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Metabolic engineering of *Saccharomyces cerevisiae* for production of ginsenoside

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Abstract

Metabolic engineering of *Saccharomyces cerevisiae* for production of ginsenoside

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Ginsenoside is a unique tri-terpene secondary metabolite of genus *Panax* also known as popular bioactive components that help with various physiological phenomena and diverse pharmacological activities. Due to revealed efficacy for various diseases and disorders; anti-inflammation, hepatoprotection, tumor cell apoptosis, anti-aging, memory enhancing, cardiovascular enhancing and preventing metabolic disorders such as diabetes and obesity, ginsenosides considered as important ingredient for modern medicine. However, traditional farming-based ginsenoside production circumstance limited ginsenoside production qualitative and quantitative.

In this study, metabolic engineering of the yeast *Saccharomyces cerevisiae*

was used for the efficient production of skeletal terpenes of ginsenosides, dammarenediol-ii (DMD-ii) and protopanaxadiol (PPD).For the production of dammarenediol-ii and protopanaxadiol in *S. cerevisiae,* we introduced multiple heterologous genes; dammarenediol synthase (*DDS*), codon optimized protopanaxadiol synthase (*PPDS*) from *Panax ginseng* and NADPH-cytochrome P450 reductase from *Arabidopsis thaliana (ATR1).* Overexpression of truncated 3-hydroxy-3-methylglutaryl-CoA reductase (*tHMG1*), squalene synthase (*ERG9*), 2,3-oxidosqualene synthase (*ERG1*) genes were performed to increase the precursor flux by strengthening the innate mevalonate pathway. The genes lipid phosphate phosphatase (*LPP1*) and diacylglycerol pyrophosphate (*DPP1*) were deleted to construct *dpp1Δlpp1Δ* strain that prevented the transition of farnesyl pyrophosphate (FPP), the critical terpenoid precursor, to farnesol. The *dpp1Δlpp1Δ* strain produced 61.44 mg/L of DMD-ii or 25.12 mg/L of PPD from 20 g/L of glucose in minimal SC medium.

Finally, multiple copies of the gene *DDS1* were integrated into delta site of yeast chromosome to construct CEN.PK2-1C *dpp1Δlpp1Δ*δ::*DDS1* strain that produced 14.74 mg/L of DMD-ii from 20 g/L of glucose in SC medium without further gene manipulation.

Keywords: Saccharomyces cerevisiae, Metabolic engineering, Ginsenoside, Dammarenediol, Protopanaxadiol

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Chapter 1. Introduction

1. 1. Introduction outline

1. 1. 1. Ginsenosides

Ginsenosides, also known as saponin, are well-known bioactive components of ginseng. Ginsenosides, the terpenoid family second metabolite, are mostly found in the roots of plant genus *Panax*, ginseng, which is a popular medicinal plant and had been used as a core material for oriental medicine or a panacea in oriental cultures since ancient period [1]. Moreover, in current period, ginseng extract including ginsenosides are used commonly as a component of cosmetic products in beauty industry or as a material for restorative and functional food in health supplement industry.

Over the past decades, massive discovery of new species, genes, metabolic pathway and metabolites were found via updates of technology. Many drugs derived from natural products especially plant originated second metabolite are also being discovered. The plant secondary metabolite derived substance such as salicylic acid isolated from willow bark, Taxol isolated from taxus bark and artemisinin from Artemisia are commercially used as a pharmaceutical substrate, for anti-inflammatory, anti-tumor and anti-malarial drug respectively, and are treated as highly valued bio-chemical compounds [2-4].

Following the trend of medical and pharmacological field, ginsenoside are reconsidered as a valuable material for various applications of clinical drugs. Nowadays, from many researches, ginsenosides are proven as physiologically active substances by inducing anti-inflammation, hepatoprotection, tumor cell apoptosis,

inhibiting tumor cell proliferation, preventing metabolic disorders such as diabetes and obesity, and exhibiting diverse pharmacological effects on the central nervous, endocrine, cardiovascular and immune systems [5-8]. Moreover, multiple types of ginsenosides are already being used for clinical application. In China, ginsenoside Rg3 are used as the major component of anti- cancer and anti-tumor drugs. In South Korea, ginsenoside Rg3 and Rh2 are prescribed to cancer patients as supportive-care agent in form of ginseng dry extract [9].

The word “Ginsenoside” is a combination of two letters ‘ginseng’ and ‘glycoside’, ginsenoside (or panaxoside) is a natural glycoside, a steroid based substance with sugar moiety attached to the triterpene backbone, which is a terpenoid with three basic isoprenoid skeletons. Ginsenosides are classified in two types; the dammarane family and the oleanane family. The dammarane ginsenosides, the most popular ginsenoside group that accounts more than 90% of total ginsenoside kinds, consist of a four-ringed steroid like structure (Figure 1). Once again, dammarane family ginsenosides are classified in protopanaxadiol (PPD), protopanaxatriol (PPT) and ocotillol type. Since ocotillol type ginsenosides only exist in trace amounts, PPD and PPT type ginsenosides are the major ginsenoside types of dammarane family [5,10]. Dammarenediol-ii (DMD-ii) is basic precursor of PPD and PPT. PPD formed by DMD-ii bonding with two hydroxyl groups; carbon number 3 and 20 of DMD-ii bonding with hydroxyl groups for to form PPD, then, carbon number 6 of PPD bonding with hydroxyl groups for to form PPT (Figure 2). Finally, hydroxyl groups of each substrates replaced by various sugar moieties form various kinds of ginsenosides [10,11].

In ginseng, DMD-ii derived ginsenosides are biosynthesized through the mevalonate pathway. Acetyl-CoA, a product from glycolysis is a precursor of terpene, which is metabolized into acetoacetyl-CoA to 2, 3-oxidosqualene, mediated by the

enzymes involved in the mevalonate pathway. Then, 2, 3-oxidosqualene are transformed into DMD-ii by dammarenediol synthase. To synthesize PPD, the basic skeleton of ginsenoside, DMD-ii form hydroxyl groups on its 3rd and 20th carbon by protopanaxadiol synthase (PPDS), *CYP716A47*. Further, PPD are converted to PPT by protopanaxatriol synthase (PPTS), *CYP716A53v2*, via hydroxylation of the 6th carbon atom [10,12-14]. Moreover, since PPDS and PPTS are cytochrome P450 protein, a cytochrome P450 reductase is essential for PPDS to form hydroxylation of DMD-ii and PPD. Finally, sugar moieties attached to the carbon replace the hydroxyl groups, by glucuronidation activity of uridine 5'-diphospho-glucuronosyltransferase (UGT) [11].

