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PRODUCTION OF OLIGO(*CIS*-1,4)ISOPRENE IN RECOMBINANT *SYNECHOCYSTIS* SP. PCC 6803 THROUGH THE INSERTION OF RUBBER BIOSYNTHESIS-RELATED GENES

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PRODUCTION OF OLIGO(*CIS*-1,4)ISOPRENE IN RECOMBINANT *SYNECHOCYSTIS* SP. PCC 6803 THROUGH THE INSERTION OF RUBBER BIOSYNTHESIS-RELATED GENES

by

SAM KA KEI

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LIST OF SYMBOLS AND ABBREVIATIONS

℃	Degree Celsius
%	Percentage
s	Second
min	Minute
g	Gram
mg	Miligram
µg	Microgram
L	Liter
mL	Milliliter
nmol	Nanomole
µL	Microliter
v/v	Volume per volume
w/v	Weight per volume
AACT	Acetoacetyl-CoA thiolase
AgTFA	Silver trifluoroacetate
$CaCl_2$	Calcium chloride
$CDCl_3$	Chloroform
CDP-ME	4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol
CDP-ME2P	2-phospho-4-(cytidine 5' –diphospho)-2-C-methyl-D-erythritol
CMK	CDP-kinase

$Co(NO_3)_2$	Cobalt(II) nitrate
CPT	*Cis*-prenyltransferase
Ctb6f	Cytochrome b6/f
$CuSO_4$	Copper(II) sulfate
DCW	Dry cell weight
DHB	2,5-dihydroxybenzoic acid
DMAPP	Dimethylallyl pyrophosphate
DXP	1-deoxy-D-xylulose 5-phosphate
DXR	DXP reductoisomerase
DXS	Deoxy-D-xylulose-5-phosphate (DXP) synthase
EDTA	Ethylenediaminetetraacetic acid
FPP	Farnesyl pyrophosphate
Fd	Ferredoxin
GGPP	Geranylgeranyl pyrophosphate
GPP	Geranyl pyrophosphate
H_3BO_3	Boric acid
HDR	HMBPP reductase
HDS	1-hydroxyl-2-methyl-2-butenyl 4-diphosphate (HMBPP) synthase
HMBPP	1-hydroxyl-2-methyl-2-butenyl 4-diphosphate
HMGS	3-hydroxy-3-methylglutaryl-CoA (HMG) reductase
HMG-CoA	3-hydroxyl-3-methylglutaryl-CoA
H_2SO_4	Sulfuric acid

IPP	Isopentenyl pyrophosphate
IPPI	Isopentenyl diphosphate Delta-isomerase
K_2HPO_4	Dipotassium phosphate
KOH	Potassium hydroxide
LB	Luria bertani
LRP	Large rubber particle
MCT	MEP cytidyltransferase
MEP	2-C-methyl-D-erythritol-4-phosphate
ME-2,4cPP	2-C-methyl-D-erythritol 2,4-cyclodiphosphate
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
MK	Mevalonate kinase
$MnCl_2$	Manganese(II) chloride
MPDC	Diphosphomevalonate decarboxylase
MVA	Mevalonate
MVAP	Mevalonate-5P
MVAPP	Mevalonate-5PP
Na_2CO_3	Sodium carbonate
Na_2DETA	Disodium salt of ethylenediaminetetraacetic acid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
Na_2MoO_4	Sodium molybdate

$NaNO_3$	Sodium nitrate
$Na_2S_2O_3$	Sodium thiosulfate
OD	Optical density
PMK	Phosphomevalonate kinase
PSI	Photosystem I
PSII	Photosystem II
SRP	Small rubber particle
SRPP	Small rubber particle protein
TAE	Tris-acetate-EDTA
TPT	*Trans*-prenyltransferase
$ZnSO_4$	Zinc sulfate

PENGHASILAN OLIGO(*CIS*-1,4)ISOPRENA DALAM SYNECHOCYSTIS SP. PCC 6803 REKOMBINAN MELALUI PENYELITAN GEN BERKAITAN BIOSINTESIS GETAH

ABSTRAK

Getah asli daripada *Hevea brasiliensis* secara dominannya, terdiri daripada 1,4-poliisoprena yang mempunyai lebih daripada 10,000 unit isoprena berulang $(C_5H_8)_n$ dalam konfigurasi *cis*. Biosintesis getah asli dimangkinkan oleh *cis*-prenyltransferase (CPT) bagi pempolimeran isopentenil pirofosfat (IPP) dengan substrat allylic yang lain untuk membentuk rantaian panjang poliisoprena dengan berat molekul yang tinggi. CPT dikatakan berperanan sebagai enzim utama dalam pemangkinan tindak balas pempolimeran tersebut. Selain itu, "farnesyl pyrophosphate" (FPP) telah dicadangkan berfungsi sebagai faktor pemula penting yang terlibat dalam biosintesis getah dan ini telah disokong oleh kajian terdahulu. Malah, "small rubber particles" (SRPs) yang terkandung dalam susu getah telah terbukti memberi kesan yang lebih jelas ke atas kadar biosintesis getah berbanding dengan "large rubber particles" (LRPs). Oleh sebab "Small rubber particle protein" (SRPP) adalah komponen yang terdapat dalam SRPs, SRPP dicadang sebagai salah satu komponen utama yang terlibat dalam biosintesis getah. Hipotesis menyatakan bahawa sianobakteria, bakteria fotosintesis boleh menjadi organisma yang berpotensi bagi penghasilan oligo(*cis*-1,4)isoprena, yang merupakan terbitan daripada getah asli. Di antara kepelbagaian jenis sianobakteria, model organisma *Synechocystis* sp. PCC 6803 adalah strain yang telah banyak dikaji, di mana genom lengkapnya adalah yang pertama dijujuk dan diterbitkan. Maklumat penting yang diperoleh adalah tapak jalan 2-*C*-metil-$_D$-eritriol-4-fosfat (MEP) asli berfungsi di dalam sitosol bagi menjana prekursor yang diperlukan dalam biosintesis

