OD_{532} - OD_{600})] - (0.56 \times OD_{450})}{W}$$

The lysed cell suspension was used for the determination of SOD and CAT activity by using kit protocol supplied Sigma Aldrich, USA (WST-1 reagent and CAT assay kit, respectively).

2.2.5 Energy conversion efficiency analysis

According to H-Y Ren, et al. [72] report, the potential of lipid production had been determined under various microalgal culture modes by calculating total energy conversion efficiency (TECE) from light to lipids as following equation.

$$TECE = \frac{HV\ of\ extracted\ lipids}{input\ light\ energy} \times 100\%$$

where TECE unit is % and HV of lipids is estimated as 36.3 kJ/g, which the values could be varied depending on different microalgal species. The input light energy was calculated by the equation in **Appendix D**, consequently in 18.88 kJ.

2.2.6 Statistical analysis

The results are presented as mean of three replicate values, with the error bars showing standard deviations (means ± SD, n = 3). Statistical significance ($p < 0.01$-0.1) was analyzed by t-test comparisons using graph pad software.

2.3 Results and Discussions

2.3.1 Phosphorus limitation conditions

In general, the nutrient limitation is method used for lipid induction in microalgae for decades, especially nitrogen and phosphorus limitation [73]. As mentioned above, nitrogen deprivation is strongly inducing the lipid content but also negatively affecting the cell biomass accumulation in *Chlorella* sp. [74]. The phosphorus limitation has been less studied and also could elevate lipid content and unsaturated fatty acids in some microalgae species, including *Chlorella* sp. [48, 73, 75]. Thus, the increase of unsaturated fatty acids is desirable for biodiesel production aspect, which could be achieved from phosphorus limitation in microalgae cultivation [76, 77].

The isolated *Chlorella* obtained a lipid content 30-40% (stationary phase), which within the range of other *Chlorella* (2-55%) reports [78]. In this study, we chose the stationary phase *Chlorella* as initial cells for lipid production due to the higher lipid content and well growing under stress conditions [79, 80]. The lipid content in normal condition became steady after 7-day production phase. The lipid content was increased from 43 to 56% within 1 day and higher than control approximately 21% under P deprivation (**Figure 1**). The result showed that is higher than some

previous *Chlorella* studies under P deprivation (0%P) i.e. *Chlorella* sp. BUM11008 (31.9%) [74], *C. zofingiensis* (44.7%) [81] and *C. pyrenoidosa* (32.77%) [82] and other *Chlorella* sp. (13.9%) [83]. Thus, the 0%P could not effectively elevate lipid content in some *Chlorella* [80]. The phosphorus plays important roles in ATP and NADPH production that is required to drive lipid synthesis and photosynthetic activity [84]. Therefore, the 0%P could severely decline the chlorophyll content and lead to low biomass [85]. As expect, the biomass was reduced under 0%P and resulting in the lower lipid production than control, which the biomass was slightly declined and remains steady after 3-day cultivation. However, the lipid content could be less affected because the reduction of phospholipids have not decreased the total lipids but could alter the lipid composition, e.g. diacylglyceryl-trimethylhomoserine (DGTS), diacylglyceryl-hydroxymethyltrimethyl-β-alanine (DGTA) and diacylglyceryl-carboxyhydroxy-methylcholine (DGCC), in plastid membranes of several green microalgae, including *Chlorella* [73, 86]. For examples, the 0%P cultivation of green alga *Monodus subterraneus* also reduced the phospholipid but could increase triacylglycerols (TAG), which mainly in digalactosyldiacylglycerol (DGDG) and DGTS [87]. According to L Alipanah, et al. [88], they had studied on the changes in expression of genes associated with lipid

metabolism under 0%P in diatom *Phaeodactylum tricornutum*. They observed the downregulation of fatty acid synthesis pathway, which leads to the decrease in membrane lipid synthesis and the lowering cell division. Additionally, the upregulation of phospholipid:diacylglycerol acyltransferase (PDAT) and phospholipase genes was detected, which acted to degrade the phospholipids and then release into diacylglycerol (DAG) and phosphatidic acid (PA) resulting in high TAG accumulation. Thus, this study could suggest that the isolated *Chlorella* sp. could accumulate high lipid content under P deprivation. However, the biomass is still low then the further optimization of nutrients is required.

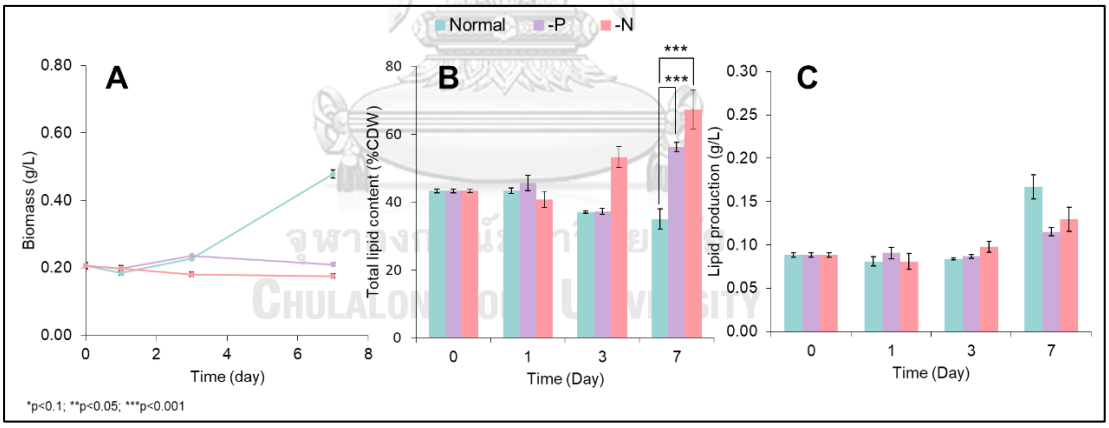


Figure 1. The effects of *Chlorella* cultivation under normal, N and P derivation in BG11 conditions. (**A**) Biomass (g/L) (**B**) Total lipid content (%DCW) (**C**) Lipid production (g/L). All the data are expressed as mean ± standard deviation from cultures that were run in triplicate. Total lipid content is significantly greater in -P

and -N for *t*-test; The *p*-values are 0.000775 and 0.007706, respectively (indicated by asterisks).

2.3.2 Effects from the combination of phosphorus limitation and BG11-based metals stress

The optimized key macro- and micro-nutrient based in BG11 medium could differently alter the lipid content in each microalga. The limitation of phosphate and the supplement metal ions – i.e. Fe^{3+} and Co^{2+} – could elevate the lipid content in *Chlorella* [89]. In this study, we have combined both of 0%P and metal addition in *Chlorella* cultures in order to increase the lipid content. Both of Fe^{3+} and Co^{2+} addition conditions demonstrated the increase of lipid content with 10% and 19% over the control, respectively. However, the lipid production was lower than control due to the inhibited growth from stressed conditions. Interestingly, the Co^{2+} conditions showed the higher lipid content than Fe condition reaching 69% at day-1 cultivation whereas Co^{2+} also stronger suppressed the cell growth.

Fe^{3+} is one of the most appropriate heavy metals used to increase the lipid content in microalgae [90]. Interestingly, the addition concentration of Fe has not only improve the lipid content but could also increase the cell growth in green algae [67, 91]. In this study, we applied

$FeCl_3$ as an extra iron supplement source because of its strong influence in lipid content in green algae [92]. The combined Fe^{3+} stress and P deprivation condition could negatively affect the cell growth but not completely inhibited (**Figure 2A**). P Singh, et al. [93] have studied on the physiological effects of *Chlorella sorokiniana* under N and P limitation combined with various stress conditions. Because Fe^{3+} and Mg^{2+} both play significant role in photosynthesis, thus maximum quantum efficiency of photosystem II (F_v/F_m) was improved under metal stress condition. Consequently, the chlorophyll content was increased when the metal concentrations (Fe, Mg, Ca) were also increased. Additionally, green alga *Ankistrodesmus faculatus* KJ671624 has been studied on biomass and lipid content under various stress combinations i.e. N, P and Fe^{3+} [94]. They also found that the highest lipid content (59.6%) was achieved under N limitation (750 mg/L), P deprivation and Fe^{3+} addition (9 mg/L) and the biomass was declined approximately 2-fold from the control (BG11). Notably, a sufficient Fe amount could prevent a severe drop in biomass production in several microalgae even were under the essential nutrient limitation (i.e. N and P) [58, 92, 94].

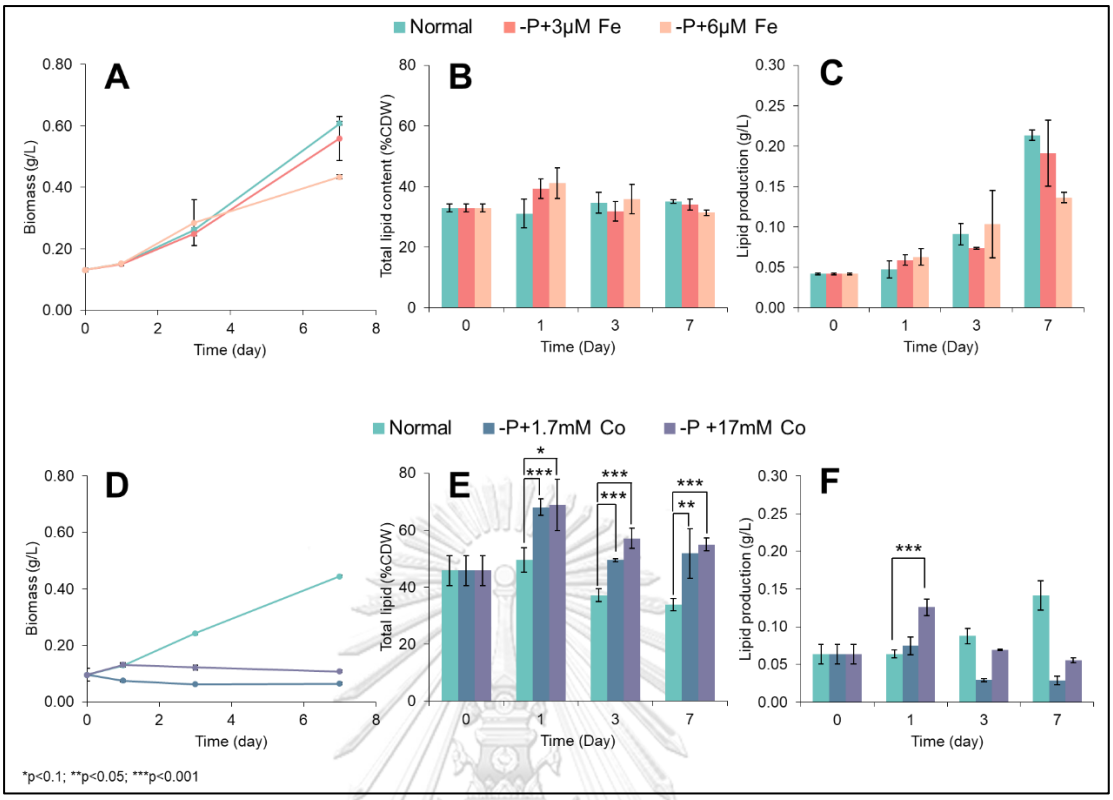


Figure 2. The effects of *Chlorella* cultivation from P deprivation with BG11-based metals (Fe and Co) treatment conditions. (**A**) and (**D**) Biomass (g/L) (**B**) and (**E**) Total lipid content (%DCW) (**C**) and (**F**) Lipid production (g/L). Fe; (**A-C**), Co; (**D-F**). All the data are expressed as mean ± standard deviation from cultures that were run in triplicate. Total lipid content and lipid production are significantly greater in -P with 1.7 and 17 mM of cobalt for *t*-test; The *p*-values are between 0.00065 to 0.051568 (indicated by asterisks).

In this study, 17 mM cobalt nitrate (100X of normal BG11) was applied to the P deprived BG11 leading to the improvement of lipid content and the reduction of biomass (**Figure 2D and 2E**). Unlike Fe^{3+} studies, M Battah, et al. [59] reported that Co^{2+} could also increase lipid

content but slightly suppress the biomass in *C. vulgaris* even presented in small amount (≥3 µM) added in bold basal medium (BBM). Moreover, M Li, et al. [95] also found that ≥10 µM of Co^{2+} could inhibit the cell growth of marine microalga *Pavlova viridis* and markedly increased antioxidant enzymes and non-enzyme antioxidative substances over control value showing a strong oxidative stress occurred. Additionally, SE Plekhanov, et al. [96] had studied on several metal effects in *C. pyrenoidosa* and observed the rapidly decrease of F_v/F_m under 0.1-10 mM of Co^{2+} treatment within 30 min. Thus Co^{2+} can induce toxicity in *Chlorella* and other green microalgae and has strongly inhibited the growth and photosynthesis when presented in too high concentrations [95, 96]. Therefore, the 19% of lipid content had increased over the control, but it was still lower than previous study (22%; M Battah, et al. [59]). Although the combined Co^{2+} with P deprivation might cause a lethal effect to the *Chlorella* even incubated for 1 day. Obviously, Fe and Co both play significant roles in photosynthesis and microalgal growth, where Fe is the most essential heavy metal of PSII [93]. Hence, these results clearly revealed that the positive influence of only Fe on the photosynthetic physiology and lipid content of microalgae whereas inappropriate concentration of Co could turn the negative effects to

microalgal growth. The incubation of *Chlorella* in presence of Co should be further adjusted properly with shorter time or alternatively lower concentration to allow better lipid production.

2.3.3 Lead stress combined with phosphorus limitation and oxidative stress determination

Pb^{2+} has been also studied that could trigger the lipid content in microalgae and growth when presents in proper amount [62, 97]. In this study, the P limitation (50%P) and P deprivation (0%P) conditions have been studied along with Pb^{2+} additional cultures to observe altered lipid content. The controls were different in each experiment – i.e. normal BG11 (100%P), P limitation (50%P; 115 µM of PO_4) and P deprivation (0%P), which the Pb^{2+} supplement samples revealed the declined trends in lipid content compared with controls (**Figure 3B, 3E, 3H**). Interestingly, the Pb^{2+} supplemented cultures indicated the better growth than controls in 50%P and 0%P conditions and elevated to the improved lipid production with 0.16 and 0.13 g/L, respectively. Additionally, the 50%P without Pb^{2+} addition could elevate the total lipids into 60% in 1 day. Obviously, the culture with presence of Pb^{2+} could trigger the biomass production of *Chlorella* when phosphate is limited. Thus, the P

limitation and Pb^{2+} addition combinations could positively affect to the changes in lipid production in *Chlorella*.