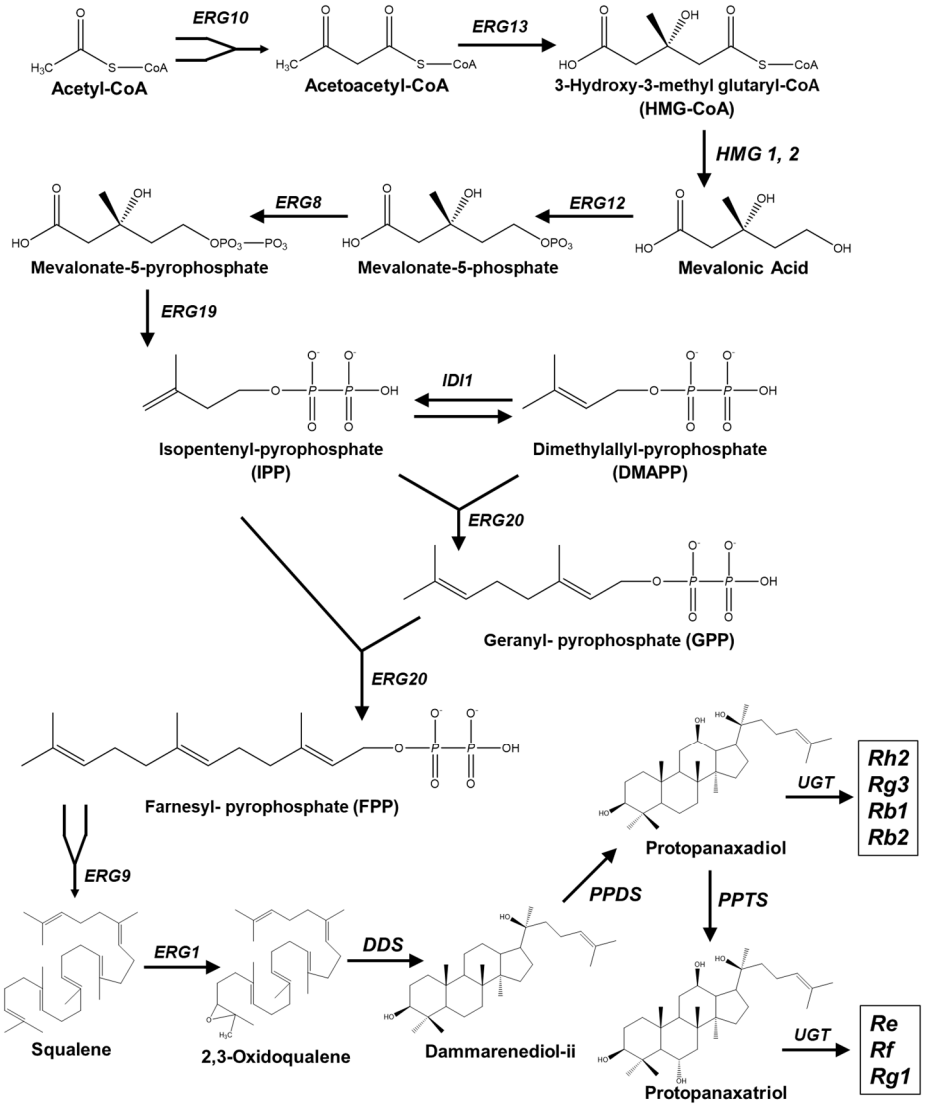
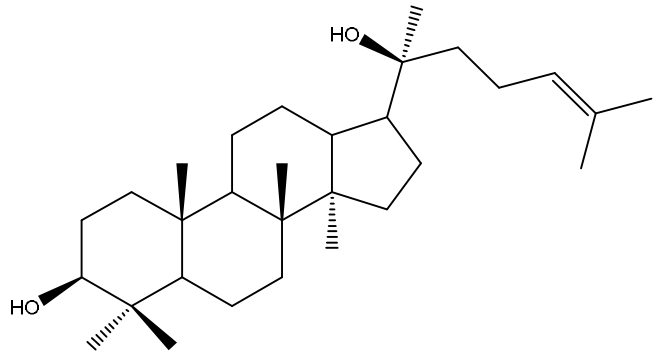


Figure 1. Biosynthesis pathway of dammarane family ginsenosides

A



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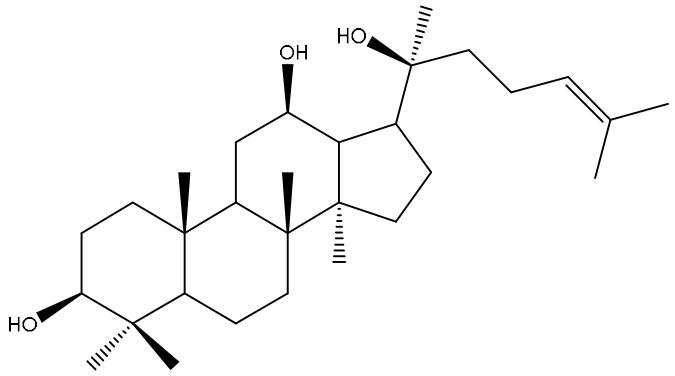


Figure 2. Structure of dammarenediol-ii and protopanaxadiol

A. Chemical structure of dammarenediol-ii ($C_{30}H_5O_2$)

B. Chemical structure of protopanaxadiol ($C_{30}H_5O_3$)

1. 1. 2. *Saccharomyces cerevisiae* as a platform for ginsenoside production

Mainly, ginsenoside products are manufactured directly from ginseng roots by pretreatment processes such as drying, extracting and purifying [1]. However, due to the long length of cultivation time required for the growth of commercially viable ginseng roots (5-7 yrs), long rotational farming terms for replantation of *panax* plants (≥ 5 yrs), labor intensive uncommon farming method and difficulty of separation of desire ginsenoside, the production of ginsenosides is still considered difficult fatigue [15]. Many health aid materials and pharmaceutical compounds are produced through chemical synthesis process. However, despite the advances in synthetic organic chemistry, it is not possible to A to Z chemically synthesize natural products in a technological and an economical manner. Synthesizing terpenoid, like ginsenosides, by chemical process involves a complex set of chemical reactions, materials, energy and, mostly, it is not very cost efficient [4,16].