getah, iaitu IPP dan dimetilalil pirofosfat (DMAPP) yang bertujuan bagi menggalakkan penjanaan oligo(*cis*-1,4)isoprena. Oleh itu, *Synechocystis* sp. telah dipilih sebagai organisma utama dalam kajian ini. Bagi mengenalpasti penghasilan oligo(*cis*-1,4)isoprena, gen yang berkaitan dengan biosintesis getah, iaitu *HbCPT2, HbFPP,* dan *HbSRPP* telah diklon dan diekspres dalam *Synechocystis* sp. Secara ringkas, gen yang berkaitan dengan biosintesis getah daripada *H. brasiliensis*, termasuk *HbCPT2*, *HbFPP* dan *HbSRPP* diamplifikasi daripada cDNA yang berasal daripada lateks yang disebabkan oleh pengekspresan gen tersebut adalah lebih tinggi dalam lateks berbanding dengan tisu lain. Seterusnya, produk PCR yang terhasil seterusnya diligasi ke dalam vektor integratif yang telah dibina, iaitu pTKP2031V_{ANN}-HbCPT2 and pTKP2031V_{ANN}-HbCPT2+FPP+SRPP dengan gen *HbCPT2*, dan *HbCPT2, HbFPP*, dan *HbSRPP* masing-masing. Kemudian, kedua-dua plasmid ditransformasi ke dalam genom *Synechocystis* sp. dan seterusnya diekspreskan. RT-PCR dilakukan bagi memeriksa pengekspresan gen dalam rekombinan *Synechocystis* sp. Ekstrak daripada strain rekombinan yang positif dianalisis oleh MALDI-TOF MS. Menariknya, hasil analisis MALDI-TOF-MS telah menunjukkan bukti pertama tentang kehadiran oligo(*cis*-1,4)isoprena dalam *Synechocystis* rekombinan. Oligo(*cis*-1,4)isoprena telah dikenalpasti dalam strain SyC yang mempunyai penyelitan *HbCPT2* berdasarkan pengesanan 68 Da di antara selang 925-993. Peningkatan berat molekul oligo(*cis*-1,4)isoprena telah dijumpai dalam rekombinan SyCFS dengan pengesanan 68 Da di antara selang 1,029 hingga 1,259 di mana ini memberikan bukti tentang korelasi positif antara *HbCPT2, HbFPP,* dan *HbSRPP* dalam menggalakan biosintesis oligo(*cis*-1,4)isoprena. Berdasarkan hasil daripada kajian pertama ini, *Synechocystis* sp. dicadangkan memiliki potensi untuk menjadi penghasil oligo(*cis*-1,4)isoprena melalui proses fotosintesis. Oleh sebab kajian ini merupakan kajian pertama yang

mengkaji tentang gen yang berkaitan dengan biosintesis getah dalam *Synechocystis* sp., hasil keputusan daripada kajian ini boleh dijadikan sebagai maklumat atau panduan yang penting untuk penyelidikan masa depan.

PRODUCTION OF OLIGO(*CIS*-1,4)ISOPRENE IN RECOMBINANT *SYNECHOCYSTIS* SP. PCC 6803 THROUGH THE INSERTION OF RUBBER BIOSYNTHESIS-RELATED GENES

ABSTRACT

Natural rubber of *Hevea brasiliensis* is composed predominantly of 1,4-polyisoprene with more than 10,000 repeating isoprene units $(C_5H_8)_n$ in *cis*-configuration. Biosynthesis of natural rubber is catalyzed by *cis*-prenyltransferase (CPT) for the polymerization of isopentenyl pyrophosphate (IPP) with other allylic substrates to form long-chain of high molecular weight polyisoprene. CPT was suggested as the key enzyme catalyzing polymerization reaction. Besides that, farnesyl pyrophosphate (FPP) was proposed as the main initiator involved in rubber biosynthesis which was supported by previous evidence provided. Moreover, SRPs found in latex had been proven to have profound effect on rubber biosynthetic rate than LRPs. As small rubber particle protein (SRPP) is component found in SRPs, SRPP was suggested to be one of major component involved in rubber biosynthesis. It is hypothesized that photosynthetic cyanobacteria can be the potential organisms for oligo(*cis*-1,4)isoprene production, which is derivative of natural rubber. Among the diverse types of cyanobacteria, model organism *Synechocystis* sp. PCC 6803 was the well-studied strain, in which its full genome was the first to be sequenced and published. More importantly, the native 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathway operates in the cytosol to generate the rubber biosynthesis precursors, namely IPP and DMAPP, promoting the generation of oligo(*cis*-1,4)isoprene. Therefore, *Synechocystis* sp. was chosen as the host organism in this study. To examine oligo(*cis*-1,4)isoprene production, genes related to rubber biosynthesis, including *HbCPT2*,

HbFPP, and *HbSRPP* were cloned and heterologously expressed in the *Synechocystis* sp. Briefly, rubber biosynthesis-related genes from *H. brasiliensis* including *HbCPT2*, *HbFPP*, and *HbSRPP* were amplified from cDNA of latex because these rubber biosynthesis-related genes are highly expressed in latex as compared to others *Hevea's* tissues. The resulting PCR products were ligated into the constructed integrative vector pTKP2031V_{ANN}, generating two new version of plasmids namely pTKP2031V_{ANN}-HbCPT2 and pTKP2031V_{ANN}-HbCPT2+FPP+SRPP with *HbCPT2*, and three inserts *HbCPT2*, *HbFPP* and *HbSRPP*, respectively. Hereafter, these two constructed plasmids were transformed and heterologously expressed in *Synechocystis* sp. RT-PCR was performed to check for the gene expression in recombinant *Synechocystis* sp. The cell extracts from positive recombinant strains were analyzed using MALDI-TOF MS. Interestingly, MALDI-TOF-MS analysis result had provided first evidence on the presence of oligo(*cis*-1,4)isoprene in recombinant *Synechocystis* strains. Oligo(*cis*-1,4)isoprene was detected in SyC strain with *HbCPT2* insert based on the detection of 68 Da in between 925 to 993 interval. The improvement on the molecular weight of oligo(*cis*-1,4)isoprene synthesized was found in recombinant strain SyCFS based on the detection of 68 Da in between 1,029 to 1,259 interval, providing an evidence about the positive correlation of *HbCPT2*, *HbFPP*, and *HbSRPP* in promoting the oligo(*cis*-1,4)isoprene synthesis. According to the results from this first time study, *Synechocystis* sp. was proposed to be a potential carbon-neutral oligo(*cis*-1,4)isoprene producer using photosynthesis. Since this is the first effort to express rubber biosynthesis-related genes in *Synechocystis* sp., results from this study provide crucial background information that can be employed for further development.