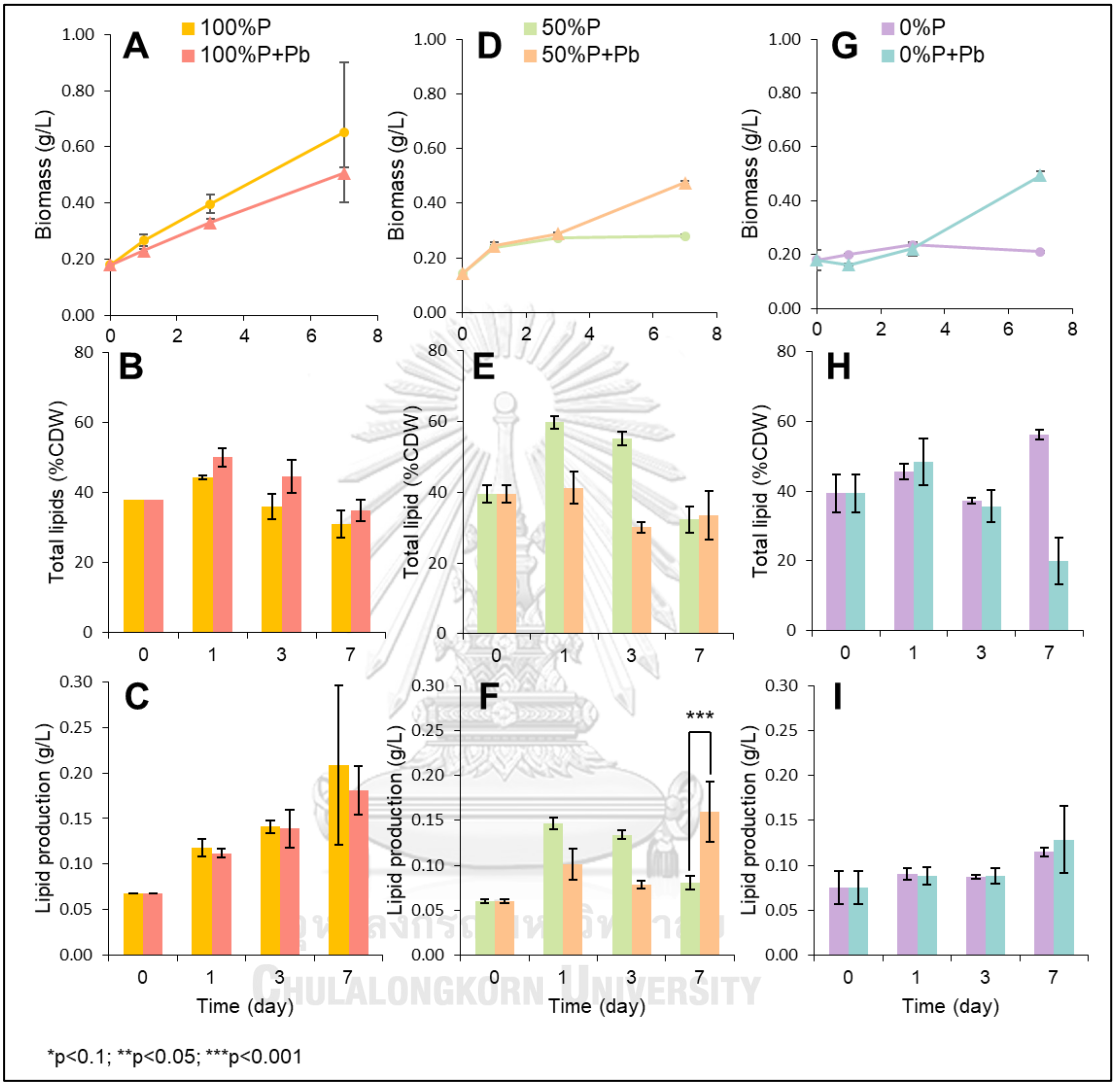


Figure 3. The effects on *Chlorella* cultivation from non BG11-based metals i.e. Pb treatment in different P concentration conditions. (**A**, **D**, **G**). Biomass (g/L) (**B**, **E**, **H**). Total lipid content (%DCW) (**C**, **F**, **I**). Lipid production (g/L). 100%P; (**A-C**), 50%P; (**D-F**). 0%P; (**G-I**). All the data are expressed as mean ± standard deviation from cultures that were run in triplicate. Lipid production is

significantly greater in 50%P with 1 µM of lead for *t*-test; The *p*-value is 0.000783 (indicated by an asterisk).

Besides the P deprivation, P limitation (50%P) is also could increase the lipid content and alter the fatty acids composition in green microalgae. K Liang, et al. [48] had studied effect of phosphorus in *Chlorella* sp., which could enhance a lipid content reaching to 23.60% at 32 µM phosphorus. Furthermore, the fatty acid profiles were also different in each concentration, especially stearate acid (C18:0) decreasing 15.34% from 240 to 16 µM. P Singh, et al. [93] had investigated the effects on lipid content and biomass of microalgae, which biomass could be reduced either under limitation or repletion of P condition [93]. In addition, they also found that the heavy metal stress could induce the cell growth and lipid content even under macronutrients limitation condition as same as this Fe^{3+} study. This could be a reason why the Pb cells could grow better under P limitation. Unlike Fe^{3+} and Co^{2+}, Pb^{2+} is heavy metal that non-essential micronutrient for microalgae growth and found as toxicity to environment. Therefore, the lower range of Pb has been studied in microalgae such as 0.01-500 µM [97, 98]. Thus, the unharmful levels of Pb could induce some pigment contents in microalgae range from 0.01-10 µM addition [98]. Additionally, A Bajguz

[97] had reported on the effects of heavy metals inhibitory in *C. vulgaris*. Consequently, the cell density was reduced when increase a concentration of Pb treatment from 1 to 100 µM whereas the cells number and chlorophyll content of 1 µM Pb condition were barely different from control. Moreover, lead treatment also revealed the highest accumulation in cellular *C. vulgaris* within first 24 h. Then A Piotrowska-Niczyporuk, et al. [62] had been studied Pb adverse effects in green alga *Acutodesmus obliquus* but it showed different trends of Pb accumulation from *C. vulgaris*, which the cell growth and contents of pigments of *A. obliquus* were suppressed when at lower concentration of Pb (≥0.1 µM) than *C. vulgaris*. Recently, T-L Pham, et al. [99] has studied on lipid production combined with removal of lead (0.05-10 mg/L) from wastewater by *Scenedesmus* sp. Consequently, the cell concentration was rapidly dropped when increase of Pb concentration from 1 to 2 mg/L and beginning to a stationary phase after 5 days. And the maximum lipid contents (31.1% and 30.8%) were achieved at 0.5 and 1 mg/L of Pb. Thus, the 0.1-10 µM of Pb have been selected to be used as the selected concentration for *Chlorella* cultivation in this study. The results shown that the maximum lipid content was observed under presence of 1 µM and the decrease of biomass in 10 µM of Pb conditions (data not shown),

which was corresponding to A Bajguz [97] study. In addition, the oxidative stress treatment is also necessary for distinguishing the Pb toxicity and the stimulation of lipid content to this isolated *Chlorella*. And I also determine the fatty acid profiles of Pb addition conditions for further used in biodiesel production and wastewater treatment aspects.

Oxidative stress treatment was performed to improve the lipid content. After 24 h treatment of Pb displays variations in the H_2O_2, MDA, SOD and CAT contents and it was shown in **Figure 4**. H_2O_2 is the important marker to determine the ROS effects of cells after Pb treatment. After the treatment H_2O_2 level was elevated when compared to the control. Oxidative treatment showed some positive effects on lipid content. During oxidative stress, O_2^- reduced in chloroplast electron transport and forms H_2O_2 [100]. The presence of ROS (oxidants) stimulate the signaling molecules and progresses the physiological responses and cell growth [101]. In the present study, it is obvious that the Pb treatment increases the H_2O_2 as a response of oxidative stress.

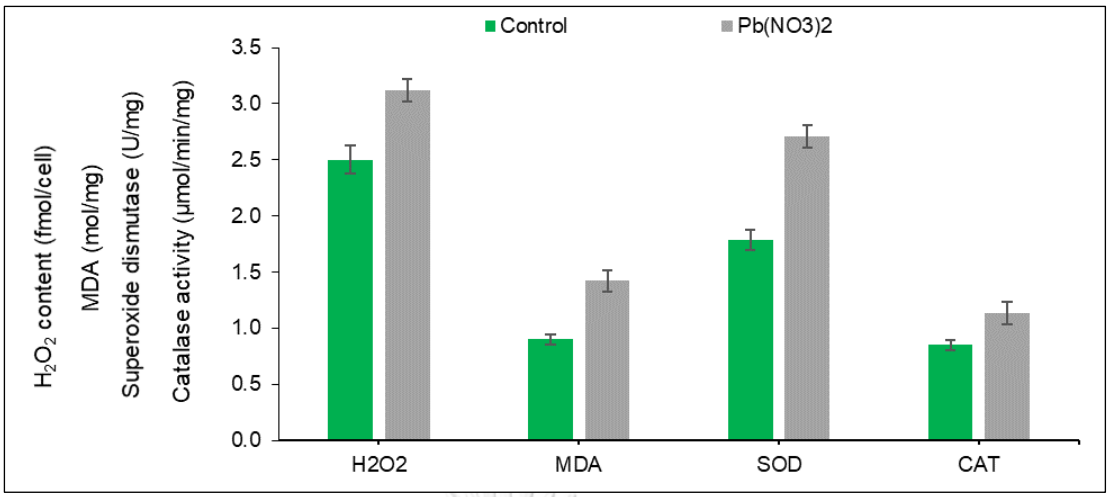


Figure 4. H_2O_2 and MDA levels, SOD and CAT activities on 10 µM of Pb supplementation. All the data are expressed as mean ± standard deviation from cultures that were run in triplicate.

To determine the effect of ROS, MDA is the important marker to study the effect of ROS. During oxidative stress, poly unsaturated fatty acids get oxidized and generate MDA and hence it is considered as an oxidative marker. Cell walls get damaged and the cells try to recover itself by lipid peroxidation [102]. In the present study, MDA level was increased after Pb treatment and it confirms that the cells able to defend against the oxidative stress.

In response of H_2O_2 and MDA, cell itself produce antioxidant enzymes to neutralize the effects of ROS. The antioxidant enzymes SOD and CAT were analyzed and shown in **Figure 4**. SOD is an important metalloenzyme which efficiently scavenges the superoxide molecules

[71]. In the present study, Pb treatment increased the ROS, and the cells were well responded against ROS by SOD and CAT enzymes. Both the enzyme activity was enhanced after the oxidative treatment. Due to the action of SOD and CAT enzymes, ROS-mediated electrons flow towards lipid synthesis to neutralize the effect of ROS and it was evident with the lipid content after the Pb treatment.

2.3.4 Fatty acid profiles and biodiesel properties under Pb conditions

The fatty acid compositions and biodiesel properties are determined from day-1 *Chlorella* samples (in FAME forms) under different phosphorus and Pb concentrations (**Table 1**). The ratio of SFAs and UFAs had showed in high value compared with other microalgae because of high content of palmitic acid (C16:0) and stearic acid (C18:0), where the others are mixers of C18:2, C18:3 and low content of C20:0 (≤0.01%) [10]. In this study, the reduction of the decanoic acid (C10:0) and stearic acid (C18:0) was observed under the decrease of P concentrations although the C16:0 have been increased, especially under presence of Pb (54.57%). Under 0%P condition, the UFAs are higher and different in compositions compared with other conditions, which majorly found as palmitoleic acid (C16:1). Hence, phosphorus limitation could increase UFAs in *Chlorella* sp., especially MUFAs [73, 74].

Interestingly, higher amount of MUFAs could obligate the balance between CN and CFPP properties of biodiesel [76]. Additionally, the SFAs content and length could directly affect the cloud point (CP), which the different SFAs mixture is more favorable than a high single SFA component due to their independent crystallization. Furthermore, I also attempted to test the nitrogen limitation and found the high amount of UFAs in both with and without Pb (data not shown) as similar to previous reports [10]. However, the low levels of phosphorus could improve a quality of biodiesel by reducing damage to catalytic converters in operational systems [49]. Then we had determined the key properties which affect the quality of biodiesel: SV, IV, CN, CP, KV, Density, and HHV were analyzed as shown in **Table 1**, which each property has followed to SK Hoekman, et al. [103].

Saponification value. The SV is a measure of the average molecular weight of all fatty acids present. Maximum SV was obtained under 100%P with Pb supplement condition at 2.37 g KOH/g lipid. In contrast, the Pb addition demonstrated the higher SV than non-Pb cultures in each phosphorus-limited condition. Thus, the addition of 1 µM Pb could positively alter the SV in all the three concentrations – i.e. 100%, 50% and 0% of phosphorus condition, which could increase from controls at 10.14, 4.78 and 4.17 g KOH/g, respectively.

Iodine value. Iodine value is a measure of total unsaturation of biodiesel, and this value is used to represent oxidative stability [104]. The increase of IV could negatively affect engine performance because high alkyl double bonds lead to formation of insoluble sediments from degradation. According to the European biodiesel standard, IV is limited to 120 g I_2/100 g. The results showed that the value of all conditions within the limits. In all the cases, the value was not significantly different, except under P deprivation without Pb that obtained the highest value at 38.75 g I_2/100 g whereas higher than the others 18.06-22.18 g I_2/100 g.

Table 1. Fatty acid profiles and biodiesel properties as per ASTM D675 and EN14214 under combined stress conditions. Fatty acid profiles of *Chlorella* treated with 1 µM of Pb under normal and P-limited BG11 for maximum lipid production. Fatty acids were measured after 1 days of incubation. All the data are expressed as mean ± standard deviation from cultures that were run in triplicate; Values are given as percent (%) of total fatty acids. SFA Saturated fatty acid, UFA unsaturated fatty acid**.** n.d. Not detected. [a]Phosphate concentration. [b]C18:2 and C18:3 total %.