Bakers' yeast, *Saccharomyces cerevisiae,* has been commonly used as a leavening agent in baking bakery products. It converts fermentable sugars that are present in dough into carbon dioxide and ethanol. In the case of a yeast, it has several advantages over other microbes. *S. cerevisiae* has a higher resistance ability to intermediate product of mevalonate pathway such as IPP and FPP than other industrial microorganism such as *Escherichia coli* [3,5]. There is a great potential for this yeast to be industrially utilized by controlling its metabolic engineering productivity. Because yeast is a eukaryotic micro-organism, it is well tolerated in industrial fermentation environments and grows well at low pH, which not only helps to prevent contamination during growth in industrial fermentation conditions, but also has the advantage of being free from infection by bacteriophages. Among many yeast

strains, genetical and physiological characteristics of *S. cerevisiae* have been studied. Thus, many genetic manipulation tools exist, which makes the construction of production system of plant terpenes such as ginsenoside possible. *S. cerevisiae* also shares a common mevalonate and fungi-sterol biosynthetic pathway from the precursor, acetyl-CoA. In addition, the production of ginsenosides require a process of hydroxylation step made by the membrane proteins cytochrome P450 enzymes. Since *S. cerevisiae* is a eukaryotic microorganism, membrane proteins like cytochrome P450 can be expressed or overexpressed through the endoplasmic reticulum [11,17].

1. 1. 3. Research trends of ginsenoside production

Since ginsenoside is a plant originated terpene, for most case, engineering approach for ginsenoside production relies on the chemical addition or the genetic modification of plant platforms; genus *Panax* plants cell or ginseng gene integrated fast growing commercial plants such as tobacco and rice. The study of Gao group showed increasing of total ginsenoside contents of *Panax ginseng* by 4.76 fold greater than control group after treating 10 mg/L of methyl jasmonate (MJ) during 24 hour culturing. Similarly, Peak group showed 2.9 fold increased ginsenoside contents from 25 days of 5 L bioreactor suspension culture of *P. ginseng* cells with daily treatment of 200 mg/L MJ from day 15 to end [18,19]. For plant platform other than *P. ginseng*, fast growing commercial plant Tobacco and rice were used. Choi Group of integrated *DDS* into transgenic tobacco using *tobacco mosaic virus* (TMV) and showed DMD-ii formation in tobacco about 20-30 μg/g DCW (dry cell weight) [20]. Later, they integrated *PPDS* and *UGT* gene to produce 1.55-2.64 μg/g DCW ginsenoside compound K in transgenic tobacco [21]. On the other hand, 83–115 μg/g DCW of oleanolic acid the precursor of oleanane family ginsenosides were produced in

transgenic rice named Taijing9 by Zheng group [22].

Engineering of microorganisms to produce heterologous molecules had been developed over decades. Instead of slow growing plant platform, few microbial were been used to produce ginsenosides. For the most industrial use microbial *E. coli*, several various attempts were tried. Lu group reconstituted DMD-ii biosynthetic pathway in *E.coli* by co-expression of squalene synthase, squalene epoxidase, NADPH-cytochrome P450 reductase from *S. cerevisiae*, and *Methylococcus capsulatus*, *A. thaliana* respectively. 8.63 mg/L DMD-ii was produced by this trial [23]. Other than producing ginsenoside form the bottom of scratch, most of tryouts in *E.coli* were converting crude ginsenoside extract to complete form or different form ginsenosides by expressing *β*-glucosidase from various origins. Im group showed that highly expressed β-glucosidase from *C. glutamicum* in *E. coli* converted ginseng extract including 50 g of PPD into 24.5 g of Rh_2 [24]. Oh group convert highly sugar moiety branched ginsenoside Rb1 and Re to low sugar moiety branched ginsenoside Rg3 and Rg2 with molar conversion yields of 100% using β-glucosidase of *Gordonia terrae* [25].

Production of ginsenoside by engineering of microorganisms mainly performed in yeast. Discoverer of *DDS*, Ebizuka group confirmed formation of DMD-ii in *S. cerevisiae* by expressing the plasmid pYES2□PNA harboring *DDS* in *S. cerevisiae* GIL77 strain. Formation of DMD-ii was confirmed by GC-MS and NMR analysis system but quantitative analysis was not performed [12]. Likewise, Choi group, first tested activity of PPDS and PPTS in yeast *S. cerevisiae* [13,14]. Since the performance of DDS, PPDS and PPTS was first test in yeast *S. cerevisiae* via plasmid expression, many metabolic engineering attempts were tried. Zheng group produced precursors of ginsenosides DMD-ii and PPD in yeast. The *DDS*, codon optimized *PPDS* (PPDSo) from *P. ginseng* and *CPR* from *A. thaliana (ATR1)* was introduced to

S. cerevisiae BY4742 strain via overexpressing within strong constitutive promoter plasmid. Further, ergosterol pathway of BY4742 was strengthen by overexpression of genes involved; gene of squalene synthase (*ERG9*), 2,3-oxidosqualene synthase (*ERG1*) and truncated 3-hydroxy-3-methylglutaryl-CoA reductase (*HMG1*), which form of *HMG1* not getting feedback inhibition of ergosterol, were overexpressed within strong constitutive promoter plasmid to construct ZD-PPD-018 strain. Engineered yeast strain ZD-PPD-018 produced 1189 mg/L (8.40 mg/g DCW) of PPD together with 1548 mg/L (10.94 mg/g DCW) of DMD-ii from 192 hr of 3 L reaction volume fed-batch fermentation in 5 L bioreactor using two phase fermentation technique [26]. Zheng group once again produced precursors of ginsenosides using yeast. β-amyrin synthase from *Glycyrrhiza glabra,* oleanolic acid synthase from *Medicago truncatula*, *DDS*, *PPDSo*, *PPTS* and *ATR1* was introduced to *S. cerevisiae* strain BY4742 and a *tHMG1, ERG9* and *ERG1* were overexpressed. Engineered yeast strain produced 17.2 mg/L PPD, 15.9 mg/L PPT and 21.4 mg/L Oleanolic acid via 168 hr of 100 mL batch flask culture [17]. Yuan group of Tianjin University obtained the highest PPD production. ZD-PPD-018 strain was used, but this time, *PPDSo* was modified through transmembrane domain truncation and connected with *ATR1* by amino acid linker, forming *PPDSo-ATR1* fusion protein. The fusion enzymes showed 4.5-fold increased catalytic activity, and 71.1% increased PPD production compared with *PPDSo* and *ATR1* co-expression. Engineered yeast strain with fusion enzyme produced 1489 mg/L of PPD from 192 hr of 3 L reaction volume fed-batch fermentation in 5 L bioreactor. Surprisingly, fusion of two enzyme conferred 96.8% of DMD-ii to PPD conversion which known as rate limiting step of ginsenoside production [27].