CHAPTER ONE

INTRODUCTION

1.1 Background of Research

Natural rubber is a high molecular mass plant-derived polyterpene which constituted of hydrocarbons with more than 10,000 repeating isoprene units $(C_5H_8)_n$ in 1,4 *cis*-configuration (Epping et al., 2015, Schmidt et al., 2010). Natural rubber is synthesized and stored in the latex which is the cytoplasm of highly specialized articulated cells, known as laticifers (Chao et al., 2015, Wititsuwannakul and Wititsuwannakul, 2005). Natural rubber is an indispensable biopolymer because it is used in more than 40,000 products due to its excellent physicochemical properties (*eg*. elasticity, resistance to abrasion and resilience) which cannot be achieved by synthetic rubber (Mooibroek and Cornish, 2000). To date, it has been reported that more than 20,000 plant species can produce rubber (Konno, 2011, Hagel et al., 2008), including some well-known rubber plant species which can synthesize large quantity of rubber with high molecular weight such as para rubber tree (*Hevea brasiliensis* Muell Arg.), guayule (*Parthenium argentatum*), and Russian dandelion (*Taraxacum* sp.) (van Beilen and Poirier, 2007). However, only the rubber tree (*H. brasiliensis*) is still prevalent as main source of renewable latex to produce commercial rubber products worldwide because of its high yield and high quality of latex (Berthelot et al., 2014a). Nowadays, demand for rubber is increasing, however, there are several problems in *H. brasiliensis* cultivations such as disease outbreak, lack of plantation fields, and slow growth of rubber tree which led to the developing of a new alternative source of natural rubber.

In the present study, *Synechocystis* sp. PCC 6803 (*Synechocystis* sp.) was utilized as a candidate to study the expression of rubber biosynthesis-related genes for oligo(*cis*-1,4)isoprene production. Among the diverse cyanobacterial species, *Synechocystis* sp. is the most-extensive studied and best-characterized strain, where its complete genome sequence, including four endogenous plasmids, pSYSM:120 kb, pSYSX:106 kb, pSYSA:103 kb, and pSYSG:44 kb, were sequenced and published (Kaneko et al., 2003, Kaneko et al., 1996). *Synechocystis* sp. is able to perform plant-liked oxygen evolving photosynthesis and it also represents a promising host organism for sustainable products production owing to its minimal nutrients requirements, amenable to genetic manipulation and naturally competent (Lau et al., 2015, Yu et al., 2013, Angermayr et al., 2009). Many biotechnological applications have used *Synechocystis* sp. as a photosynthetic host organism to synthesize diverse types of plant secondary metabolites, such as isoprene, limonene, and caffeic acid. Besides that, MEP pathway operates in *Synechocystis* sp. (Ershov et al., 2002) and the presence of isopentenyl pyrophosphate (IPP) isomerase provide both IPP and dimethylallyl pyrophosphate (DMAPP) which are essential building blocks involved in rubber biosynthesis (Qu et al., 2015, Barkley et al., 2004, Poliquin et al., 2004, Steinbüchel, 2003), which led to *Synechocystis* sp. becoming a potential platform that can be utilized in this study.

Therefore, the objective of this study were:

1. To clone three rubber biosynthesis-related genes from *H. brasiliensis* and introduce these genes into the genome of photosynthetic cyanobacterium *Synechocystis* sp. PCC 6803.
2. To analyze the oligo(*cis*-1,4)isoprene production in recombinant *Synechocystis* sp.

CHAPTER TWO

LITERATURE REVIEW

2.1 Terpenoids

Terpenoids or isoprenoids constitute the largest family of organic chemicals in nature with at least 40,000 members isolated from plants, animals and microbes. Although they exist in all organisms, most of them are natural plant products. In plants, they function as hormones, electron carriers, light-harvesting pigments in photosynthesis, mediators of polysaccharide synthesis, repellents, pollinators and structural components of membranes which are essential for plant growth, development and metabolism. Terpenoids have diverse physical and chemicals properties in nature, they are either lipophilic or hydrophilic, volatile or non-volatile, chiral or achiral, cyclic or acyclic. Owing to their diverse biological functions and wide range of physical and chemical properties, terpenoids are being extensively used in the fields of pharmaceuticals, agrochemicals and as flavorings, fragrances and cosmetics (Bohlmann and Keeling, 2008, McGarvey and Croteau, 1995).

Terpenoids are derived from the C_5 universal precursor, isopentenyl pyrophosphate (IPP) and its allylic isomer dimethylallyl pyrophosphate (DMAPP) (McGarvey and Croteau, 1995). IPP and DMAPP, also known as isoprene unit which are synthesized from two distinct pathways: (i) mevalonate (MVA) pathway which is cytosolic in nature, operates in eukaryotes, archaea and some bacteria; (ii) non-mevalonate pathway or also referred to as 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathway, which is plastidial in nature and this metabolic pathway has been found in

most eubacteria, plant and algae in parallel with a cytosolic MVA pathway (Pattanaik and Lindberg, 2015, Bohlmann and Keeling, 2008). Biosynthesis of terpenoids takes place via condensation of additional IPP moieties through various action of prenyltransferase to generate higher order terpenoid building blocks: monoterpenoids ($C_{10}H_{16}$), sesquiterpenoids ($C_{15}H_{24}$) and diterpenoids ($C_{20}H_{32}$) by using geranyl pyrophosphate (GPP) (C_{10}), farnesyl pyrophosphate (FPP) (C_{15}) or geranylgeranyl pyrophosphate (GGPP) (C_{20}) as the precursors, respectively. Moreover, condensation of these intermediates can also formed even higher order of terpenoids. For example: triterpenoids ($C_{30}H_{48}$) and tetraterpenoids ($C_{40}H_{64}$) are derived from the pairwise condensation of FPP and GGPP, respectively, and these intermediates can be further condensed to give rise to an undefined number of five-carbon precursors yielding polyterpenoids $(C_5H_8)_n$ (Bohlmann and Keeling, 2008). The carbon chain length of terpenoids vary from short chain GPP (C_{10}) to very long chain natural rubber ($C_{>10,000}$) (Takahashi and Koyama, 2006). The classification of terpenoids is according to the number of five-carbon isoprene units incorporated into their skeleton: hemiterpenes C_5 (1 isoprene unit), monoterpenes C_{10} (2 isoprene units), sesquiterpenes C_{15} (3 isoprene units), diterpenes C_{20} (4 isoprene units), triterpenes C_{30} (6 isoprene units), tetraterpenes C_{40} (8 isoprene units), polyterpenes $(C_5)_n$ where n may vary from 9 to 30,000 (McGarvey and Croteau, 1995).