Fatty acid compositions (%)						
Types of fatty acids	BG11			BG11 with Pb		
	100%[a]	50% [a]	0% [a]	100% [a]	50% [a]	0% [a]
Decanoic acid (C10:0)	8.21	7.66	n.d.	24.30	11.16	2.56
Myristic acid (C14:0)	11.86	10.88	≤0.01	3.33	11.13	12.02
Palmitic acid (C16:0)	42.27	45.86	49.24	26.19	38.59	54.57
Palmitoleic acid (C16:1)	15.52	9.32	33.36	5.27	17.15	7.61
Stearic acid (C18:0)	17.94	16.68	11.36	25.24	18.01	11.89

Oleic acid (C18:1)			≤0.01	6.92	≤0.01	10.59	≤0.01	2.30
Others[b]			1.20	2.69	6.05	5.08	3.97	8.98
SFA			83.28	81.07	60.60	79.06	78.88	81.04
UFA			16.72	18.93	39.41	18.89	21.12	18.89
Factors	**EN**	**ASTM**	**Biodiesel properties**					
SV	NA	NA	226.50	224.14	215.32	236.64	228.92	219.49
IV	120	NA	16.57	17.95	38.75	19.35	20.69	17.74
CN	>51	>47	66.67	66.61	62.93	65.00	65.49	67.18
CP	NA	NA	18.82	19.13	20.91	8.78	15.31	23.71
HHV	NA	NA	38.90	38.97	39.21	38.56	38.82	39.09
Viscosity	3.5-5.0	1.9-6.0	3.47	3.54	3.70	3.18	3.37	3.68
Density	0.86-0.90	NA	0.87	0.87	0.87	0.87	0.87	0.87

Units of factors. SV (mg KOH/g)**,** IV (g I_2/100g)**,** CP (°C), HHV (MJ/kg), KV (mm^2/s), Density (g/cm^3).

Cetane number. Cetane number is an important property determining the combustion quality, and ignition delay time. A high CN ensures the ignition properties and good engine performance as well as the reduction of white smoke formation from the engine. The results demonstrated that all of the samples are higher than the minimum value of the American Society for Testing and Materials (ASTM) and European standards. The lowest CN was found in 0%P whereas the highest is also found in 0%P with the Pb addition. However, the range of CN is not significant different in each condition. Notably, this *Chlorella* revealed a higher CN (62.93-67.18) than *Chlorella* sp. in some previous studies

[105], which meant that it could be blended at higher concentrations with petroleum diesel [10]. Thus, this is the good candidate for biodiesel production.

Cloud point. Cloud point is the temperature at which the fuel starts to appear cloud and confirms the wax crystal formation which blocks the filters and fuel lines of the vehicle [106]. No limit ranges are given in ASTM due to the fact that the climate conditions in the United States vary considerably. However, a lower range of CP is more suitable. In this study, the range was rather high in each condition, except in 100%P with Pb condition (8.78°C). Additionally, the decrease of P concentrations was observed that could lead the increase of CP, where the highest CP was in 0%P with Pb at 23.71°C. However, this *Chlorella* oil still obtained a high CP when compared with other microalgae oil [105]. A number of double bonds located near the ends of carbon chains were higher than the middle of carbon chain or might have low shorter linear carbon chain than longer chain, consequently this oil have high CP [107].

Kinematic viscosity. Kinematic viscosity is essential value used to present the resistance of biodiesel flow in fuel injection system at low temperature, which samples obtaining *cis* double bond generally have higher KV than samples obtaining *trans* double bond [104]. Moreover, the unsaturated FAMEs have lower viscosities than the saturated FAMEs.

Hence, the high KV could cause to poor vaporization and atomization of the engine. For ASTM values, the KV range is 1.9–6.0 whereas the KV range in Europe is 3.5–5.0. The results demonstrated that KV values of all conditions are within the limits of both EN and ASTM. Interestingly, the KV of this *Chlorella* FAMEs was achieved at lower values (3.18-3.70 mm^2/s) than several crops biodiesels and some microalgae – e.g. *Nannochloropsis oculate*, *Dorstenia brasiliensis* and *B. braunii* [10, 108].

Density. The density of fatty acids is related to the number of carbon atoms present, which high saturated and shorter chain fatty acids could lead to high density biodiesel [104, 107]. The lower density is desirable for the fuel injection process, which correlated with amount of fuel into the engine estimated by its volume (g/cm^3). Thus, the high content of long chain unsaturated FAMEs is better for blending with biodiesel. Adopting European values, the density (g/cm^3) ranges between 0.86 and 0.90. All experiments revealed same density values at 0.87, which achieved more considerable value than vegetable oils – e.g. palm (0.897), rapeseed (0.913) and soybean (0.916) [107].

Higher heating value. Higher heating value is the amount of heat released after the complete combustion, which in a unit quantity of fuel into H_2O and CO_2 [107]. HHV could be decreased when double bonds increase and high amount of shorter chain fatty acids. However, the

selection between the lower or higher HHV compounds as biodiesel sources remains unclear. In general, HHV of microalgae biodiesel was found to be in the range of 36.6-40.4 MJ/kg, which is normally lower than that obtained from commercial diesel fuel (45.62-46.48 MJ/kg) [10, 106]. The HHV of all experiments were between 38-39 MJ/kg, which within the range as MA Islam, et al. [10] report.

2.3.5 Energy conversion efficiency

This shows that algal cells cultured in normal and stress conditions can efficiently convert the substrate into lipids. However, under the stress conditions, the TECE of cultivations were lower than normal condition due to the lower biomass production. The highest TECEs were obtained at 26.70±1.52% and 19.66±6.28% under in normal BG11 with Pb and 0%P with Fe condition, respectively **(Table 2).** Interestingly, the results have been higher than *Scenedesmus* sp. cultured under mixotrophic condition (14.6%) in previous study [72]. Interestingly, the either nutrient limitation or heavy metal stress of microalgae could allow the improvement of the energy conversion even under autotrophic cultivation. To further enhance the economic feasibility of algal biodiesel production in wastewater, it is essential to fittingly cultivate microalgae outdoors in natural conditions.

Table 2. Energy conversion efficiency from light to lipids under different conditions.

Conditions	Total lipids (g/L)	HV of lipids (kJ)	Input light energy (kJ)	TECE (%)
Normal	0.19	6.90	44.05	15.66
P dev	0.17	6.17	44.05	14.01
P dev Cobalt	0.13	4.72	44.05	10.71
P dev Iron	0.14	5.08	44.05	11.54
Pb	0.18	6.53	44.05	14.83
50%P Pb	0.16	5.81	44.05	13.19
P dev Pb	0.13	4.72	44.05	10.71

CHAPTER III: EVOLUTIONARY ENGINEERING IN *Synechococcus elongatus* PCC 7942 FOR α-FARNESENE PRODUCTION

3.1 Literature reviews

Most of studies conducted on metabolically engineered cyanobacteria for the production of photosynthetic terpenes have been done by overexpressing the key genes in the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway coupled with heterologous expression of specific terpene synthases. The key genes in the MEP pathway can be both native genes or foreign genes (from novel bacteria), which play significant roles in supplying intermediates from G3P and pyruvate, such as dimethylallyl diphosphate (DMAPP), isopentenyl diphosphate (IPP), geranyl diphosphate (GPP) or farnesyl diphosphate (FPP), and triggering the terpene synthesis pathways [32, 109]. The rate-limiting genes in the MEP pathway have been already studied based on the *E. coli* pathway in different species of cyanobacteria, which include DXP synthase (dxs), isopentenyl diphosphate isomerase (idi), HMB-PP synthase (ispG) and farnesyl diphosphate synthase (ispA) [110, 111]. For examples, metabolic engineering of Syn7942 has achieved great improvement in the isoprene production level (up to 1.26 g/L) using a strong inducible promoter (P*trc*)

with the overexpression of the key MEP pathway genes (*idi, dxs, ispG*) coupled with a fused *idi-ispS* [35].

Besides the overexpression of MEP genes, heterologous terpene synthases have been also considered. Terpene synthases are usually derived from plants and certain terpene-producing bacteria, which most of plant enzymes have been specified to be compatible for cyanobacterial expression [31]. Terpenes are derived from a prenyl diphosphate (FPP) precursor and are composed of isoprene (C5) units. Photoautotrophic cyanobacteria are exceptional microbial cell factories that can perform CO_2 fixation and facilitate direct conversion of photon-to-fuel [112-114]. Push-pull metabolic engineering of cyanobacteria has enabled the production of many different classes of terpenes such as hemiterpene (C5), monoterpene (C10), sesquiterpene (C15), diterpene (C20), triterpene (C30), and tetraterpene (C40) [31].

In this study, acyclic sesquiterpene – i.e. α-farnesene, has been produced in engineered Syn7942. α-Farnesene is a valuable volatile compound in various applications e.g. pharmaceuticals, fragrances and advanced biofuels [29, 115]. Focus on biofuel application, α-farnesene obtains desirable characteristics as biodiesel precursor – i.e. low hygroscopicity and high energy density. In fact, the natural α-farnesene is generally found in several plants such as plant pollination, apple aroma

and immune response to herbivores [115]. Obviously, it could be produced in limited amount and difficult to achieve in origin plants. Thus, the metabolic engineering of cyanobacteria could provide the promising tools to enhance the α-farnesene production. Previously, the engineered cyanobacteria had been already studied in *Anabaena* sp. PCC 7120 (Ana7120) [37] and Syn7942 [38] by heterologous expressing the codon-optimized farnesene synthase (FS) from Norway spruce and *Malus x domestica* (apple), respectively. Additionally, the overexpression of the key genes in the MEP pathway (codon-optimized *dxs-idi-ispA* from *E. coli*) under the control of *trc* promoter has also been employed in Syn7942 to increase the pools of farnesyl diphosphate (FPP) as intermediate. This strategy significantly elevated the production of α-farnesene up to 15-fold (4.6 mg/L) compared to that with a single heterologous expression of P*nir-psba1-FS* in Ana7120. In fact, Ana7120 displayed slower growth rates than Syn7942 and this might be the reason behind the low production. In addition, P Hellier, et al. [116] had studied the toxic effect of five terpenes – i.e. geraniol, geranial, linalool, farnesene and citronellene – on *Synechocystis* sp. PCC 6803 (Syn6803) with an absence of *n*-dodecane overlay, consequently in toxic effect of all terpenes (the least toxic is citronellene). Notably, the cultures of Syn6803 with a presence of *n*-dodecane indicated the enhancement in cell growth

under 0.02-1.0%v/v of farnesene. Thus, this is the alternative strategy to generate the two-phase system of the non-toxic organic solvent and cyanobacterial culture medium for terpene production. Furthermore, the development of solvent system [38] with dodecane was also helpful to collect the more volatile α-farnesene released from the cells compared with the sealed gaseous trapped system [37].

Push strategies of metabolic engineering can be applied to increase the key intermediaries by overexpressing the key enzyme(s)—such as farnesyl diphosphate (FPP), for terpene synthesis—in the methylerythritol phosphate (MEP) or mevalonate (MVA) pathway. However, pull strategy of metabolic engineering is based on improving the enzymatic abilities of terpene synthase to produce terpenes as a carbon sink from the accumulated intermediates [40, 117]. Therefore, a balance between the push and pull pathways is important when engineering a strain is not for only reducing the toxicity resulting from the accumulation of key intermediaries, but also for enhancing the titer of the final product [38, 39, 118].

However, lack of a precise regulator of target gene expression or dynamic genetic switch in engineered strains may cause an imbalance between the push and pull pathways, resulting in lower production of biochemicals or growth inhibition [119, 120]. To overcome this

limitation, adaptive laboratory evolution (ALE) has been applied to construct microbial cell factories using metabolic engineering and random mutagenesis [121-123]. This method leads to the generation of a large number of mutant libraries (commonly >100 samples), therefore a high-throughput screening technique is often employed later to rapidly screen all the samples within a short time period [124, 125]. ALE has been applied to improve the production of chemicals using natural or artificial selection by growing the microbes in a controlled condition [121]. Recently, a cyanobacterium, Syn6803 evolved to grow under extremely high concentrations of isobutanol (5 g/L) after long-term adaption in a medium containing 2 g/L isobutanol [126]. Another study obtained an evolved Syn6803 strain by culturing it in a medium containing 9 µM cadmium sulphate ($CdSO_4$) over 800 days in order to identify high cadmium tolerant strains that can be used for wastewater treatment [127].

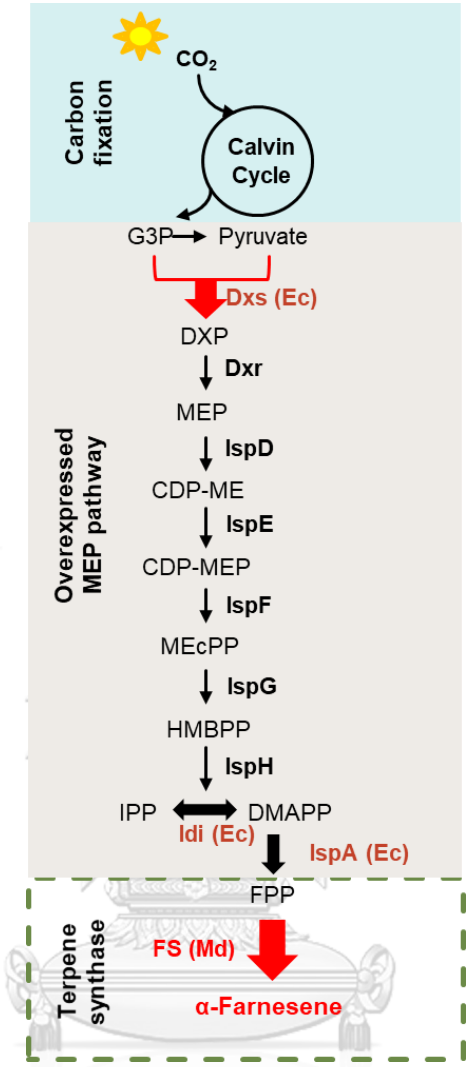


Figure 5. A schematic pathway depicting the α-farnesene production by heterologous expression of α-farnesene synthase in the Methylerythritol Phosphate (MEP)-optimized recombinant *S. elongatus* PCC 7942 (SeHL33). The strain SeHL33 overexpressing the key genes (*dxs*, *idi*, and *ispA*) of the MEP pathway has been developed to supply the pool of farnesyl pyrophosphate (FPP) [39], which is shown in orange. FPP is converted to farnesene by heterologous farnesene synthase (red).