Table 1. Overview of study for ginsenoside production

Platform	Strategy	Production	Ref.
P. ginseng	10 mg/L of methyl jasmonate treatment	4.76 fold total ginsenoside	[18]
Tobacco	integrate dammarenediol synthase from *P. ginseng* by *Tobacco mosaic virus*	20-30 μg/gDW. DMD-ii	[20]
Rice (Taijing9)	Introduce *β*-amyrin synthase *Panax japonicus*	83–115 μg/gDW oleanolic acid	[22]
E. coli	Introduce dammarenediol synthase from *P. ginseng,* squalene synthase from *S. cerevisiae,* squalene epoxidase from *M. capsulatus* and *CPR* from *A. thaliana*	8.63 mg/L DMD-ii	[23]
S. cerevisiae BY4742	Integrate dammarenediol synthase, protopanaxadiol synthase (optimized) from *P. ginseng*, *CPR* from *A. thaliana* Overexpress squalene synthase, 2,3-oxidosqualene synthase, truncated 3-hydroxy-3-methylglutaryl-CoA reductase	1548 mg/L DMD-ii 1189 mg/L PPD	[26]
S. cerevisiae BY4742	Integrate dammarenediol synthase, protopanaxadiol synthase, *β*-amyrin synthase from *G. glabra*	17.2 mg/L PPD 15.9 mg/L PPT 21.4 mg/L OA	[17]
S. cerevisiae BY4742	Integrate dammarenediol synthase, Protopanaxadiol synthase (optimized) from *P. ginseng* fused with *CPR* from *A. thaliana.* Overexpress squalene synthase, 2,3-oxidosqualene synthase, truncated 3-hydroxy-3-methylglutaryl-CoA reductase	1489 mg/L of PPD	[27]

1. 2. Objective

Through technological advances, valuable but unknown species, metabolic pathways and metabolites have been discovered. Many plant derived second metabolites are newly discovered or are reevaluated for their performances. Ginsengs are well-known valuable medicine plant, known as restorative and oriental panacea. However, its various bioactive functions and pharmacological activities in human body are only now being discovered. Ginsenosides, tri-terpene secondary metabolite of ginseng, are key components of many beneficial bioactivities effects including anticancer, anti-aging and anti-diabetes. To overcome the time consuming, labor intensive and extraction and separation difficulties of ginsenoside production through farming, engineering for target ginsenoside production using fast growing microbial are preferred.

S. cerevisiae is a useful and favorable biological platform for plant terpenoid production due to many aadvantages. Although yeast *S. cerevisiae* has overlapping metabolic pathway with ginsenoside biosynthesis, it is not naturally producing ginsenosides or its skeletal structure DMD-ii and PPD. In this study, metabolic engineering approaches such as gene overexpression, deletion, integration and heterogeneous gene introduction were used to produce ginsenoside in *S. cerevisiae*.

The purpose of this study is to construct a yeast strain that can efficiently synthesize and enrich the productivity of DMD-ii and PPD through metabolic engineering tactics.

Chapter 2. Materials and methods

2. 1. Chemicals, strains and media

Bacto-Yeast extract, Bacto-peptone, Bacto-tyrptone were purchased form BD Bioscience (Franklin Lakes, NJ U.S.A), glucose was purchased from Affymetrix (Santa Clara, CA U.S.A), yeast nitrogen base without amino acids and amino acids were purchased from Sigma-Aldrich (St. Louis, MO U.S.A). DMD-ii authentic sample was purchased from CoreScience (Seoul, South Korea), and PPD authentic sample was purchased from Sigma-Aldrich (St. Louis, MO U.S.A)

In this study, all gene manipulation was done with *Escherichia coli* DH5α [F-Φ80*lacZ*ΔM15Δ(*lacZYA-arg*F) U169 *recA*1 *endA*1 *hsdR*17 (r_K^- m_K^+) *phoA supE*44λ^- *thi-1 gyrA96 relA1*]. Luria-Bertain (LB) medium (10 g/L typtone, 5 g/L yeast extract, 10 g/L NaCl) containing 50 μg/mL Ampicillin was used for *E.coli* culture.

Yeast *Saccharomyces cerevisiae* CEN.PK2-1C (*MATa ura3-52 trp1-289 leu2-3, 112 his3 1 MAL2-8^C SUC2*) strain obtained from EUROSCARF was used as parent strain throughout this study.

Yeast cells were cultured in YP medium (10 g/L yeast extract, 20 g/L peptone) supplemented with 20 g/L glucose (YPD), in synthetic complete (SC) medium (6.7 g/L yeast nitrogen base without amino acids, 1.4 g/L amino acid dropout mixture lacking His, Trp, Leu and Ura) supplemented with lacking auxotrophic amino acids and 20 g/L glucose, or in synthetic defined (SC-) medium (6.7 g/L yeast nitrogen base without amino acids, 1.4 g/L amino acid dropout mixture lacking His, Trp, Leu and Ura) supplemented with 20 g/L glucose and auxotrophic requirements (50 μg/L His, Trp, Ura and 120μg/mL Leu) for selection. All yeast cell experiments.

2. 2. *DPP1* and *LPP1* deletion and *DDS1* integration

The gene disruption mutants were constructed by using *loxP::URA3::loxP/Cre* recombination system. Gene deletion cassettes, *lpp1::URA3* cassette and *dpp1::URA3* cassette, were obtained by PCR from pUG72 as template, using a gene-specific primer of del_ORF_F, del_ORF_R. Obtained gene deletion cassette was integrated in to yeast cell by using Lithium acetate method, and SC- medium was used to select proper transformed yeast strain. Further confirmation of the correct integration of the cassette at the target gene locus through PCR analysis using the confirmation primers (c_ORF_F and c_ORF_R) was performed and the marker gene was removed by transformation of Cre recombinase-expression vector, pSH63.

The *dpp1Δlpp1Δ* double deletion strain was constructed by inserting *lpp1::URA3* cassette into *dpp1Δ* strain from above and marker was rescued by transform pSH63 Cre recombinase-expression vector.

For integration of *DDS1* in *lpp1* site, P_{TDH3}-*DDS1*-T_{CYC1}-*loxP-URA3-loxP* cassette amplified by PCR with del_*lpp1*_F and del_*lpp1*_R primer. Then amplified cassette was integrated in to yeast cell by using Lithium acetate method. The marker gene was removed by transformation of Cre recombinase-expression vector, pSH63. Complete of integration and rescue of maker was confirmed by auxotroph selection and PCR.

For delta-integration of *DDS1*, P_{TDH3}-*DDS1*-T_{CYC1} was cloned to delta6M. from constructed plasmid, delta2-P_{TDH3}-*DDS1*-T_{CYC1}-*loxP-kanMX–loxP*-delta1 cassette was amplified by PCR with delta_int_F and delta_int_R primer. Then amplified cassette was integrated in to yeast cell by using lithium acetate method with DMSO treatment. The complete of integration of cassette was confirmed by antibiotic, G418, selection

Table 2. List of strains used in this study.