2.2 Natural Rubber

Natural rubber is a high molecular mass biopolymer (>1,000kDa) constituted of more than 10,000 repeating isoprene units $(C_5H_8)_n$ with 1,4 *cis*-configuration as shown in figure 2.1 (Epping et al., 2015, Schmidt et al., 2010). The *cis*-configuration of isoprene unit in the rubber was discovered in 1954 using X-ray diffraction (Tanaka, 1989). To date, it has been reported that more than 20,000 plant species are known to produce latex containing rubber (Konno, 2011, Hagel et al., 2008). Among these include some well-known rubber plant species which can synthesize large quantity of rubber with high molecular weight such as the para rubber tree (*Hevea brasiliensis*), guayule (*Parthenium argentatum*), and Russian dandelion (*Taraxacum* sp.) (van Beilen and Poirier, 2007). However, only the rubber tree (*Hevea brasiliensis* Muell Arg.) is still prevalent as the main source of renewable latex for the production of commercial rubber products worldwide because of its high yield and high quality latex (Berthelot et al., 2014a). Natural rubber is an indispensable biopolymer that is used in more than 40,000 products due to its excellent physicochemical properties (*eg*. elasticity, resistance to abrasion and resilience) which cannot be achieved by the synthetic rubber (Mooibroek and Cornish, 2000).

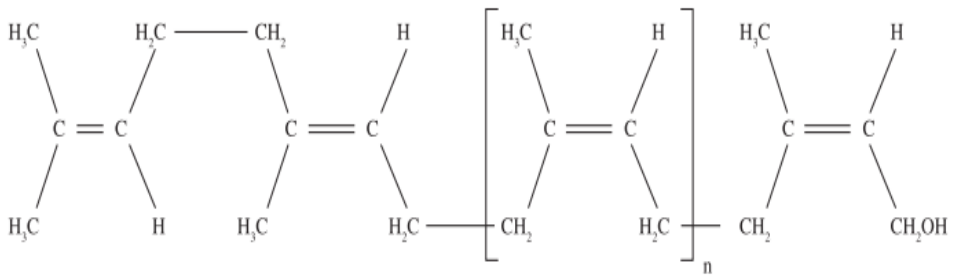

Figure 2.1 Chemical structure of polymer *cis*-1,4-polyisoprene with repeating isoprene units $(C_5H_8)_n$ shown in parenthesis (Herculano et al., 2011).

2.3 Rubber Biosynthesis

The biosynthetic pathway for polyisoprene is classified into two sections: first, the production of IPP and DMAPP from two independent MVA and MEP pathways; second, the polymerization of IPP and DMAPP to generate a higher molecular weight polyisoprene (Steinbüchel, 2003). Each section will be discussed in more detail in the following sub-section:

2.3.1 Biosynthesis of IPP and DMAPP

Both MVA and MEP pathways start from different intermediates: the MVA pathway utilizes acetyl-CoA, while MEP pathway utilizes pyruvate and glyceraldehyde-3-phosphate (Figure 2.2). MVA pathway was considered the sole metabolic pathway for biosynthesis of IPP and DMAPP until MEP pathway was discovered by Rohmer and his coworkers (Rohmer, 1999). MVA pathway initiates with the condensation of three molecules of acetyl-CoA to 3-hydroxyl-3-methylglutaryl-CoA (HMG-CoA) and catalyzed by HMG-CoA reductase to MVA. MVA is then transformed to IPP and DMAPP after two successive phosphorylation and a decarboxylation. On the other hand, MEP pathway starts with the condensation of pyruvate and glyceraldehyde 3-phosphate, catalyzed by 1-deoxy-D-xylulose 5-phosphate (DXP) synthase to produce DXP. DXP is also used for the biosynthesis of pyridoxal and thiamin and can also be converted to MEP after catalyzed by DXP reductoisomerase (DXR). 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (ME-2,4cPP) is produced after several enzymatic reactions, here after ME-2,4cPP is catalyzed by 1-hydroxyl-2-methyl-2-butenyl 4-diphosphate (HMBPP) synthase (HDS) forming HMBPP. HMBPP is then converted into IPP and DMAPP by HMBPP reductase (HDR) (Vranová et al., 2012, Takahashi and Koyama, 2006, Dubey et al., 2003). Isopentenyl pyrophosphate isomerase (IPPI) plays a major role in controlling the balance between

IPP and DMAPP (Vranová et al., 2012). These two pathways are takes place simultaneously in plant where the MVA pathway operate in cytosol and the MEP pathway operate in plastid (Pattanaik and Lindberg, 2015, Dubey et al., 2003, Rohmer, 1999).