Among the photosynthetic terpenes produced by microbes, phototrophic sesquiterpene compounds, such as β-caryophyllene [43]

(0.32 ng/L), α-bisabolene [42] (0.6 mg/L), amorpha-4,11-diene [39] (19.8 mg/L), and farnesene [38] (4.6 mg/L), have been successfully produced in metabolically engineered cyanobacterial strains. Metabolic engineering of Syn7942 with overexpressed MEP pathway genes (*dxs*, *idi*, and *ispA*) and synthetic *FS* gene has resulted in the production of 4.6 mg/L of α-farnesene (a precursor of squalene or biodiesel) from CO_2 (**Figure 5**) [38]. However, the final yield of α-farnesene was relatively low compared to either phototrophic sesquiterpene production or heterotrophic α-farnesene production [31]. Thus, in this study, modular engineering to regulate gene expression, protein engineering of farnesene synthase (FS), and evolutionary engineering were performed in Syn7942 in order to increase the production of α-farnesene from CO_2.

3.2 Materials and Methods

3.2.1 Strains and plasmid construction.

All the bacterial strains and plasmids used in this study are listed in **Table 4**. *E. coli* DH10β was used for gene cloning and was grown in Luria-Bertani (LB) medium supplemented with 50 µg/mL kanamycin at 37°C. Different ribosome binding sites (RBSs) from pSe2Bb1k-AFS (apple fruit - *AFS1*; GenBank accession number AY182241) plasmid [38] were designed using the RBS Calculator software v2.0 (Salis Lab; De Novo DNA, CA). The translation initiation rates (TIR) of the RBS sites were calculated at 10-fold different levels (i.e. 56871, 5687, 568 arbitrary unit. [a.u.]). Polymerase chain reaction (PCR) components were purchased from Phusion High-Fidelity PCR Kit (Thermo Fisher Scientific™). Briefly, the reactions were running for 30 cycles (Step 2-4), which programmed as (1) Initial denature step: 98°C, 30 s. (2) Denature step: 98°C, 10 s. (3) Annealing step: 55-68°C, 30 s. (4) Extension step: 72°C, 1.15 min. (5) Final extension step: 72°C, 10 min. (6) Cooling down: 15°C, N/A.

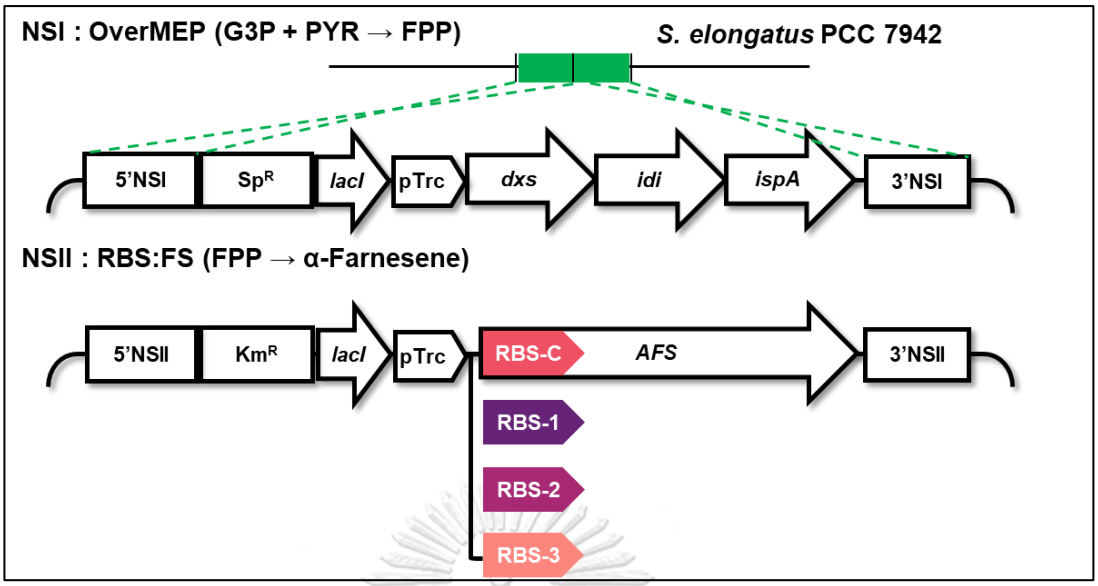


Figure 6. Schematic diagram showing the construction of *S. elongatus* PCC 7942 strains that produce α-farnesene. The *dxs-idi-ispA* genes were codon-optimized to Syn7942 from *E. coli* (given by Dr. Choi [39] from FMB laboratory) and then introduced into the neutral site I (NSI) for supplying the FPP intermediate from CO_2 (OverMEP module). The farnesene synthase gene (*AFS*) (*Malus* × *domestica* Borkh [128]) was introduced in the neutral site II (NSII) of *S. elongatus* genomic DNA (FS module) with different RBS sequences (RBS-C, RBS-1, RBS-2, RBS-3). The details of the RBS sequences are shown in **Table 3.**

For plasmid construction, we used SynBrick expression plasmids for integration into the chromosome of the Syn7942 strain[38] (**Figure 6**); this was done based on the BglBrick standard cloning method [129]. All the plasmids were constructed using pSe2Bb1k-AFS with various RBS sequences. To generate random mutations in the *AFS* gene, error-prone

PCR was performed using GeneMorph® II random mutagenesis kit (Agilent Technologies, USA) [130]. The mutation frequency (low or high) was controlled by the initial target quantity (ng) of the *AFS* gene product; for regulating the mutation frequency, both low (500 ng) and high (25 ng) amounts of target were employed in this study. After the PCR products were cleaned up, the mutated PCR products were cloned into pSe2Bb1k-RBS3-AFS, then transformed into competent *E. coli* cells. Hundreds of *E. coli* colonies from each plate were then collected with a cell scraper and resuspended in LB medium, resulting in pools of pSe2Bb1k-AFS (erPCR). The primers used for gene cloning and DNA sequencing are described in **Table 3 and Table 10**.

Table 3. RBS sequences and oligonucleotides used for gene cloning in this study.

Name	Relevant characteristics (5' → 3')	Source
RBS-1	CAAAagatctAATAAAGGAGGTTTAAAGC T**ATGgaatttcgcgtgcacctgcag**	This study
RBS-2	CAAAagatctTAGCATCGAACATAGAGAG GTCAGACATGgaatttcgcgtgcac	This study
RBS-3	CAAAagatctTGTATTCGTAGGGTACAGTT T**ATGgaatttcgcgtgcacct**	This study
RBS-R	TTGGATGCTCTTGAATTGCC	This study

Note: The restriction enzyme sites were shown as lower cases. RBS and coding regions of AFS were underlined and bold letters, respectively.

3.2.2 Transformation of engineered *S. elongatus* PCC 7942.

Transformation of Syn7942 was performed as described previously [38, 131]. Three recently engineered RBS plasmids were transformed into the strain, SeHL33, which overexpressed the key MEP pathway enzymes [38]. In addition, the pools of pSe2Bb1k-AFS (erPCR) were used for constructing pools of recombinant cyanobacteria using the strain, SeHL33. The presence of colonies was observed on BG11 agar with the addition of spectinomycin (10 µg/mL) and kanamycin (5 µg/mL), which indicated successful transformation of the resultant plasmids into Syn7942. To validate successful chromosomal integration, the genomic DNA from each strain was extracted and confirmed by PCR and DNA sequencing of targets into neutral site II (NSII). The primers used to verify the sequences were NSII-fw (5'-TAA TGT TTT TTG CGC CGA CA-3') and NSII-rv (5'-TTG GAT GCT CTT GAA TTG CC-3') for NSII. The relevant characteristics of the recombinant Syn7942 strains are described in **Table 4.**

Table 4. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	References
Strains		
E. coli DH5α	F−(80d lacZ M15) (lacZYA-argF) U169 hsdR17(r– m+) recA1 endA1 relA1 deoR96	[105]
S. elongatus PCC 7942	Wild type (ATCC 33912)	ATCC
SeHL33	*S. elongatus* PCC 7942 NSI::Bb1s-dxs-idi-ispA	[106]
SeHL32FS	SeHL33 NSII::Bb1k-AFS	[86]
FMB-1236	SeHL33 NSII::Bb1k-RBS1-AFS	This study
FMB-1237	SeHL33 NSII::Bb1k-RBS2-AFS	This study
FMB-1238	SeHL33NSII::Bb1k-RBS3-AFS	This study
FMB-1239	SeHL33 NSII::Bb1k-RBS3-AFS, laboratory adapted strain under 0.5 mM IPTG	This study
FMB-1238(erPCR)	FMB-1238 strain variants by pSe2Bb1k-AFS(erPCR) integration	This study
Plasmids		
pSe2Bb1k-GFP	pUC, Km^r, LacI, P_{trc}, BglBrick sites, NSII target sites, SyneBrick Vector	[107]
pSe2Bb1k -AFS	pUC, Km^r, LacI, P_{trc}, NSII target sites, the farnesene synthase gene (*AFS*) originated from *Malus* × *domestica* Borkh[101].; the *AFS* gene(se.co) with 3238 a.u. RBS	[86]
pSe2Bb1k-RBS1-AFS	pSe2Bb1k-AFS with 56871 a.u. RBS	This study
pSe2Bb1k-RBS2-AFS	pSe2Bb1k-AFS with 5687 a.u. RBS	This study
pSe2Bb1k-RBS3-AFS	pSe2Bb1k-AFS with 568 a.u. RBS	This study
pSe2Bb1k-AFS(erPCR)	pSe2Bb1k-RBS3-erPCR products of AFS	This study

Note: (se.co) represents that the gene sequence is codon-optimized to *S. elongatus* PCC 7942.

3.2.3 Cyanobacterial culture conditions and adaptive laboratory evolution.

Engineered strains were cultured in 100 mL BG11 medium supplemented with 10 mM MOPS (pH 7.5) and were incubated in 5% CO_2 gas and 95% (v/v) filtered air (flow rate of 10 cc/min). The cultures were incubated at 30°C with exposure to 100 µE/m^2/s continuous fluorescent light [39, 132]. In addition, the medium was supplemented with 10 µg/mL each of spectinomycin and kanamycin as a selection pressure. For the production of α-farnesene from Syn7942, 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to the culture medium 24 h after inoculation for induction of gene expression along with supplementation with 20% (v/v) dodecane in order to trap the *in situ* α-farnesene produced from the cell culture after induction, in accordance with a protocol published in a previous study [38]. For achieving adaptive laboratory evolution (ALE), 0.5 mM IPTG was added into the culture medium after 24 h of incubation, instead of 1 mM IPTG.

3.2.4 Quantification of photosynthetic α-farnesene production.

The α-farnesene produced by the cyanobacterial strains was determined using gas chromatography (GC; Agilent 7890B, USA), as previously described [38]. In brief, 200 µL samples from the dodecane

overlay in the culture were collected and diluted with 800 µL ethyl acetate containing 5 mg/L α-humulene (No. 53675; Sigma-Aldrich, USA), as an internal standard. The samples from the dodecane/ethyl acetate mixture were subsequently quantified using trans-β-farnesene (No. 73492; Sigma-Aldrich, USA) as a reference standard.

3.2.5 Quantification of inorganic pyrophosphate and determination of enzyme activity by using PiPER™ assay.

Recombinant Syn7942 strains were grown in 96-well plates containing BG11 at 30°C with exposure to 100 µE/m^2/s continuous fluorescent light. After 3 or 7 days of IPTG induction, the cell samples were prepared for the measurement of intracellular inorganic pyrophosphate (PPi) using PiPER™ assay. The preparation of the samples and solutions was performed according to the PiPER™ assay kit (Thermo Fisher Scientific™) manual. In brief, the cyanobacterial cell culture (100 µL) was centrifuged at 3667 × *g* for 10 min, the cell pellets in the 96-well plates were resuspended before lysing with B-PER™ bacterial lysis buffer (Thermo Fisher Scientific™) [133] and then, the cell extracts were continually diluted with the reaction buffer before adding a reaction solution (containing maltose phosphorylase and Amplex red reagent) to a final volume of 100 µL per well. The changes in the PPi

levels in the wells containing the mutants were measured by calculating the optical density (OD_{730}) per well every 10 min using a microplate reader (Infinite® M Nano, Tecan Life Science) and then, these results were compared with those of the control (**Figure 18**). The enzyme activity assay was also performed in accordance with the guidelines provided in the PiPER™ assay kit manual using 2 µg farnesyl-pyrophosphate (FPP, No. F6892; Sigma-Aldrich, USA) as the substrate. Quantification of the protein was done using the Quick Start™ Bradford Protein Assay (Bio-Rad).

3.3 Results and discussion

3.3.1 RBS optimization for improved α-farnesene translation

The new plasmids were redesigned and constructed with four different RBSs (56781, 5687, 3238, and 568 a.u. TIR) controlling AFS gene. The chromosomally integration was occurred in host strain (SeHL33; OverMEP module) [38] to obligate the new RBS-optimized strains, i.e. FMB-1236, 1237, 0185, and 1238, respectively (**Figure 7**). The results showed that the highest production of of α-farnesene was found in the strain with the lowest TIR (FMB-1238; 568 a.u.) with a titer of 5.66 ± 0.58 mg/L, whereas α-farnesene was not detected in the highest TIR strain (FMB-1236; 56871 a.u.) (**Figure 8**). In contrast, the FMB-1238 strain could produce α-farnesene a little higher than a control (FMB-0185; 3238 a.u.) from previous study [38]. Additionally, the PPi contents were also simultaneously determined as co-products from α-farnesene conversion, consequently in the lower PPi contents were observed in higher TIR strains.