Strain	Genotype	Reference
E. coli		
DH5α	F- Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17* (r_K^-, m_K^+) *phoA supE44* λ- *th*i-*1 gyrA96 relA1*	
S. cerevisiae		
CEN.PK2-1C	*MATa ura3-52 trp1-289 leu2-3,112 his3Δ1 MAL2-8^C SUC2*	EUROSCARF
dpp1Δlpp1Δ	CEN.PK2-1C::*dpp1Δ*::loxP *lpp1Δ*::loxP	This study
dpp1Δlpp1Δ::DDS1	CEN.PK2-1C::*dpp1Δ*::loxP *lpp1Δ*::P_{TDH3}-*DDS1*-T_{CYC1}	This study
dpp1Δlpp1Δ δ::DDS1	CEN.PK2-1C::*dpp1Δ*::loxP *lpp1Δ*::loxP *δ*::P_{TDH3}-*DDS1*-T_{CYC1}	This study

2. 3. Plasmids

Codon optimized P. ginseng C.A. Meyer CYP716A47 (PPDSo) for yeast and ATR1 were offered from professor Byung-Gee Kim (Seoul National University). Four DDS homologues; DDS1, DDS2, DDS3, DDS4, and CYP716A47 (PPDS1) , three CYP716A47 like genes; CYP716A47-2 (PPDS2), P450-2, P450-3 and ginseng originate CPRs; pgCPR1, pgCPR1a, pgCPR3, pgCPR5 selected from whole genome sequencing data of P. ginseng cultivar Chunpoong were offered from professor Tae-Jin Yang (Seoul National University).

All plasmids used and primers used for plasmid construction in this study listed in Table 3 and 4. To generate expression plasmid for yeast, ORFs of plant originate genes or mevalonate pathway genes were amplified by PCR and cloned in between restriction enzyme sites, listed on right side of their PCR primer list, of multi-cloning site on p413*GPD*, p414*GPD*, p415*GPD*, p416*GPD* plasmid which under control of *TDH3* promoter and *CYC1* terminator. *ERG9* was amplified by *ERG9*_F and *ERG9*_R primer from genomic DNA of *S. cerevisiae*. Amplified fragment was restricted by *Bam*HI and *Xho*I restriction enzyme and cloned into *Bam*HI and *Xho*I site of p414*GPD* to construct p414*GPD-ERR9* plasmid. *ERG1* was amplified by *ERG1*_F and *ERG1*_R primer from genomic DNA of *S. cerevisiae*. Amplified fragment was restricted by *Bam*HI and *Xho*I restriction enzyme and cloned into *Bam*HI and *Xho*I site of p416*GPD* to construct p416*GPD-ERR1* plasmid. t*HMG1* was amplified by t*HMG*_F and t*HMG*_R primer from genomic DNA of *S. cerevisiae*. Amplified fragment was restricted by *Bam*HI and *Xho*I restriction enzyme and cloned into *Bam*HI and *Xho*I site of p413*GPD* to construct p413*GPD*-t*HMG1* plasmid. For plant genes, *DDS1* was amplified by *DDS*_F and *DDS1*_R primer from plasmid harboring *DDS1* given by professor Yang. Amplified fragment was restricted by *Pst*I

and *Xho*I restriction enzyme and cloned into *Pst*I and *Xho*I site of p415*GPD* to construct p415*GPD-DDS1* plasmid. Similar method was used for constructing p415*GPD-DDS2,* p415*GPD-DDS3* and p415*GPD-DDS4. PPDSo* was amplified by *PPDS*_F and *PPDS*_R primer from plasmid harboring *PPDSo* given by Professor Kim. Amplified fragment was restricted by *Bam*HI and *Xho*I restriction enzyme and cloned into *Bam*HI and *Xho*I site of p414*GPD* to construct p414*GPD-PPDSo* plasmid. Similar method was used for constructing p414*GPD-PPDS1,* p414*GPD-PPDS2,* p414*GPD-P450-2* and p414*GPD-P450-3.ATR1* was amplified by *ATR1*_F and *ATR1*_R primer from plasmid harboring *ATR1* given by Professor Kim. Amplified fragment was restricted by *Bam*HI and *Xho*I restriction enzyme and cloned into *Bam*HI and *Xho*I site of p416*GPD* to construct p416*GPD-ATR1* plasmid. Similar method was used for constructing p416*GPD-pgCPR1,* p416*GPD-pgCPR1a,* p416*GPD-pgCPR3* and p416*GPD-pgCPR5.* All transformation of plasmid into yeast cell was performed using lithium acetate method with and without DMSO treatment.

Table 3. List of primer sequences used in this study.

Target DNA	Primer	Sequence	Restriction enzyme
Primers for deletion strain construction			
DPP1	del_dpp1_F	5'-ATGAACAGAGTTTCGTTTATTAAAACGC CTTTCAACATAG CAGCTGAAGCTTCGTAC GC -3'	
	del_dpp1_R	5'-TTACATACCTTCATCGGACAAAGGATGT AATTCC TCAT CCGCATAGGCCACTAGTGG AT -3'	
LPP1	del_lpp1_F	5'-ATGAACAGAGTTTCGTTTATTAAAACGC CTTTCAA CATAG CAGCTGAAGCTTCGTAC GC -3'	
	del_lpp1_R	5'-TTACATACCTTCATCGGACAAAGGATGT AATTCC TCAT CCGCATAGGCCACTAGTGG AT -3'	
Primers for deletion strain confirm			
DPP1	c_dpp1_F (+150)	5'-TAACATACGCGCAGTTCG -3'	
	c_dpp1_R (-150)	5'-CTTTCGTGTAAAGTGATGTTGGG-3'	
LPP1	c_lpp1_F (+200)	5'-CGTGGCTATTGCTCTAATTCAT-3'	
	c_lpp1_R (-200)	5'-CGACCAGATGAATCACATGTG-3'	
Primers for delta integration			
δ-site	delta_int_F	5'- TGAGAAATGGGTGAATGT -3'	
	delta_int_R	5'- TGTTGGAATAGAAATCAACTATC -3'	
Primers for plasmid construction			
DDS1	*DDS_F*	5'-GCGCTGCAGATGTGGAAGCTGAAGGTT-3'	*Pst*I
	DDS_R	5'-GCGCTCGAGTTAAATTTTGAGCTGCTGG TG-3'	*Xho*I
DDS2	*DDS_F*	5'-GCGCTGCAGATGTGGAAGCTGAAGGTT-3'	*Pst*I
	DDS_R	5'-GCGCTCGAGTTAAATTTTGAGCTGCTGG TG-3'	*Xho*I
DDS3	*DDS3_F*	5'-GCGACTAGTATGTGGAAGCTGAAGGTT-3'	*Spe*I
	DDS_R	5'-GCGCTCGAGTTAAATTTTGAGCTGCTGG TG-3'	*Xho*I
DDS4	*DDS4_F*	5'-GCGACTAGTATGTGGAAGCTAAAGATT-3'	*Spe*I
	DDS4_R	5'-GCGCTCGAGCTAAAACTTTGAACTAGACA-3'	*Xho*I
PPDSo	*PPDSo_F*	5'-GCGGGATCCATGGTGTTGTTTTTCTCC -3'	*Bam*HI
	PPDSo_R	5'-GCGCTCGAGTTAATTGTGGGGATGTAGA TG -3'	*Xho*I
CYP716A47 (PPDS1)	*PPDS1_F*	5'-GCGGGATCCATGAGGACTTTGGGACCC -3'	*Bam*HI
	PPDSo_R	5'-GCGCTCGAGTTAATTGTGGGGATGTAGA TG -3'	*Xho*I