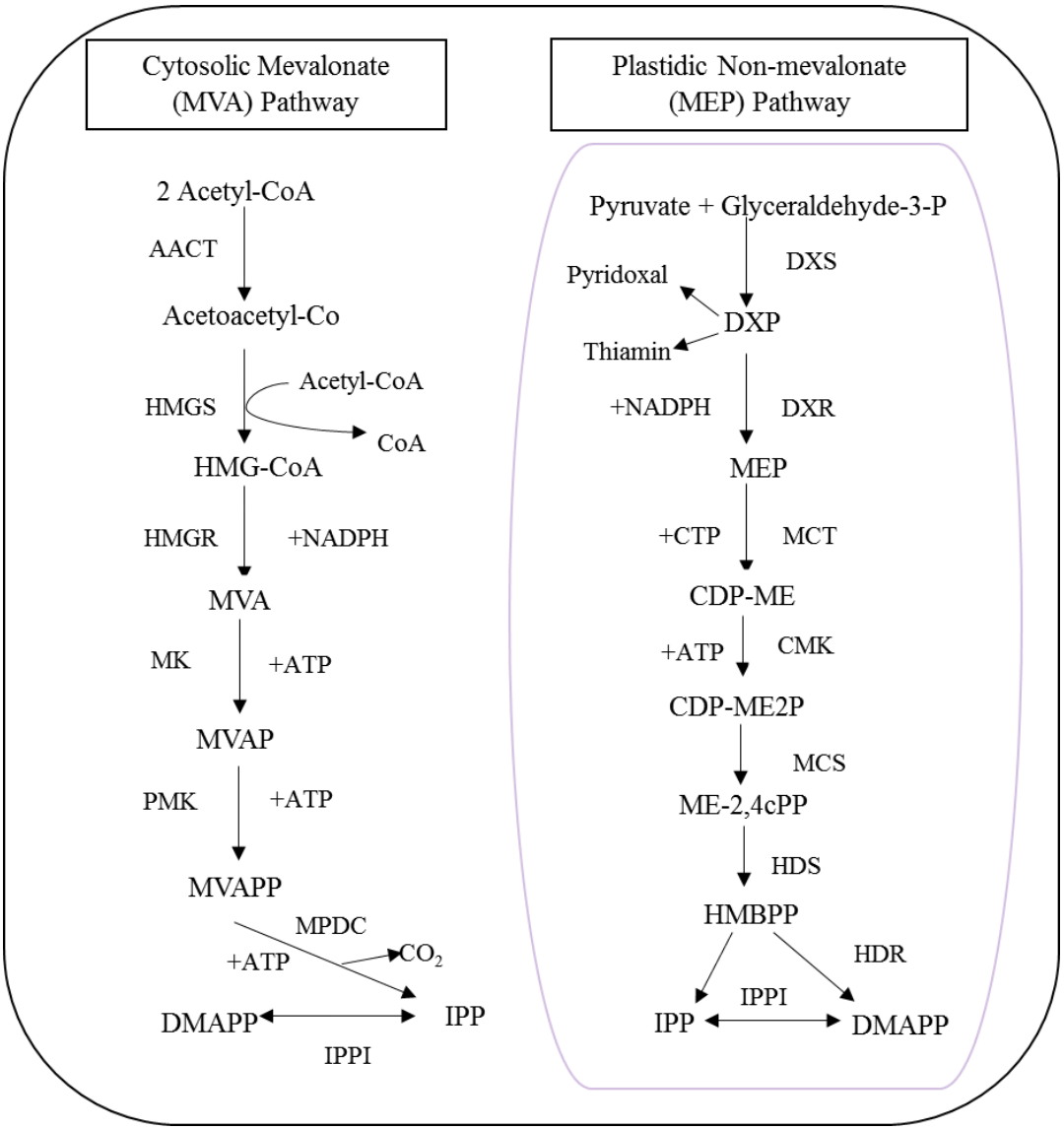

Figure 2.2 Two independent biosynthetic pathways for IPP and DMAPP and enzymes catalyzing in each step. [Left] Cytosolic mevalonate (MVA) pathway. Abbreviations: AACT: acetoacetyl-CoA thiolase; HMGS: 3-Hydroxy-3-methylglutaryl-CoA (HMG) reductase; MVA: Mevalonate; MK: Mevalonate kinase; MVAP: Mevalonate-5P; PMK: Phosphomevalonate kinase; MVAPP: Mevalonate-5PP; MPDC: Diphosphomevalonate decarboxylase; IPPI: Isopentenyl pyrophosphate (IPP, C_5) isomerase; DMAPP (C_5): Dimethylallyl pyrophosphate. [Right] Plastidic non-mevalonate (MEP) pathway. Abbreaviations: DXS: 1-Deoxy-D-xylulose-5-phosphate (DXP) synthase; DXR: DXP reductoisomerase; MEP: 2-C-methyl-D-erythritol 4-phosphate; MCT: MEP cytidyltransferase; CDP-ME: 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol; CMK: CDP-kinase; CDP-ME2P: 2-Phospho-4-(cytidine 5' – diphospho)-2-C-methyl-D-erythritol; MCS: 2-*C*-methyl-$_D$-erythritol 2,4-cyclodiphosphate (ME-2,4cPP) synthase; HDS: 1-Hydroxyl-2-methyl-2-butenyl 4-diphosphate (HMBPP) synthase; HDR: HMBPP reductase. The role of DXP for the biosynthesis of pyridoxal and thiamin is also indicated. (adapted from Roberts, 2007, Bouvier et al., 2005, Dubey et al., 2003, Steinbüchel, 2003).

2.3.2 Polymerization of IPP and DMAPP for high molecular weight polyisoprene production

Prenyltransferases (prenyl diphosphate synthases) are key enzymes contribute to the generation of higher molecular weight of polyisoprene, by using IPP and DMAPP as precursors (Steinbüchel, 2003). Generally, these enzymes catalyze the transfer reactions between prenyl groups to the corresponding acceptors such as IPP, aromatic compounds or proteins for the production of isoprenoids in nature (Kharel and Koyama, 2003, Steinbüchel, 2003). Prenyltransferases can be classified into three classes: isoprenyl pyrophosphate synthases (IPPSs), protein prenyltransferases and prenyltransferases (Liang et al., 2002). IPPSs catalyze in chain elongation by the addition of allylic prenyl groups to consecutive condensation reactions with IPP, while protein prenyltransferases catalyze the transfer of isoprenyl pyrophosphate to a protein or peptide. For the prenyltransferases, they catalyze the cyclization of isoprenyl pyrophosphate (Kharel and Koyama, 2003). Prenyltransferases are further divided into two major groups: *trans*- and *cis*-prenyltransferases where the *trans*-prenyltransferases (TPTs) catalyze product formation in *trans*-configuration. In contrast, *cis*-prenyltransferases (CPTs), catalyze the formation of products in *cis*-configuration. TPT belongs to the short chain prenyl pyrophosphate synthases groups, for example: FPP synthase and GGPP synthase. They catalyze the formation of intrinsic allylic substrates in order to form longer chain prenyl pyrophosphate by other prenyl transferases. However, CPTs catalyze the consecutive condensation of allylic prenyl groups with IPP to generate long chain prenyl pyrophosphate in *cis*-configuration (Kharel and Koyama, 2003).

TPTs catalyze the subsequent additions of IPP to DMAPP or to the condensation products for the formation of GPP (C_{10}), FPP (C_{15}) and finally GGPP (C_{20}) (Figure 2.3) (van Beilen and Poirier, 2007, Steinbüchel, 2003). *In vitro* studies demonstrated that DMAPP, GPP, FPP and GGPP can initiate polyisoprene synthesis (van Beilen and Poirier, 2007, Stubbe et al., 2005, Cornish and Siler, 1995, Madhavan et al., 1989, Archer and Audley, 1987). However, FPP was suggested to be the main initiator for rubber biosynthesis in *Hevea brasiliensis* because it has a low binding constant as compared to others allylic pyrophosphate. In addition, FPP is found in cytosol of the laticifer where rubber transferase is localized. A supporting result using NMR showed that rubber molecule from *H. brasiliensis* has a C_{15} tail, indicating that FPP probably acts as an initiator for rubber synthesis (da Costa et al., 2005, Tanaka et al., 1996). Hereafter, *in vitro* experiments from three rubber producing plants such as *Ficus elastic*, *Parthenium argentatum*, and *H. brasiliensis* also showed that FPP was the most efficient initiator involved in rubber biosynthesis (Xie et al., 2008). Many efforts have been done to understand the rubber biosynthesis pathway, but the precise biosynthesis pathway of rubber *H. brasiliensis* still remains elusive.