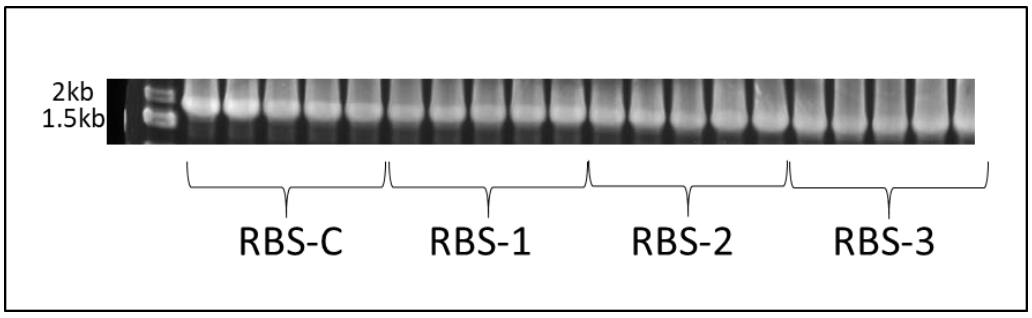


Figure 7. Gel electrophoresis of RBS-C and RBS-1, -2, -3 with AFS PCR products. The PCR products electrophoresed on a 1.5% agarose gel in 1X TAE buffer. From left: lane 1: DNA ladder, lane 2-6: RBS-C (1,788 bp), lane 7-11: RBS-1 (1,778 bp), lane 12-16: RBS-2 (1,784 bp), land 17–21: RBS-3 (1,779 bp).

Regarding protein translation, the lower TIR for AFS protein, i.e. 568-3238 a.u.) might be suitable for the expression of AFS to convert FPP into α-farnesene in SeHL33 strain. The predicted translation efficiencies by Salis calculator and UTR Designer have been reported no correlation between the predicted and experimental expression of eYFP and GFP systems in *Synechococcus* sp. PCC 7002 and Syn6803 [134, 135]. The translation initiation is a process that the ribosome recognizes the start of open reading frame (ORF) and initiates protein translation specifically to mRNAs and organisms [136]. In contrast, the translation rate of cyanobacteria is unlike to *E. coli* as they obtain slower growth rate and prefer cooler temperatures, which might be the reason behind the slower translation rate [137, 138]. Although the slower translation rate

could positively allow a more soluble and proper folded proteins by time window for the co-translation folding of each domain [139].

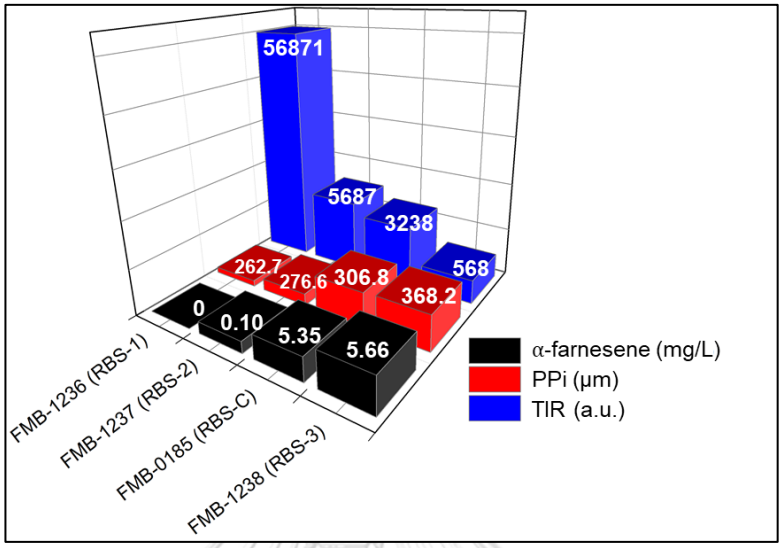


Figure 8. Correlations between α-farnesene production (mg/L; black), intracellular PPi level (μM; red), and translation initiation rate (TIR) (arbitrary unit, a.u.; blue) for the recombinant strains were calculated using the RBS calculator. The genotypes of the recombinant strains are shown in **Table 4**.

3.3.2 Generation of random AFS mutant libraries

Further, protein engineering of FS could be beneficial in increasing α-farnesene production as an alternative metabolic engineering strategy [134]. The random mutagenesis approach is one of the powerful tools to generate mutant libraries, which could be achieved by physical, chemical and biological methods along with high-throughput screening method [140, 141]. To increase the enzymatic activity of FS, random mutations

were introduced in *AFS* using Error-Prone PCR in this study due to its more specificity to the target gene than either physical (e.g. gamma, X-rays and UV-C exposure) or chemical (Chemical mutagens act as alkylating agents, cross-linking agents, and polycyclic aromatic hydrocarbons (PAHs)) techniques [141, 142].

For the generation of *AFS* mutant libraries, 3.15 to 4.59 × 10^2 and 6.88 to 7.6 × 10^3 CFU/μg *E. coli* transformants were used. A pool of integration plasmids with *AFS* mutations was transformed into the FMB-1238 strain. Over 500 Syn7942 colonies were collected and cultured. As the specific PPi levels and AFS activities that measured following a PiPER™ assay correlated with the α-farnesene production, we analyzed the cultures growing in the 96-well microtiter plates using this method to compare the α-farnesene production among selected mutants (**Figure 9** and **10**). The titer of the best α-farnesene producing strain among mutant was lower than that of the control (FMB-1238). It is possible that hundreds of strains used for the testing might produce low titer as more than 100,000 strains were tested for industrial strain development using fluorescence-activated cell sorting (FACS) system [125]. For instance, the combination of Error-Prone PCR using GeneMorph® II with FACS had been already successful in phototrophic purple bacterium *Rhodobacter capsulatus* to re-engineer *nifH* variants purposed for

improving H_2 production [143]. After sequencing 20 *E. coli* variants per library (selected from ~4 × 10^6 clones per library), they could not investigate the obvious mutational incidences and then approximately 8 × 10^5 *R. capsulatus* clones per library were achieved after mating process. Only 0.024% of total population had shown higher cell emitting fluorescence than the main population, thus large number of libraries is necessary for FACS. Moreover, the random mutation could occur undesirable consequences – e.g. frame shift mutation or amino acid substitutions, which could lead to yield inactive proteins. As proof, the point mutation of amino acids at position 330 (D→A) and 487 (S→A) could be resulted in a loss of sesquiterpene synthase activity on *AFS* enzyme due to inactivated metal binding sites – i.e. Mn^{2+}/Mg^{2+} and K^+, respectively [144, 145]. On the other hand, the substitution of S487K by site-directed mutagenesis could enhance 4-fold on sesquiterpene synthase activity of *AFS* in the absence of potassium [145]. The reduction of initial DNA concentration as a substrate of error-prone PCR might also increase the chance of mutations [130, 134]. In addition, a FACS-assisted α-farnesene screening system has not been developed yet due to lack of a fluoresce-based biosensor. Thus, the limitations in high-throughput screening (HTS) for α-farnesene production could contribute to the failure of strain selection [146].

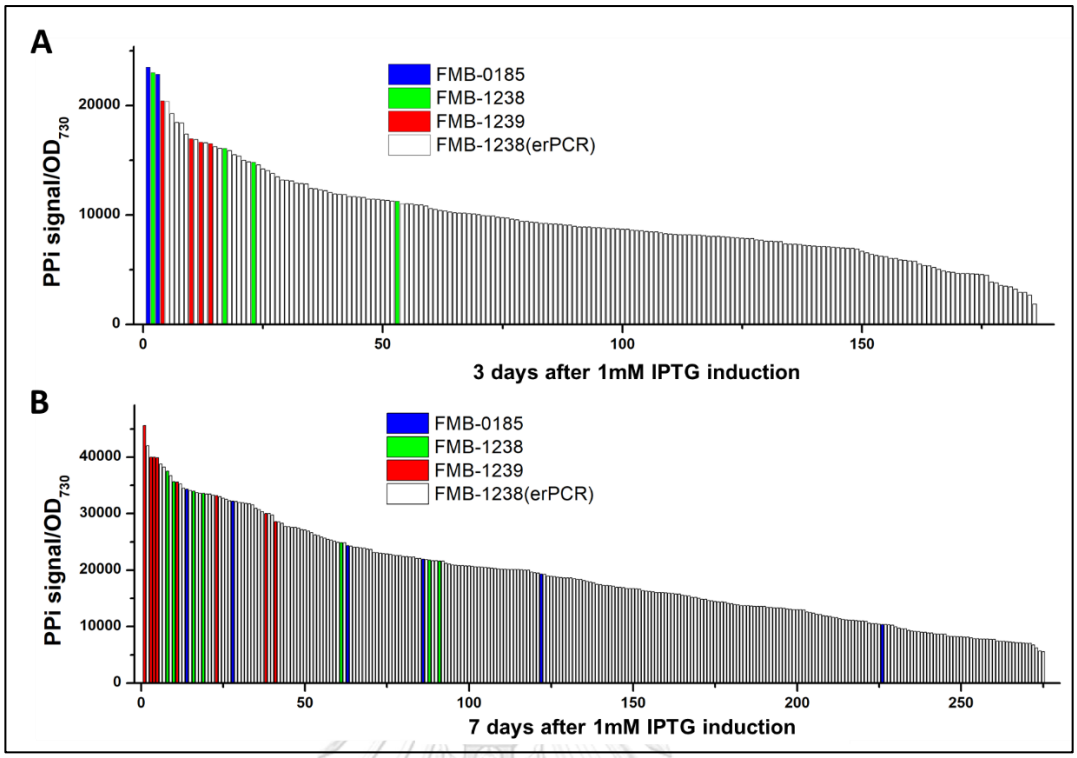


Figure 9. Measurement of specific PPi levels in the α-farnesene producing strain. The PPi levels in the cultures (**A**) 3 days after induction and (**B**) 7 days after induction were analyzed using the PiPER™ assay kit. The strains used for this assay were the cyanobacterial mutants generated using error-prone PCR library (FMB-1238 (erPCR), white bar), metabolically engineered strains (FMB-0185, blue; FMB-0128, green), and the evolved strain (FMB-1239, red). The PPi signals were normalized to the optical density at 730 nm.

3.3.3 Push-and-pull strategy and engineered RBS-Syn7942 fitness

In a 'push-and-pull strategy' of metabolic engineering [40, 147, 148] that supplies the key intermediaries and increases the rate of formation of the final product, controlling the accumulation of the key

intermediate could be essential for enhancing the production if the key intermediate is toxic. The pull strategy was not effective for α-farnesene in Syn7942. Interestingly, different specific PPi levels were observed on day 7 after induction in both FMB-0185 or FMB-1238 strains, compared to their levels on day 3 days after induction (**Figure 9 and 10**). Based on this, we speculated that the cell growth and production of α-farnesene could be imbalanced in recombinant strains due to the formation of a toxic intermediate, FPP.

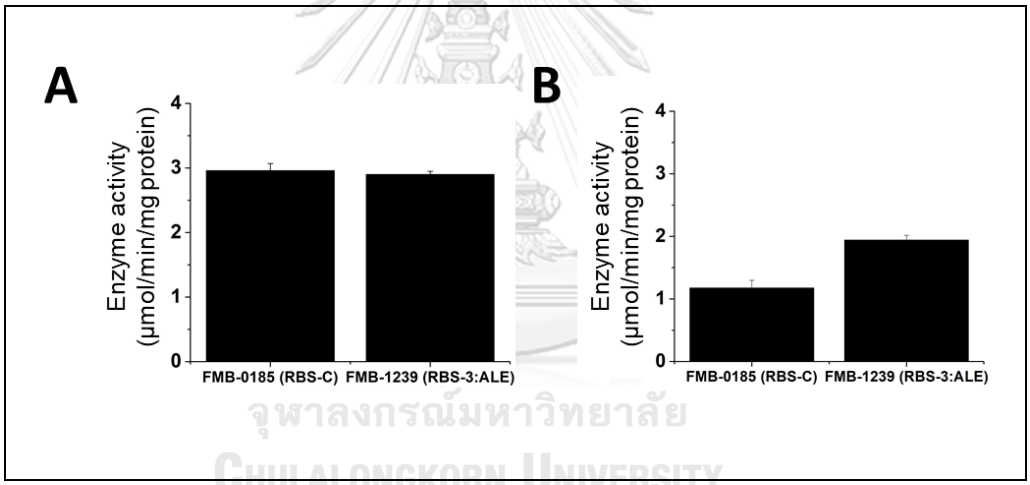


Figure 10. Enzyme activity of FS in control and ALE strains. The FS activities of (**A**) day-3 and (**B**) day-7 after induction were analyzed using the PiPER™ assay kit with the substrate, FPP.

The correlation of the predicted TIR and gross differences in expression should be tested by the most tightly controlled IPTG induction system for engineered Syn7942 [134]. Then, we investigated the

imbalanced gene expression systems for α-farnesene production in the recombinant strains by lowering the IPTG concentration, which led to lower expression of *AFS* and the key MEP enzymes. Then the induction with 0.5 mM IPTG was investigated that had caused severe growth inhibition in all four cyanobacterial strains (FMB-0185, FMB-1236, FMB-1237, FMB-1238), all of which had different FS activities as demonstrated by the measured PPi levels (**Figure 11**). This growth inhibition could be due to the accumulation of FPP resulting from imbalanced gene expression [116, 149]. Interestingly, the growth of only the FMB-1238 strain recovered and improved after 10 days (**Figure 11**) while the other cyanobacterial cells did not grow at all. Subsequently, the laboratory evolved strain FMB-1238, which was renamed as FMB-1239, was transferred to fresh BG11 medium under the normal induction conditions (1 mM IPTG). Compared to the production of cyanobacterial farnesene in other strains, FMB-1239 showed the maximum productivity with 1197 µg/L/d (7.18 ± 0.36 in 6 days), which was higher than that of the control (SeHL32FS; 600 µg/L/d) [38] as well as *Anabaena* sp. PCC 7120 (20.4 µg/L/d) [37] by approximately 2- and 59-fold, respectively. In this study, the evolved strain exhibited increased production rates during log (2 to 6 days) to early stationary (6 to 10 days) phases. Moreover, as the growth rate in the evolved strain was comparably higher than that of

the other strains for up to 20 days, long-term production of the enzyme by this strain could be employed at an industrial scale (**Figure 11**). Thus, the adaptive laboratory evolved strain (FMB-1239) showed better cell growth and 2-fold higher enzyme production (12.99 ± 1.66 mg/L), compared to that of the other strains used in this study (**Figure 12**).