CYP716A47-2 (PPDS2)	*PPDS2_F*	5’-GCGGGATCCATGAGAACTTTGGGACCC -3’	*Banm*HI
	PPDS_R	5’-GCGCTCGAGTTAATTGTGGGGATGTAGA TG -3’	*Xho*I
pgP450-2	*P450-2_F*	5’-GCGCTGCAGATGGATTATTTCCTCTACATT -3’	*Pst*I
	P450-2_R	5’-GCGGTCGACCTAGGGATTAGGGAAGAGG -3’	*Sal*I
pgP450-3	*P450-3_F*	5’-GCGGGATCCATGGAACTCTTCTATGTCC -3’	*Banm*HI
	P450-3_R	5’-GCGCTCGAGTTAGGCTTTGTGAGGAAAT-3’	*Xho*I
ATR1	*ATR1_F*	5’-GCGCCCGGGATGACTTCTGCTTTGTATG -3’	*Sma*I
	ATR1_R	5’-GCGGTCGACTCACCAGACATCTCTGAG-3’	*Sal*I
pgCPR1	*CPR 1_F*	5’- GCGGGATCCATGCTGAAAGTGTCTCCC -3’	*Bam*HI
	CPR1_R	5’-GCGCTCGAGTTACCATACATCACGCAG-3’	*Xho*I
pgCPR1a	*CPR 1_F*	5’- GCGGGATCCATGCTGAAAGTGTCTCCC -3’	*Bam*HI
	CPR1_R	5’-GCGCTCGAGTTACCATACATCACGCAG-3’	*Xho*I
pgCPR3	*CPR1_F*	5’-GCGGGATCCATGCTGAAAGTGTCTCCC -3’	*Bam*HI
	CRP3_R	5’-GCGCTCGAGTTACCATACATCACGCAG-3’	*Xho*I
pgCPR5	*CPR5_F*	5’-GCGGGATCCATGGCTGAAAGTTTGAAC -3’	*Bam*HI
	CPR5_R	5’-GCGCTCGAGTTACCATACATCCCGGAG -3’	*Xho*I
tHMG1	*tHMG_F*	5’-GCGGGATCCAAAAATGGACCAATTGGTGA AGACT -3’	*Bam*HI
	tHMG_R	5’-GCGCTCGAGTTAGGATTTAATGCAGGTG AC -3’	*Xho*I
ERG9	*ERG9_F*	5’-GCGGGATCCATGGGAAAGCTATTACAATTG-3’	*Bam*HI
	ERG9_R	5’-GCGCTCGAGTCACGCTCTGTGTAAAGT-3’	*Xho*I
ERG1	*ERG1_F*	5’-GCGGGATCCATGTCTGCTGTTAACGTTG -3’	*Bam*HI
	ERG1_R	5’-GCGCTCGAGTTAACCAATCAACTCACCAAAC -3’	*Xho*I

Table 4. List of plasmids used in this study.

Plasmid	Relevant characteristics
pUG72	DNA-template for amplification of *loxP-URA3-loxP* gene auxotrophic marker gene
INTpUG72M	DNA-template for amplification of MCS-*loxP-URA3-loxP* gene auxotrophic marker gene
pSH63	Plasmid containing the Cre-recombinase; *TRP1* marker gene
delta6M	DNA-template for amplification of *delta2*-MCS-*loxP-kanMX-loxP-delta1* gene antibiotic marker gene
p413*GPD*	Plasmid with *GPD* promoter and *CYC1* terminator; *HIS3* marker gene
p413TEF	Plasmid with TEF1 promoter and *CYC1* terminator; *HIS3* marker gene
p414*GPD*	Plasmid with *GPD* promoter and *CYC1* terminator; *TRP1* marker gene
p415*GPD*	Plasmid with *GPD* promoter and *CYC1* terminator; *LEU2* marker gene
p416*GPD*	Plasmid with *GPD* promoter and *CYC1* terminator; *URA3* marker gene
p414*GPD-PPDSo*	Plasmid with the cloned *PPDSo* gene of *P. ginseng* under control of *GPD* promoter and *CYC1* terminator, *TRP1* marker gene
p414*GPD-CYP716A47 (PPDS1)*	Plasmid with the cloned *CYP716A47* gene of *P. ginseng* under control of *GPD* promoter and *CYC1* terminator, *TRP1* marker gene
p414*GPD-CYP716A47-2 (PPDS2)*	Plasmid with the cloned *CYP716A47-2* gene of *P. ginseng* under control of *GPD* promoter and *CYC1* terminator, *TRP1* marker gene
p414*GPD-pgP450-2*	Plasmid with the cloned *pgP450-2* gene of *P. ginseng* under control of *GPD* promoter and *CYC1* terminator, *TRP1* marker gene
p414*GPD-pgP450-3*	Plasmid with the cloned *pgP450-3* gene of *P. ginseng* under control of *GPD* promoter and *CYC1* terminator, *TRP1* marker gene
p415*GPD-DDS1*	Plasmid with the cloned *DDS* gene of *P. ginseng* under control of *GPD* promoter and *CYC1* terminator, *LEU2* marker gene
p415*GPD-DDS2*	Plasmid with the cloned *DDS2* gene of *P. ginseng* under control of *GPD* promoter and *CYC1* terminator, *LEU2* marker gene

p415*GPD-DDS3*	Plasmid with the cloned *DDS3* gene of *P. ginseng* under control of *GPD* promoter and *CYC1* terminator, *LEU2* marker gene
p415*GPD-DDS4*	Plasmid with the cloned *DDS4* gene of *P. ginseng* under control of *GPD* promoter and *CYC1* terminator, *LEU2* marker gene
p416*GPD-ATR1*	Plasmid with the cloned *ATR1* gene of *A. thaliana* under control of *GPD* promoter and *CYC1* terminator, *URA3* marker gene
p416*GPD-pgCPR1*	Plasmid with the cloned *pgCPR1* gene of *P. ginseng* under control of *GPD* promoter and *CYC1* terminator, *URA3* marker gene
p416*GPD-pgCPR1a*	Plasmid with the cloned *pgCPR1a* gene of *P. ginseng* under control of *GPD* promoter and *CYC1* terminator, *URA3* marker gene
p416*GPD-pgCPR3*	Plasmid with the cloned *pgCPR3* gene of *P. ginseng* under control of *GPD* promoter and *CYC1* terminator, *URA3* marker gene
p416*GPD-pgCPR5*	Plasmid with the cloned *pgCPR5* gene of *P. ginseng* under control of *GPD* promoter and *CYC1* terminator, *URA3* marker gene