Ultrastructural analysis revealed that the rubber particles are mainly globular in shape resembling the structure of lipid droplets that contain a hydrophobic core of polyisoprene surrounded by a phospholipid monolayer which includes a mixture of lipids, proteins and other molecules (Schmidt et al., 2010). Rubber particle is the specific organelle where rubber biosynthesis occurs. It is suspended in the cytoplasm of highly differentiated laticifer cells that localized in the bark phloem of *H. brasiliensis* (Wang et al., 2015). The rubber particles in *H. brasiliensis* can be divided into large rubber particles (LRPs) and small rubber particles (SRPs) (Singh et al., 2003) where SRPs have higher rubber biosynthesis activity than LRPs (Ohya et al., 2000,

Archer et al., 1963). Rubber particles in *H. brasiliensis* are the most complicated particles so far (Cornish, 2001) which composed of 186 identified proteins (Dai et al., 2013). Previous studies proposed the rubber particles are originated from rough endoplasmic reticulum (Chrispeels and Herman, 2000), but the precise development of rubber particles in *H. brasiliensis* has not been fully elucidated.

Rubber synthase, also known as the rubber transferase or rubber polymerase, is a rubber synthesizing *cis*-prenyltransferase (CPT) enzyme (E.C. 2.5.1.20) that catalyze the consecutive *cis*-1,4-condensation of cytoplasmic IPP with the allylic pyrophosphate as an initiator (Figure 2.3) (Schmidt et al., 2010, Kharel and Koyama, 2003, Cornish, 2001, Siler and Cornish, 1993, Archer et al., 1963). Rubber synthase is an integral membrane-bound protein exists on the rubber particles surface (Figure 2.3). This localization enables the enzyme to interact with both the hydrophilic FPP and IPP molecules in the cytoplasm and depositing the hydrophobic polymer chain extruded from the enzyme into rubber particles core (van Beilen and Poirier, 2007). Two cDNA clones (designated as HRT1 and HRT2) encoding *cis*-prenyltransferase were isolated from latex of *H. brasiliensis* for *in vitro* rubber transferase assays study. Results from this finding showed that HRT2 catalyzed the formation of longer chain polyprenyl product than HRT1 *in vitro* and the catalytic activity of HRT2 was increased proportionally by the addition of washed bottom fraction particles from latex (Asawatreratanakul et al., 2003).

Apart from rubber transferase, one of the crucial proteins have also been reported as rubber-biosynthesis related protein, namely small rubber particle protein (SRPP) (Dai et al., 2013, Oh et al., 1999, Dennis and Light, 1989). SRPP is one of the major protein which composed of rubber particles (Berthelot et al., 2014b, Xiang et al., 2012). SRPP is one of the most abundant rubber particle bound-protein which had

been found to be highly expressed in the *Hevea* latex (Chow et al., 2007, Han et al., 2000). Based on transcriptome analyses, SRPP gene is expressed predominantly in the laticifers (latex) (Aoki et al., 2014, Chotigeat et al., 2010, Chow et al., 2007, Ko et al., 2003, Han et al., 2000), suggesting that this protein may be directly involved in rubber biosynthesis. Many experiments have been carried out and the involvement of SRPP proteins in rubber (*cis*-1,4-polyisoprene) polymerization was studied using *in vitro* biosynthesis rubber assays of incorporation of [^{14}C]-IPP into whole latex or washed rubber particles (Oh et al., 1999, Dennis and Light, 1989). SRPP is proposed as one of the hydrophobic supporting protein which is associated with the surface of rubber particle as shown in proposed model schematized in (Figure 2.3) (Berthelot et al., 2014b, Berthelot et al., 2012). The SRPP from *H. brasiliensis* was associated with the small rubber particles (SRPs) (Berthelot et al., 2014b) and the evidence from *in vitro* studies revealed that SRPs had higher rubber biosynthesis activity than large rubber particles (LRPs) because of the higher activity of rubber transferase in SRPs (Rojruthai et al., 2010, Ohya et al., 2000, Archer et al., 1963). Proteomic analysis for the high yield of latex producing ethylene-treated *H. brasiliensis* revealed that the number of SRPs synthesized in latex was higher than LRPs, suggesting crucial role of SRPs in rubber biosynthesis (Wang et al., 2015). To date, the exact function of SRPP is still remain unclear. Therefore, more efforts are necessary to unravel the exact roles of rubber proteins in rubber biosynthesis.