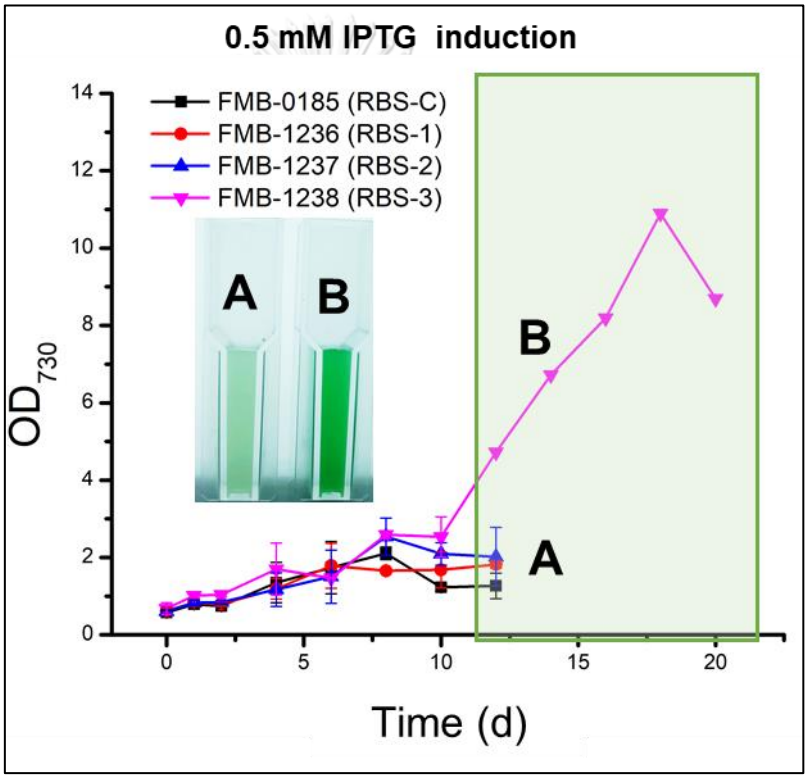


Figure 11. Adaptive laboratory evolution (ALE) of cyanobacteria for α-farnesene production. The growth of the engineered cyanobacterial strains after induction with 0.5 mM IPTG. The blue box shows the time period of adaptive evolution. The image of cuvettes was taken for the samples **A** and **B**.

Based on a previous TIR study, FMB-1239 showed the lowest RBS strength for *AFS* expression, which is equal to the value of FMB-1238. In parallel, the highest specific PPi value was obtained from the evolved strain (FMB-1239) (**Figure 9**). Although the specific enzyme activities of FMB-0185 and FMB-1239 were at the same levels at 3 days after induction, the specific enzyme activity of FMB-1239 was higher than that of FMB-0185 at 7 days after induction (**Figure 10**). Thus, we believe that the evolved strain might have adapted a gene balancing mechanism for the key MEP enzyme and FS gene to eliminate the FPP accumulation. To confirm the evolved strain, the native, overexpressed MEP pathway genes (*dxs*, *idi*, and *ispA*) and *AFS* were sequenced otherwise that no mutation occurs. Further whole-genome sequencing is required to identify the mutations that resulted in the enhanced cellular fitness and α-farnesene production. In addition, RNA-sequencing analysis could also be useful for understanding the changes in the chromosomal DNA in the evolved strain and explain the key genetic points or segments affecting α-farnesene production-related pathways.

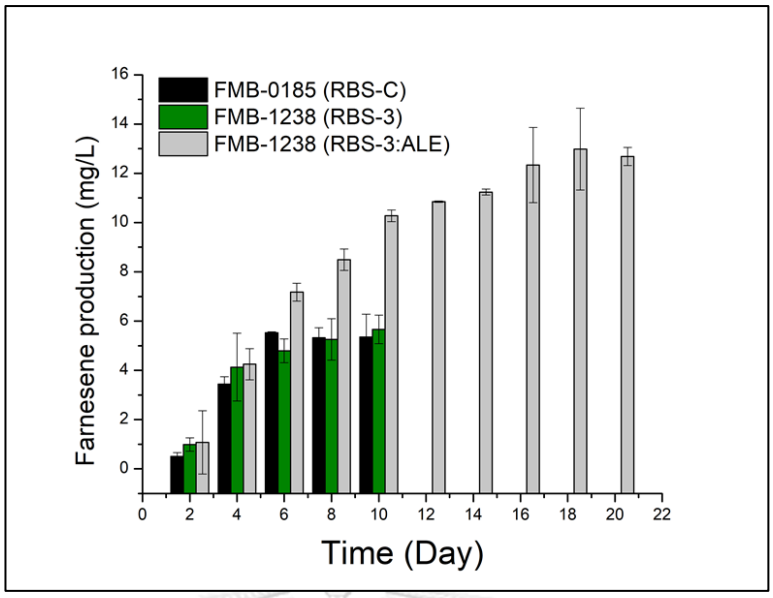


Figure 12. The growth of engineered cyanobacterial strains and the evolved strain after induction with 1 mM IPTG. α-farnesene production from the cyanobacterial cells was determined. The growth and farnesene yield (mg/L) of the recombinant strains were measured. The genotypes of the recombinant strains are presented in **Table 4**. All the data are expressed as mean ± standard deviation from cultures that were run in triplicate. N.D., not detected; LOD, 0.045 mg/L; LOQ, 0.136 mg/L.

CHAPTER IV

Conclusions

Microalgae as feedstock for biodiesel production are obviously more effective and productive than vegetable oils used in terms of environmental and sustainable aspects. Besides the reduction of CO_2 emission, microalgae could also absorb and survive under heavy metals stress or limited nutrients conditions e.g. wastewater or harsh environment. In this study, the isolated green alga *Chlorella* was studied under P limitation and heavy metals (Fe, Co and Pb) stress conditions to achieve an enhancement of lipid content for biodiesel application with good quality. Based on the results, the highest increase of lipid content was observed with 19% over control under Co stress and P deprivation, but the biomass was strongly inhibited. Interestingly, an isolated *Chlorella* sp. showed a good biomass production reaching 0.49 g/L when adding Pb with P deprivation condition with 0.13 g/L of lipid production. Thus, 1 µM of Pb is not harmful to this *Chlorella* but could favorably trigger the cell growth. The fatty acid profiles revealed the increase of UFAs under sole P deprivation but most in MUFAs i.e. C16:1. Under Pb stress, there is no significant enhancement in PUFAs whereas the biodiesel quality was slightly better than non-Pb conditions. However, the

biodiesel properties were all in good values in accordance with the EN 14214 and ASTM D6751 standards. Notably, the energy conversion efficiency from stress conditions could provide good TECE% as comparable to mixotrophic conditions in previous study. In contrast, this study has found a desirable *Chlorella* and could enhance the lipid content and production under various stress conditions over some previous studies (**Table 5**). Hence, the Pb supplement conditions could provide the suitable environment in this *Chlorella* for biodiesel production. Moreover, this is the first study on Pb stress with P limitation combinations in *Chlorella* for biodiesel production purpose as the reduction of phosphorus could improve the quality of biodiesel.

Several heterotrophic microbes have been studied on metabolic engineering of FPP-derived biofuel production as potential microbial cell factories. In this study, a cyanobacterium Syn7942 has been successfully developed for improvement in α-farnesene production by synthetic biology tools and high throughput analysis, such as the 96-well plate-based screening method. Moreover, the evolutionary engineering can be also used synergistically with metabolic engineering to improve α-farnesene yield reaching at 12.99 mg/L, which was more than 2-fold higher than previous study. The alternative synthetic biology tools are expectantly established in further studies to produce efficient

cyanobacteria-based bio-solar cell factories, such as dynamic switches that regulate carbon fluxes and genetic elements triggered by intracellular cellular responses. In conclusion, the physiological and genetic modification of microalgae and cyanobacteria could offer the promising tools to enhance biodiesel precursors.

Table 5. Literatures of different types of nutrient limitation and heavy metals stress to induce lipids in microalgae, including this study.

Microalgae	Medium	Macronutrients	Heavy metals	Lipid content	References

Scenedesmus sp.	50%BG11	P 0.1mg/L	-	53.00%	[150]
Scenedesmus sp.	BG11	-	Fe 1.2 mg/L, Mg 7.3 mg/L, Ca 0.98 mg/L, EDTA 1 mg/L	47.40%	[91]
Chlorococcum pamirum	BG11	-N and -P	-	64.50%	[151]
Isochrysis galbana	modified *f/2*	1%P	-	≈40.00%	[84]
Chlorella vulgaris	BBM	-	Co 3 µM	25.30%	[59]
Scenedesmus sp.	BG11	-	Fe 12 mg/L	≈40.00%	[67]
C. vulgaris	BBM	-P	-	53.20%	[152]
C. sorokiniana	BG11	-N and -P	Fe 15 mg/L, Mg 125 mg/L, Ca 18 mg/L, EDTA 3 mg/L	48.67%	[93]
Raphidocelis subcapitata	Chu10	-	Pb 1.20 and 12.06 µM	5.33 and 5.89 pg/cell	[153]
Scenedesmus sp.	COMBO	-	Pb 0.5 mg/L	31.10%	[99]
Chlorella sp.	BG11	-P	$FeCl_3$ 6 µM	41.02%	This study
			Co 17 mM	68.93%	
			Pb 1 µM	48.52%	
		-	Pb 1 µM	50.02%	

APPENDICES

Appendix A
The BG11 medium recipe

Table 6. BG11 medium recipe.

Components	Amount	Final concentration
$NaNO_3$	1.5 g	17.6 mM
K_2HPO_4	0.04 g	0.23 mM
$MgSO_4 \cdot 7H_2O$	0.075 g	0.3 mM
$CaCl_2 \cdot 2H_2O$	0.036 g	0.24 mM
Citric acid	0.006 g	0.031 mM
Ferric ammonium citrate	0.006 g	0.021 mM
EDTA (disodium salt)	0.001 g	0.0027 mM
Na_2CO_3	0.02 g	0.19 mM
Trace metal mix A5	1.0 mL	
$Na_2S_2O_3 \cdot 5H_2O$ (agar media only, sterile)	49.8 g/200 mL dH_2O	1 mM
Agar (if needed)	10.0 g	
Distilled water	1.0 L	

*pH adjustment by (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (HEPES) at pH 7.5.

Table 7. Trace metal mix A5 recipe.

Components	Amount
H_3BO_3	2.86 g
$MnCl_2 \cdot 4H_2O$	1.81 g
$ZnSO_4 \cdot 7H_2O$	0.222 g
$NaMoO_4 \cdot 2H_2O$	0.39 g
$CuSO_4 \cdot 5H_2O$	0.079 g
$Co(NO_3)_2 \cdot 6H_2O$	49.4 mg
Distilled water	1.0 L

Appendix B
TAE buffer preparation

Working solution

1X 0.04 M Tris acetate

0.01 M EDTA

Concentrated stock solution (per liter)

242 g Tris base

57.1 mL glacial acetic acid

100 mL 0.5M EDTA (pH8.0)

Appendix C
Biomass, lipid content and lipid production calculation

According to A-y Liu, et al. [154] calculation, Biomass (*B*) was reported as grams of dried biomass in per liter of culture and lipid content (*C*) was reported as percentage of dry mass (% of DCW), while lipid production (*P*) was reported as grams per liter using the following equation for calculation:

$$P = CB$$

where *P* is lipid production (mg/L); *C* is lipid content (% of DCW); *B* is biomass (g/L).

Total lipid content calculated as percent dry weight by:

$$\text{Total lipids content (\%DCW)} = \frac{\text{g dried lipids}}{\text{g dried biomass}} \times 100$$

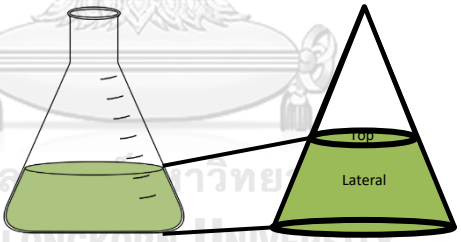
Appendix D
The input light energy calculation

The input light energy was calculated as H-Y Ren, et al. [72] studies as follows:

$$Input\ light\ energy = \frac{(Light\ intensity \times Area\ of\ light\ exposed \times Time)}{1000}$$

where the unit of input light is kJ, light intensity is W/m^2, area of light exposed is m^2 and time is s.

This study, the light intensity of 9.09 W/m^2 is chosen and the duration time of experiment was 3 days or 72 h. The effective area of light intercepted in each flask was calculated as figure below [113]:



The calculation of 500 mL Erlenmeyer flask is based on the average area of top and lateral area is used for energy calculation, which the area of light exposed is calculated equal 0.008012 m^2. Therefore, the input light energy in this experiment is 18.88 kJ.

Appendix E
Preliminary studies in *Chlorella* cultivation and pretreatment

The preliminary studies were not principally used in this study but only for observing the minor effects on *Chlorella* from pretreatment of cells by microwave (**Figure 13**) and different growth rates from 2 medium cultures (**Figure 14**), where each condition had been done only single sample.

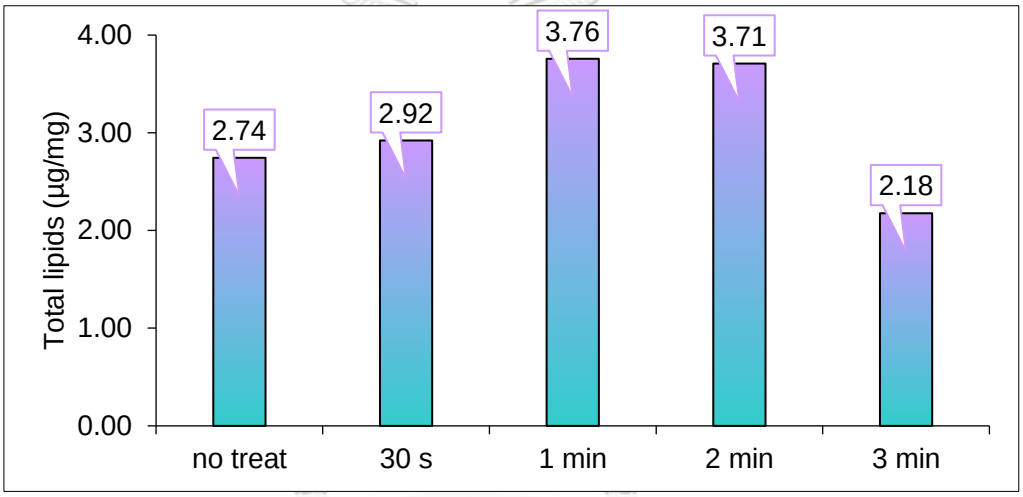


Figure 13. Pretreatment of *Chlorella* by microwave. Total lipids from samples disrupted with highest temperature at different time points by microwave. Note: Dry cells are all collected from same day and same weights (day-18) and extracted the total lipid for twice times because of soap formation.