2. 3. Culture conditions and analytic methods

Culture condition

In this study, the transformed yeast was inoculated in a 5mL selective medium in 50 mL flask for first inoculation. Using first inoculant, yeasts were inoculated in 10 mL selective medium at optical density of OD_{600} = 0.2 using a 100 mL flask, and cultured at 30 ° C, 170 rpm. 4 mL cell cultured media was harvested after 120 hr culture. Cell pellet obtained by centrifuge was used for further analysis.

Sample extraction

From 4 mL cell cultured media, cell pellet was harvest by centrifugation 3,000 rpm 5 minutes. Cell pellet was treated with 1 mL of 3 N HCl in boiling water bath for 3 minutes and cooled in ice bath for 3 minutes. After centrifugation at 14,000 rpm for 1 minute, the precipitate was wash with 1 mL distilled water. After another centrifugation at 14,000 rpm for 1 minute, the precipitate was unpelleted in 1 mL methanol and vortex for 3 minutes, then centrifuged at 14,000 rpm for 5 minute. The supernatant of sample was obtained for further analysis.

Liquid chromatography

Sample was analyzed by HPLC, using UltiMate 3000 HPLC system (Thermo Scientific, Dionex) equipped with UltiMate 3000 UV/Vis detection system (Thermo Scientific, Dionex) and Agilent XDB-C-18 column (5 μm*, 4.6 * 250 mm) maintained at 30 ℃. The mobile phase consisted of 90 % acetonitrile consisting 0.1 % TFA and 10% water consisting 0.1 % TFA, flow rate of 0.6 mL/min. The UV wavelength detection was monitored at 203 nm absorbance.

Individual ginsenoside precursor, DMD-ii and PPD, was monitored and identified by

retention time and spectra peak comparison with its authentic sample. Quantification of each ginsenoside precursor was done by using responding calculated standard curves, the linear range points of each precursor were 10, 50, 100, 150, 200 mg/L and the regression correlation values (r^2) of curves were over 0.999 (Figure 3).

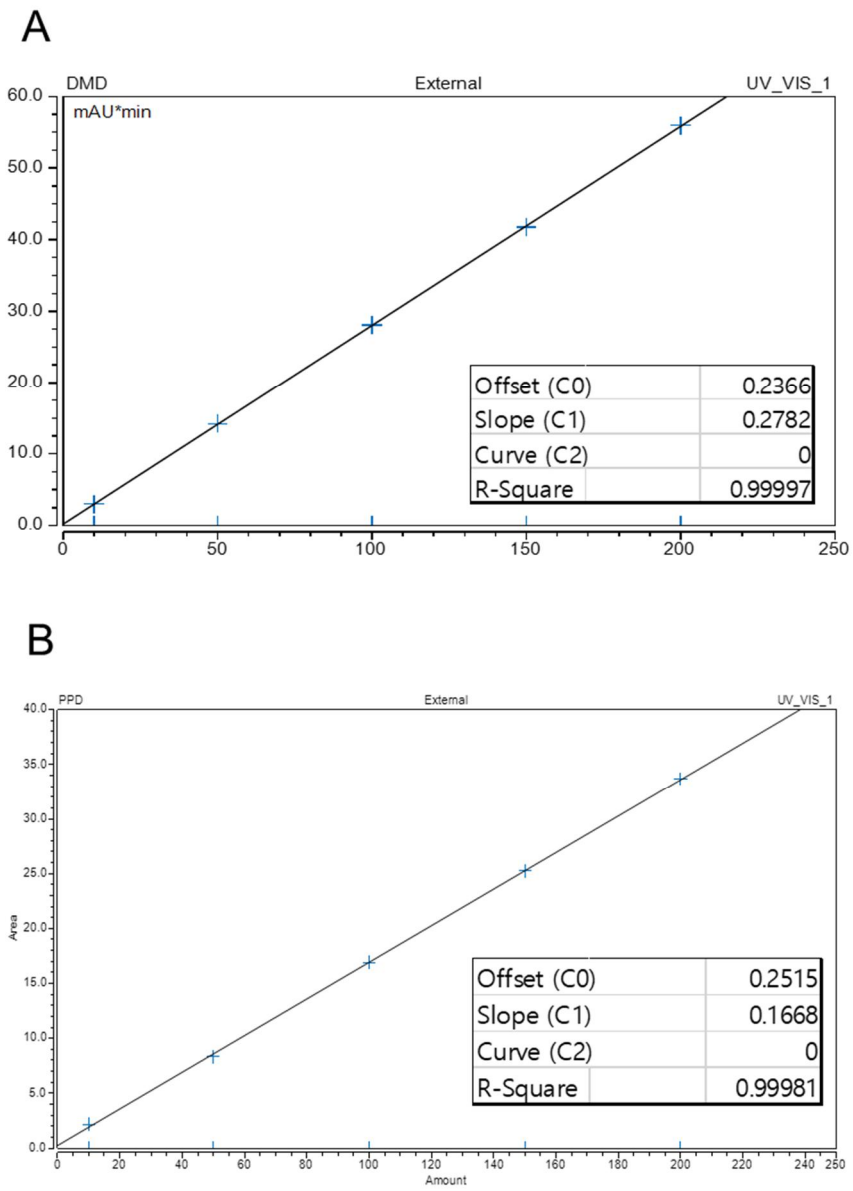


Figure 3. Standard curve of dammarenediol-ii and protopanaxadiol

A. Standard curve of dammarenediol-ii ($C_{30}H_5O_2$)

B. Standard curve of protopanaxadiol ($C_{30}H_5O_3$)

Chapter 3. Result and discussion

3. 1. Introduction of dammarenediol synthase

The dammarenediol-ii (DMD-ii) is the basic terpenoid skeleton for almost dammarane family ginsenosides. DMD-ii formation is done by dammarenediol synthase using 2, 3-oxidosqualene as precursor. Although *S. cerevisiae* has mevalonate/ergosterol pathway the innate metabolic pathway of yeast overlapping with ginsenoside biosynthesis pathway till 2, 3-oxidosqualene, the yeast itself cannot naturally conduct biosynthesis of DMD-ii. Therefore, to produce DMD-ii in *S. cerevisiae,* introduction of DDS is necessary. Four DDS homologues, which isolated and amplified directly from whole genome sequenced DNA of *P. ginseng,* have been given by Professor Yang (Figure 4).