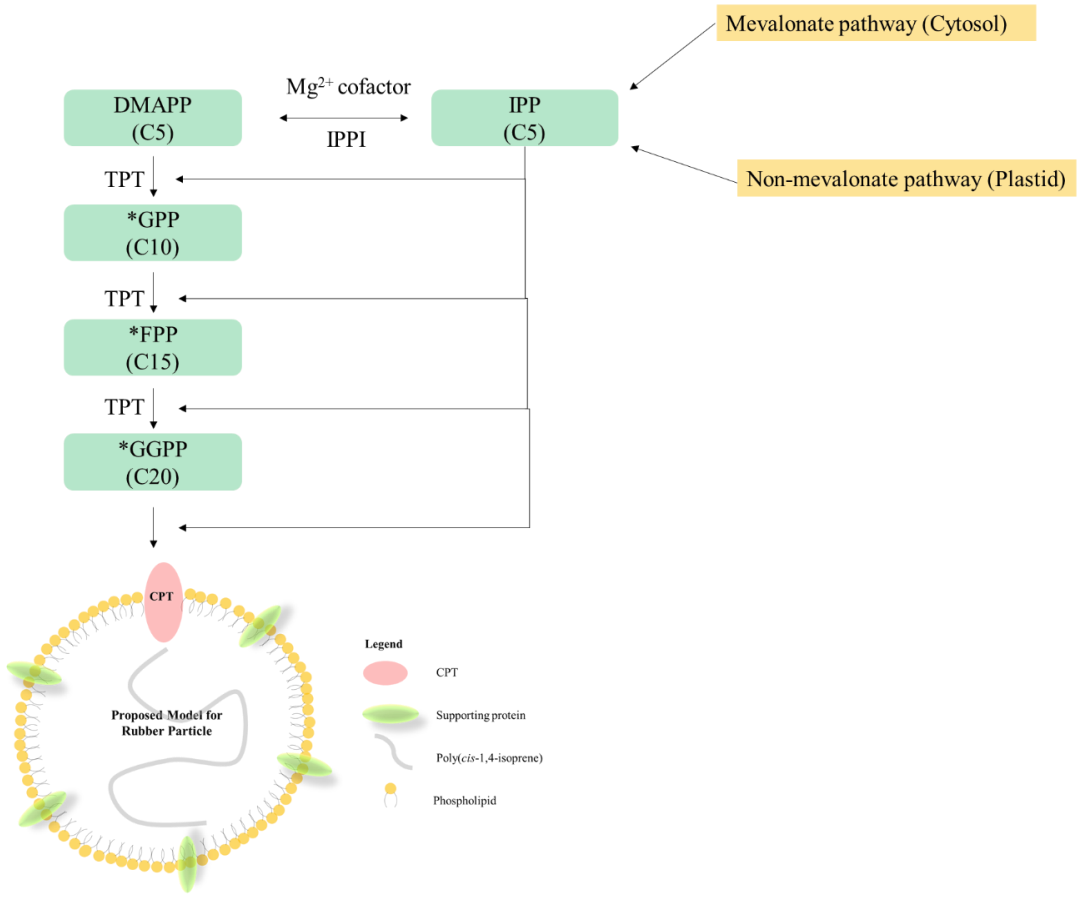

Figure 2.3 Natural rubber biosynthesis pathway together with the proposed model of rubber particle. The IPP (monomer) and its stereoisomer DMAPP are synthesized from cytosolic mevalonate pathway or plastidic non-mevalonate pathway. The IPP and DMAPP are condensed to form several allylic pyrophosphates such as GPP, FPP and GGPP, catalyzed by *trans*-prenyltransferases (TPTs). CPT catalyzes the polymerization of IPP with allylic pyrophosphates to produce rubber. Prenyltransferases require IPP (monomer), Mg^{2+} cofactor and allylic pyrophosphates for chain elongation. Abbreviations: IPP: isopentenyl pyrophosphate; IPPI: isopentenyl pyrophosphate isomerase; DMAPP: dimethylallyl pyrophosphate; TPT: *trans*-prenyltransferase; GPP: geranyl pyrophosphate; FPP: farnesyl pyrophosphate; GGPP: geranylgeranyl pyrophosphate; CPT: *cis*-prenyltransferase; * indicates allylic pyrophosphate. (adapted from Gronover et al., 2011, Kharel and Koyama, 2003, Steinbüchel, 2003)

2.4 Cyanobacteria

Cyanobacteria, also known as blue-green algae, are the evolutionarily oldest prokaryotes that have been found in microfossils originated from 2 to more than 3.5 billion years ago (Schopf, 1993, Carr and Whitton, 1982 , Knoll and Barghoorn, 1977). They are the only known Gram-negative unicellular prokaryote endowed with complex photosynthetic system which are capable of performing plant-liked oxygenic photosynthesis (Lau et al., 2015, Knoop et al., 2010, Mulkidjanian et al., 2006, Stanier and Bazine, 1977). In addition, they can also fix carbon dioxide in the atmosphere via Calvin-Benson cycle (Smith, 1983). Cyanobacteria are considered as the origin of life as they are the pioneer oxygenic phototrophs of the planet Earth (Kulasooriya, 2011, Carr and Whitton, 1982). In cyanobacteria, photosynthesis and respiration are performed simultaneously in the same compartment, however, some cyanobacteria can also fix nitrogen in atmosphere (Vermaas, 2001). They are the only prokaryotes which posses a classical circadian clock mechanism (Golden et al., 1997) and overwhelming evolutionary studies indicated that the eukaryotic photosynthesis had originated from endosymbiosis of cyanobacteria (Raven and Allen, 2003, Löffelhardt and Bohnert, 1994). Cyanobacteria are metabolically flexible as they can switch rapidly from one mode to another (Stal, 1995). They are also diverse in morphology and they show different forms in nature such as unicellular, filamentous, planktonic or benthic, and colonial (cocoid) ones (Lau et al., 2015). They colonize wide range of ecological niches and are widespread in various environments such as marine, freshwater, and terrestrials (Paerl et al., 2000, Whitton, 1992).

Cyanobacteria have powerful phototrophic metabolism to utilize CO_2 in the atmosphere and convert it into biomass and organic compounds (Pisciotta et al., 2010). Owing to their powerful phototrophic metabolism, intensive research on cyanobacteria is ongoing as they represent potential "cell factories" for generating renewable biofuels and other chemical compounds, driven by cellular photosynthesis. Examples on the application of cyanobacteria for the synthesis of industrial products including isoprene (Bentley et al., 2014), alk(a/e)nes (Wang et al., 2013b), fatty alcohol (Qi et al., 2013), isopropanol (Kusakabe et al., 2013), 1,2-propanediol (Li and Liao, 2013), sucrose (Xu et al., 2013), acetone (Zhou et al., 2012), 1-butanol (Lan and Liao, 2012), ethanol (Gao et al., 2012), fatty acids (Liu et al., 2011), isobutyraldehyde (Atsumi et al., 2009), and isobutanol (Atsumi et al., 2008) have been reported (Figure 2.4).

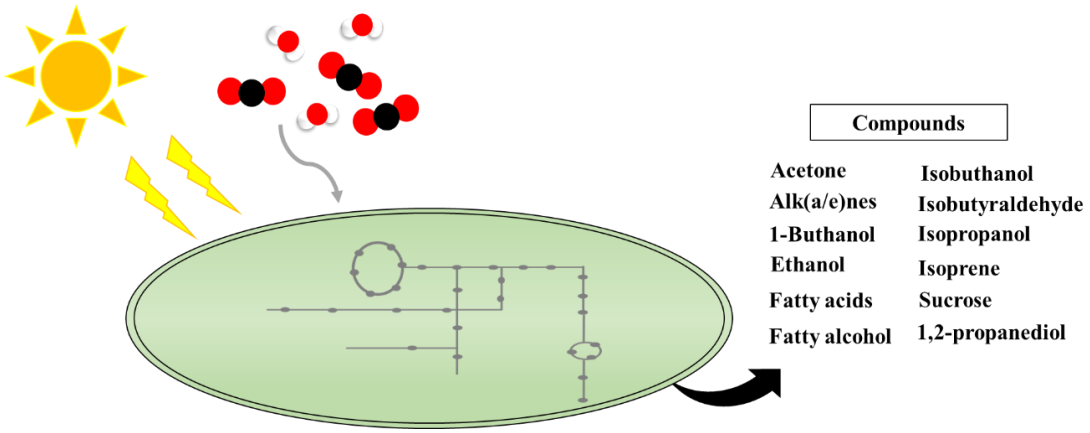

Figure 2.4 A schematic representation of various industrial compounds synthesized in cyanobacteria driven by cellular photosynthesis.