The pretreatment of microalgal cells by microwave and ultrasonic had been already studied on their effects on lipid extraction [155]. The results demonstrated that the increased length of treatment time could

improve the lipid extraction yield (%) to the maximum at 11.6% in both methods. However, when the lipid yields remained stable after 75 and 1200 s pretreated with microwave and ultrasound, respectively.

The difference of components concentration between BG11 and Modified Chu13 are shown as a table below:

Table 8. The comparison of component final concentrations in BG11 and M.Chu13 media.

Components	Final concentration	
	BG11	M. Chu13 [156]
$NaNO_3/KNO_3$*	17.6 mM	1.98 mM*
K_2HPO_4	0.23 mM	0.23 mM
$MgSO_4 \cdot 7H_2O$	0.3 mM	0.41 mM
$CaCl_2 \cdot 2H_2O$	0.24 mM	0.54 mM
Citric acid	0.031 mM	0.52 mM
Ferric ammonium citrate	0.021 mM	0.038 mM
EDTA	0.0027 mM	-
Na_2CO_3	0.19 mM	-

The both of growth rates were observed for 24 days and found that slightly increasing for 3 days. In contrast, the growth became to exponential phase (3-12 days) only in BG11 medium whereas the exponential phase was reached slower (9-18 days) but could elevate higher cell density in M.Chu13 medium.

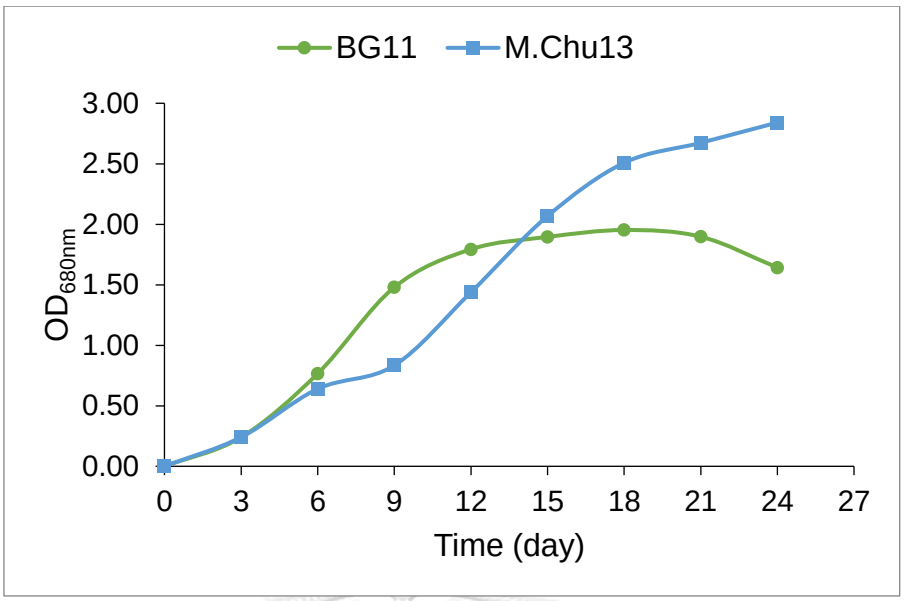


Figure 14. The comparison of *Chlorella* growth in BG11 and M.Chu13 medium for 24 days.

Appendix F
Fatty acid profiles and biodiesel properties under -N and Pb

Table 9. Fatty acid profiles and biodiesel properties as per ASTM D675 and EN14214 under combined -N and Pb stress conditions. Fatty acid profiles of *Chlorella* treated with 1 µM of Pb under normal and P-limited BG11 for maximum lipid production. Fatty acids were measured after 1 days of incubation. All the data are expressed as mean ± standard deviation from cultures that were run in triplicate; Values are given as percent (%) of total fatty acids. SFA Saturated fatty acid, UFA unsaturated fatty acid**.** n.d. Not detected. [a]C18:2 and C18:3 total %.

Fatty acids composition (%)									
N concentration			100%	50%			0%		
Pb (µM)			0	0.1	1	10	0.1	1	10
Decanoic acid (C10:0)			8.21	8.57	n.d.	9.75	n.d.	n.d.	10.04
Myristic acid (C14:0)			11.86	16.78	≤0.01	12.98	≤0.01	≤0.01	13.08
Palmitic acid (C16:0)			42.27	40.27	59.84	42.00	55.41	51.52	36.86
Palmitoleic acid (C16:1)			15.52	13.06	26.57	13.08	12.88	26.07	17.24
Stearic acid (C18:0)			17.94	13.13	9.55	17.21	17.64	12.53	9.83
Oleic acid (C18:1)			≤0.01	13.78	≤0.01	≤0.01	≤0.01	≤0.01	10.88
Others[a]			1.20	0.94	4.04	4.98	14.07	9.89	2.08
SFAs			83.28	78.75	69.39	81.94	73.23	64.05	69.81
UFAs			16.72	26.84	30.61	18.06	26.77	35.96	30.20
Factors	**EN**	**ASTM**	**Biodiesel properties**						
SV	NA	NA	226.50	238.87	215.98	227.79	212.41	214.17	228.21
IV	120	NA	16.57	25.43	30.16	17.54	25.42	34.92	28.87
CN	>51	>47	66.67	63.43	64.79	66.32	66.28	63.93	63.72
CP	NA	NA	18.82	16.19	26.48	17.10	24.82	22.11	14.40
HHV	NA	NA	38.90	41.06	39.21	38.86	39.33	39.26	38.82
KV	3.5-5.0	1.9-6.0	3.47	3.67	3.73	3.42	3.92	3.78	3.33
Density	0.86-0.90	NA	0.87	0.92	0.87	0.87	0.87	0.87	0.87

Units of factors. SV (mg KOH/g)**,** IV (g I_2/100g)**,** CP (°C), HHV (MJ/kg), KV (mm^2/s), Density (g/cm^3).

Appendix G
Map of Se2Bb1k-AFS vector

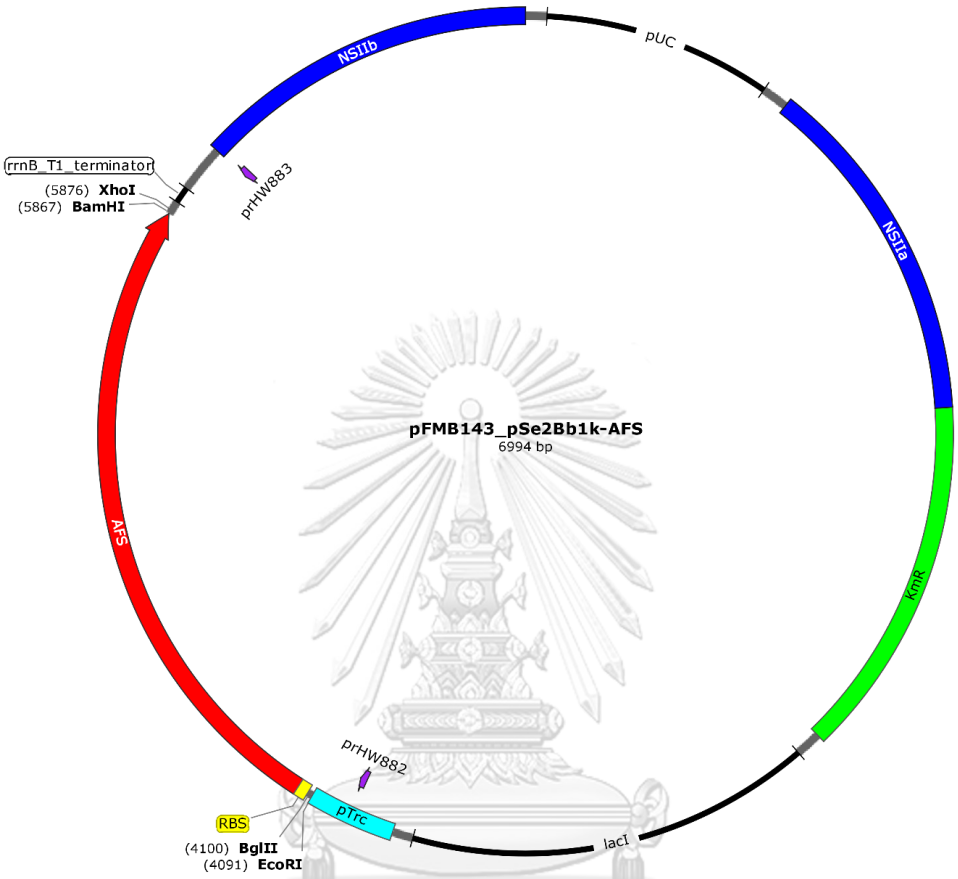


Figure 15. Map of Se2Bb1k-AFS vector. Acknowledged locations of primers, neutral sites, ORFs are including: forward (prHW882) and 3' reverse (prHW883) primers (purple arrows). Neutral site II a and b (dark blue sites). Kanamycin resistance gene (green site). *Trc* promoter (light blue site). RBS (yellow). AFS gene (red arrow). LacI system is used for controlling AFS expression. This vector is derived from HJ Lee, et al. [38].

Appendix H
The list of primers used in this study

Table 10. Oligonucleotides used for DNA sequencing.

Name	Relevant characteristics (5' → 3')	Source
Promoter checking		
Native MEP		
dxs-proF	GGGTCGACAGACTGAGCCCA	This study
dxs-proR	TGTCGTTGAGCACGACCAAC	This study
dxr-proF	CGAGACCGGCCAAACCAG	This study
dxr-proR	CAGTTACTTGCGACAGCCGT	This study
ispD-proF	GCACTCAAATTCCAGCCTCC	This study
ispD-proR	GCTCAACGACGACATCAATC	This study
ispE-proF	CGCTTCCAGTTCTGGTTGTG	This study
ispE-proR	TTGGCCTTCGTAAAGTTGG	This study
ispF-proF	TGGGATCGAAACGTACGCTG	This study
ispF-proR	CGCCTCCTCCACGGACTGAT	This study
ispG-proF	CTCGCGCAGGCTGGTGACTA	This study
ispG-proR	CTCAGAAGCCGATAAAGAC	This study
ispH-proF	CTTTCCGCAGCTCCAAG	This study
ispH-proR	GAGCTGGTGGCGACCGTCTC	This study
idi-proF	CAACAAGCCCTTCGGCTCCA	This study
idi-proR	TGCCTGTGAACGTCGGTACC	This study
ispA-proF	TCTGACGACCGATTCGAC	This study
ispA-proR	CCTGGAATGGGTTGAGATG	This study
NSI		
UP-pTrcI	CGACAGGTTTCCCGACTGGA	This study
Pr_AFS-R1	CAGCCGTTGCGCAGGTATTC	This study
NSII		
Up-pTrcI	CGACAGGTTTCCCGACTGGA	This study
DXS-R1	TAAAGGCCAGGTGCAGGCGGG	This study
Gene checking		
Native MEP		
7942_dxs_F	ACGTTCACTGCAGCCAGCAG	This study

7942_dxs_R	CATGATCAGTCCAGGTCTTG	This study
7942_dxr_F	TCGCCTCCCTCGCTTCAGCA	This study
7942_dxr_R	TAGACCTCAGGCACATCGAT	This study
7942_ispD_F	ATCGGGGTAAGCTGAAGTC	This study
7942_ispD_R	GCACTCAAATTCCAGCCTCC	This study
7942_ispF_F	ATCTAGGCCACATGACGTCA	This study
7942_ispF_R	TGGGATCGAAACGTACGCTG	This study
7942_ispE_F	ATCAGATTGCGCCCGAAGC	This study
7942_ispE_R	GAGCTAGTGCTGGATGGGAT	This study
7942_ispG_F	TCGCCCAAGGGTTGGTCGAT	This study
7942_ispG_R	GCGAAAACCGCCTATCAGGA	This study
7942_ispH_F	GCAATTGGCGATCGCGGCTT	This study
7942_ispH_R	GTGGAGTCGAGAGTCACGAT	This study
7942_idi_F	CAGCACATGCCAGTTCAGAC	This study
7942_idi_R	GTTGGACCAATGACCGTGGC	This study
7942_ispA_F	GTCAAGCTGAAGGTGGGCAC	This study
7942_ispA_R	CGGTAGCGAAATAGCGATCG	This study
NSI		
DXS-F1	CGTGTCGCTCAAGGCGCACT	This study
DXS-R1	TAAAGGCCAGGTGCAGGCGGG	This study
idi-F1	AACCCGTGCCCGTGCTGA	This study
idi-R1	TACACCAGAAAGGGGCGCAG	This study
ispA-F1	ATCGATGCCACCCCCTGGG	This study
ispA-R1	AGCCGAACGCCCTAGGTATA	This study
NSII		
PrHW882F	TAATGTTTTTTGCGCCGACA	FMB lab collection
PrHW883R	TTGGATGCTCTTGAATTGCC	FMB lab collection
Pr_AFS-F1	CACCACTTTGCCCACCTGAA	This study
Pr_AFS-R1	CAGCCGTTGCGCAGGTATTC	This study
Pr_AFS-F2	CTACCTGATCAACCAGCGCC	This study
Pr_AFS-R2	CATCGCCATCTTTGTACAGG	This study

Appendix I
The protein structure and domains of AFS

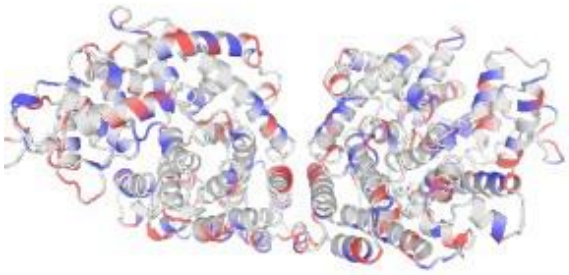
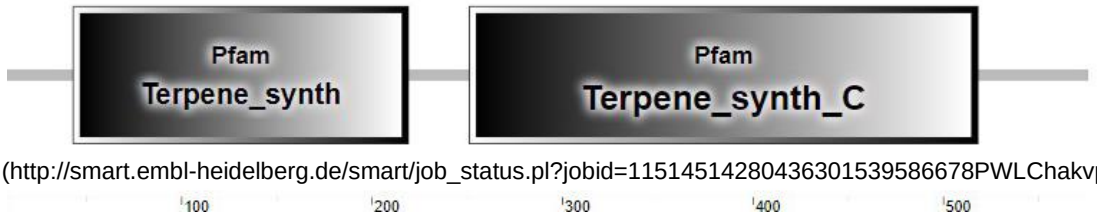


Figure 16. Swiss model of (E,E)-α-farnesene synthase (Q84LB2) of *Malus domestica* (Apple).