2.4.1 *Synechocystis* sp. strain PCC 6803

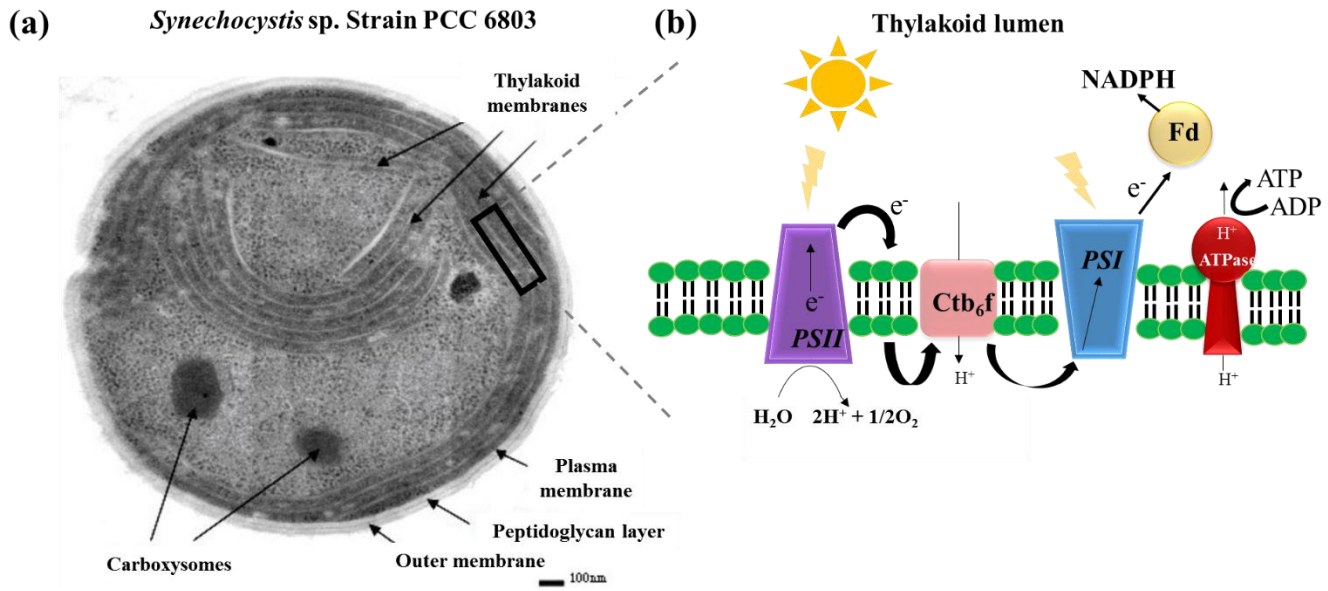

Figure 2.5 Overview of *Synechocystis* sp. organization. (a) Thin section electron micrograph of *Synechocystis* sp. cell to show the cell components as well as the thylakoid membrane system. Cell envelope of *Synechocystis* sp. is made up with plasma membrane, peptidoglycan layer, and outer membrane. Most of the thylakoid membranes are localized around the cell as concentric layers proximal to the plasma membrane (Liberton et al., 2006). (b) A schematic representation of the light-dependent electron transport chain in thylakoid lumen. *Synechocystis* sp. has an internal system of thylakoid membranes where electron transfer chain reactions of photosynthesis and respiration occur. Abbreviation: PSII: Photosystem II; Ctb_6f: Cytochrome b6/f; PSI: Photosystem I; Fd: Ferredoxin. (adapted from Wlodarczyk et al., 2016, Ducat et al., 2011, Knoop et al., 2010). Both photosystems PSI and PSII are major pigment-proteins complexes embedded in the thylakoid membranes (Barber, 1987). Photon energy drives water splitting, resulting in NADPH and ATP production which are necessary for carbon fixation and other cellular processes (Vermaas, 2001).

Among the diverse cyanobacterial species, *Synechocystis* sp. PCC 6803 (hereafter referred to as *Synechocystis* sp.) is the most-extensively studied and best-characterized strain, where its complete genome sequence, including four endogenous plasmids (pSYSM:120 kb, pSYSX:106 kb, pSYSA:103 kb, and pSYSG:44 kb) were sequenced and published (Kaneko et al., 2003, Kaneko et al., 1996). *Synechocystis* sp. is a non-nitrogen-fixing type unicellular cyanobacterium with multiple (6 to 12) copies of genomes, initially isolated from a California freshwater lake in 1968 (Yu et al., 2013, Matsui et al., 2007, Stanier et al., 1971). Although the *Synechocystis* sp. is originated from freshwater, it can also be cultivated in high salt conditions (Iijima et al., 2015) and could be found in biofilm near costal area (Gram et al., 2002). It has a spherical cell shaped (Liberton et al., 2006) and multiply by binary fission (Flores and Herrero, 2010). The overview of *Synechocystis* sp. organization are showed in figure 2.5.

Synechocystis sp. possesses several advantages as host for the synthesis of industrial products. Unlike heterotrophic bacteria, it only requires minimal nutrients to grow (*eg.* sunlight, water, carbon dioxide and minerals) (Lau et al., 2015). It is a fast-growing cyanobacterium with minimal doubling time of seven to eight hours (Angermayr et al., 2009). Most importantly, *Synechocystis* sp. is naturally competent. It has the ability to uptake the exogenous DNA spontaneously at high efficiency through homologous recombination into its genome, making it amenable to genetic manipulation (Kufryk et al., 2002, Vermaas, 1996, Williams, 1988). Another advantage is *Synechocystis* sp. has versatile carbon metabolism which can be cultivated under photoautotrophic, mixotrophic and heterotrophic conditions (Yu et al., 2013, Angermayr et al., 2009). Therefore, *Synechocystis* sp. is a representative model organism for basic and applied researches including mutagenesis, molecular biological and biophysical studies (Li and Sherman, 2000).

2.4.1(a) MEP pathway in *Synechocystis* sp. for terpenoids production

In *Synechocystis* sp., MEP pathway operates in the cytosol (Ershov et al., 2002) and it is the exclusive native route for terpeniods production (Figure 2.6). The presence of IPP isomerase provide both IPP and DMAPP which can be combined to form monoterpenes (C10), sesquiterpenes (C15), and diterpenes (C20) building blocks for terpenoids formation (Englund et al., 2015). The genes encoding enzymes involved in the MEP pathway that have been identified from the genome information of *Synechocystis* sp. are summarized in Table 2.1 (Pattanaik and Lindberg, 2015). Information on the *Synechocystis* sp. genome annotation and pathway mapping are available from KEGG (http://www.genome.jp/kegg-bin/show_organism?menu_type=pathway_maps&org=syn) and CyanoBase (http://genome.microbedb.jp/cyanobase/Synechocystis).