SW Pechous, et al. [128] had reported that the RR(X_8)W motif might play a role in reaction of acyclic sesquiterpene synthase and yield α-farnesene.



(http://smart.embl-heidelberg.de/smart/job_status.pl?jobid=1151451428043630153958667 8PWLChakvpj)

Figure 17. Domains within Malus domestica protein AFS1_MALDO (Q84LB2).

Domain1: terpene synth; Range 43-219 aa. Domain2: terpene synth C; Range 251-518 aa. PDB domain: 33-575 aa. SCOP domain: 547-574 aa. Low complexity region: 427-440 aa.

Appendix J
The optimization of PiPER assay in this study

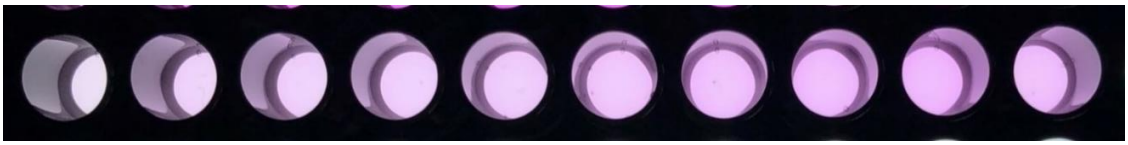
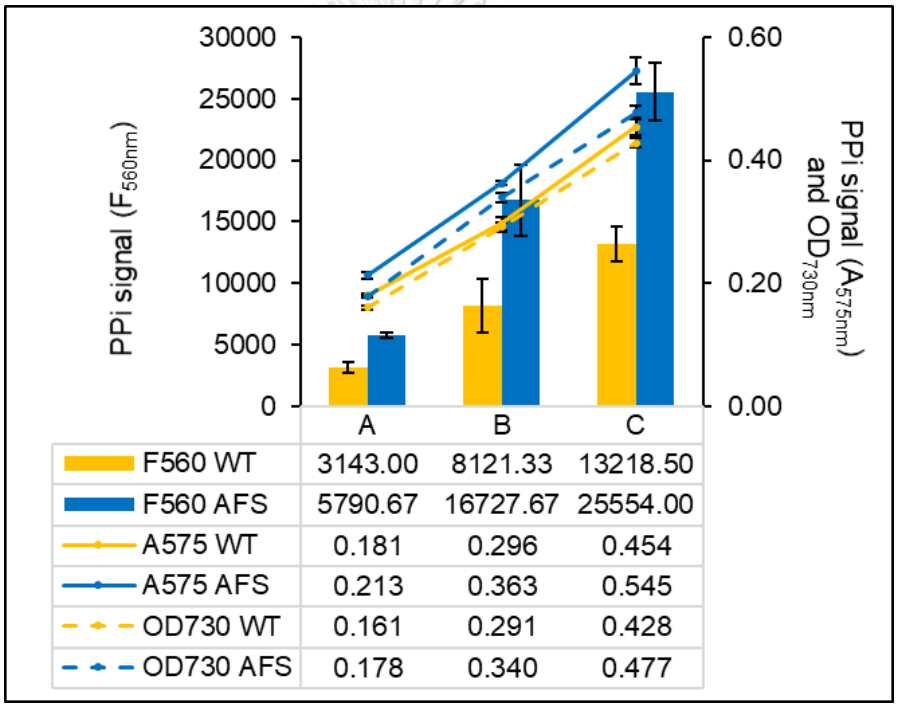


Figure 18. An image of PPi standard from PiPER assay. From left: 0, 1, 3, 5, 7, 10, 12, 15, 17 and 20 µM of PPi.



	A	B	C
F560 WT	3143.00	8121.33	13218.50
F560 AFS	5790.67	16727.67	25554.00
A575 WT	0.181	0.296	0.454
A575 AFS	0.213	0.363	0.545
OD730 WT	0.161	0.291	0.428
OD730 AFS	0.178	0.340	0.477

Figure 19. An optimization of culture and Tris-HCl solution ratios. The fluorescence and absorbance achieved from PPi signals were measured by microplate reader (Infinite® 200 PRO, Tecan). The three ratios were tested; (**A**) 1:3 (**B**) 2:3 (**C**) 1:1. All the data are expressed as mean ± standard deviation from cultures that were run in triplicate.

Appendix K
The scheme of generation of random mutants and PPi analysis

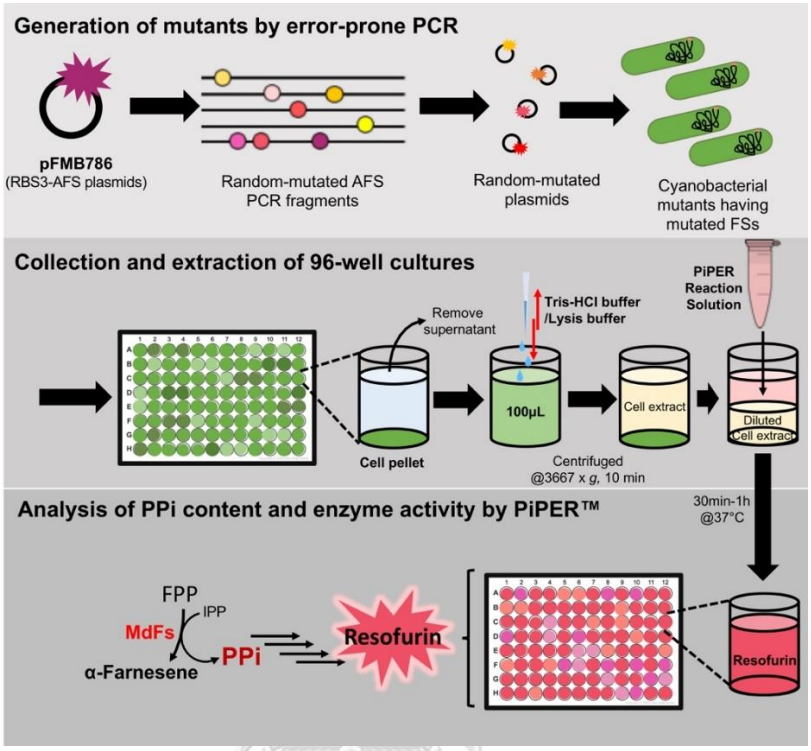


Figure 20. Scheme of generation of random mutants and PPi analysis.

Generation of mutants by error-prone PCR. The template used in error-prone PCR was pFMB786 (described in **Table 4**). Error-prone PCR was performed by using GeneMorph® II Random Mutagenesis Kit (Agilent) and following its manual instruction, which high- and low-rate of mutation were obtained from 25 and 500 ng of initial template, respectively. Randomly mutated PCR libraries were cut and ligated with pSe2Bb1k plasmid backbone before transformed into *E. coli* DH10β**.** Random-mutated plasmids were continually collected and transformed

into SeHL33. The colonies selection was done with cultivation on BG11 supplemented with spectinomycin and kanamycin.

Collection and extraction of 96-well cultures. Each selective colony was inoculated in 96-well microplate along with controls until reaching OD_{730} >1 and then transferred 100 µL of culture to new 96-well plates (Clear 96-Well Plate, Greiner Bio-One) containing BG11 with 1 mM IPTG addition. Preparation of samples before fluorescence screening was firstly collecting cell pellets and then removing supernatants. Cell pellets were then washed and lysed by mixing with pH 7.5 Tris-HCl and lysis buffer (B-PER™, Thermo Fisher Scientific™) respectively. Diluted cell extracts from day-3 and -8 after induction were transferred to new 96-well plate (µClear® 96 Well Plate, Greiner Bio-One) for PPi signal detection and OD_{730} measurement.

Analysis of PPi content and enzyme activity by PiPER™. PiPER™ reaction solution must be prepared fresh before use (described in the instruction) and added into 96-well plate containing cell extracts (with 100 µL in total volume). The reaction was run for 30 – 60 min at 37°C in microplate reader. Resofurin fluorescence was measured and calculated at 560 nm with 590 nm excitation wavelength. Note that the enzyme activity assay is described in second part of instruction.

Appendix L
The repeated experiments of ALE cultivation

To confirm the potential of ALE strain in farnesene production, the repeated cultivation (SET2) was parallelly observed, which the starter in SET2 was obtained from 20-day cell culture from SET1.

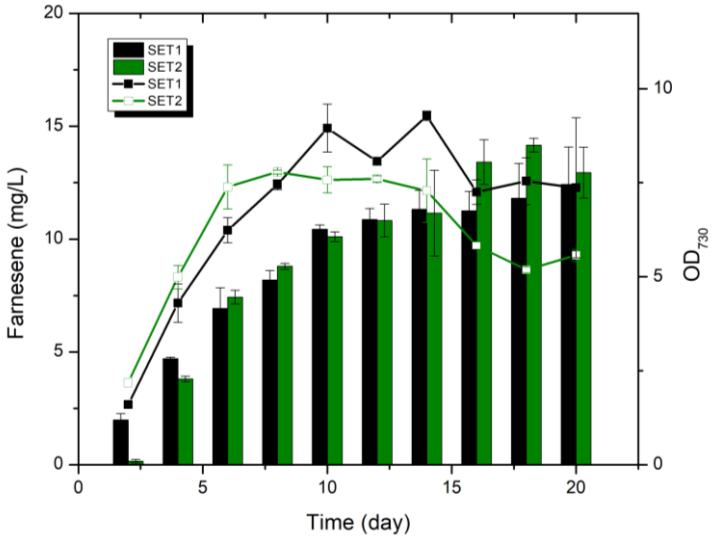


Figure 21. The repeated cultivation of ALE strain. 20-day cell culture were re-inoculated twice to confirm the efficiency of α-farnesene production with total 40 days. The second set can produce α-farnesene up to 14 mg/L. All the data are expressed as mean ± standard deviation from cultures that were run in duplicate.

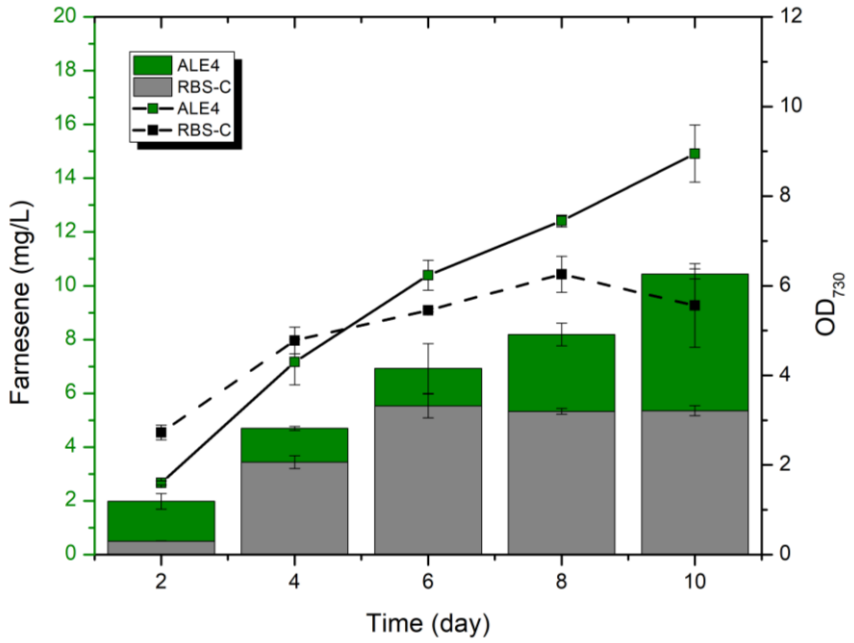


Figure 22. The comparison of control and ALE strain. ALE strain obtains 2-fold higher ability of α-farnesene production than control in 10-day cultivation, including growth rate. All the data are expressed as mean ± standard deviation from cultures that were run in duplicate.

Appendix M
The determination of *dxs* and *AFS* expression level by RT-qPCR

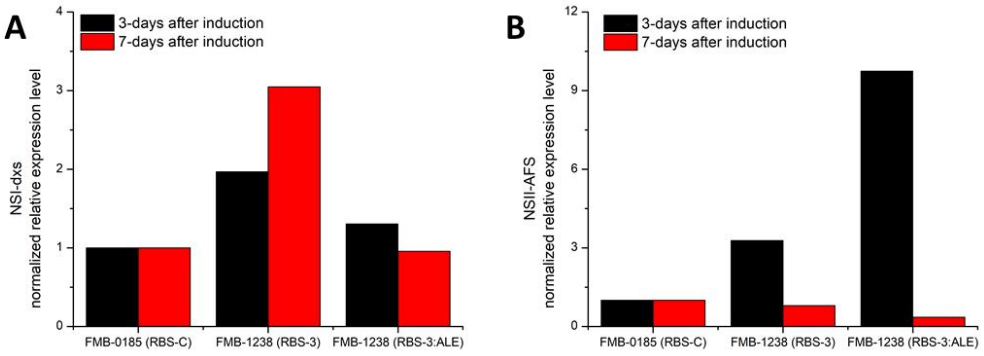


Figure 23. The relative mRNA expression level of *dxs* and *AFS* in control, RBS-3 and ALE strain. The 3 and 7-days induction of each strain were determined as mid-log and late-log stage in order to observe the changes of *dxs* (*E. coli*) and *AFS* expression. USA). Quantitative real-time PCR was performed using a TaqMan™ Gene expression Master Mix reagent (Applied Biosystem, USA) on a 7300-system instrument (Applied Biosystem, Foster City, CA, USA) in accordance with the manufacturer's instructions. The *rrsB* gene encoding 16S RNA ribosomal RNA was used as housekeeping gene (5'TAMRA TaqMan™). The amplification conditions were as follows: 10 min denaturation at 95 °C; 40 cycles at 95 °C for 15 s, at 60 °C for 1 min.

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