The four DDS homologues were singly cloned in p415*GPD* plasmid, having *LEU2* auxotrophic marker, constructing p415*GPD-DDS1*, p415*GPD-DDS2*, p415*GPD-DDS3* and p415*GPD-DDS4*. The constructed plasmid transformed into wild type (WT) *S. cerevisiae* CEN.PK2-1C strain by lithium acetate method. For control group, p415*GPD* plasmid was transformed into CEN.PK2-1C *WT* strain. Then, transformed yeasts were cultivated in 10 mL SC-Leu media containing 2% glucose for 120 hr.

All transformed yeast showed similar cell density after 120 hr culture. Control group showed no production of DMD-ii. *DDS1* showed 16.47 mg/L and *DDS2* showed 12.56 mg/L of DMD-ii production. *DDS3* and *DDS4* did not showed production of DMD-ii (Figure 5). Since *DDS1* showed best production performance, *DDS1* was selected for further experiments.

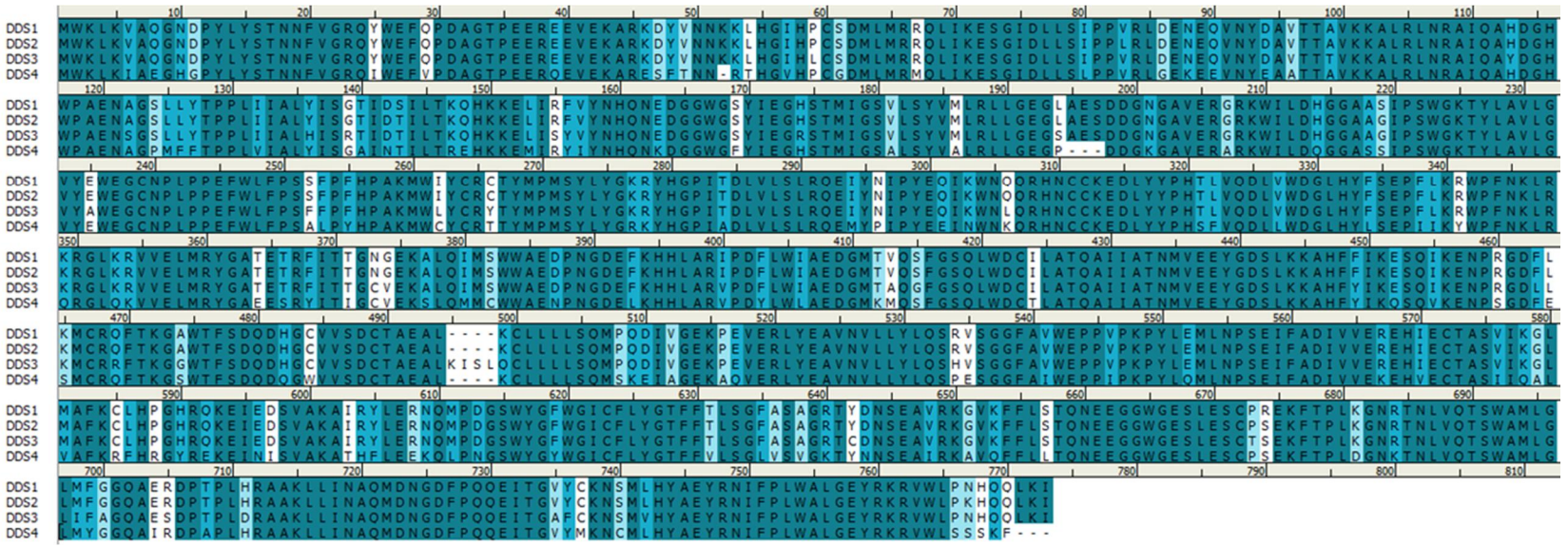


Figure 4. Protein sequence of dammarenediol synthase homologues

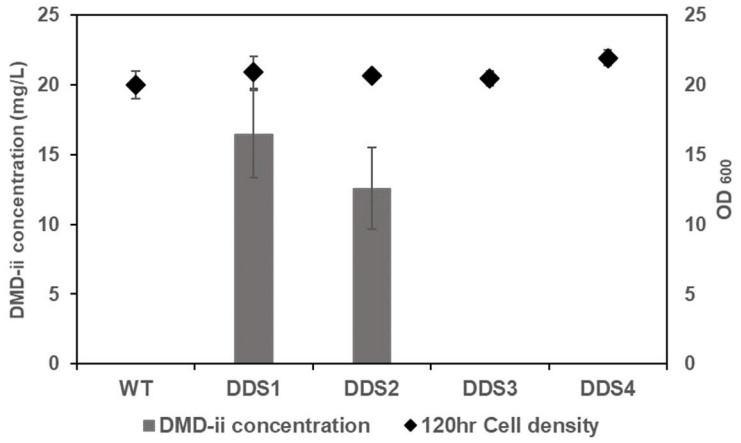


Figure 5. Dammarenediol-ii production by four dammarenediol synthase homologues in *S. cerevisiae*

3. 2. Overexpression of innate pathway genes: *ERG9*, *ERG1*, and *HMG1*

Since DMD-ii is made from 2, 3-oxidosqualene which is an intermediate substrate of mevalonate/ergosterol pathway of yeast, strengthening the innate pathway of yeast could be one necessary step of DMD-ii production using recombinant yeast. *ERG1* is the gene that coding squalene epoxidase (or monooxygenase) which synthesize 2, 3-oxidosqualene by catalyze the cyclization of squalene, and *ERG9* is coding squalene synthase that synthesize squalene by catalyze the condensation of 2 two farnesyl pyrophosphate (FPP). *ERG1* and *ERG9* were chosen to overexpress among many genes in ergosterol pathway. In the later part of mevalonate pathway, especially flux right after FPP formation, terpene precursors could be used for many various substrate synthesis and strengthening *ERG9* and *ERG1* would help the accumulation of 2, 3-oxidosqualene [26]. Among the genes in ergosterol pathway before mevalonate formation, *HMG1* was chosen for overexpression target. *HMG1* and *2*, coding 3-hydroxy-3-methylglutaryl-coenzyme A reductase, are well-known metabolic flux gate keeper of mevalonate pathway; *HMG1* genes are prone to feedback inhibition by ergosterol. To overcome this bottleneck limitation, truncated form of *HMG1* was introduced. The truncated form of *HMG1* (*tHMG1*) represent *HMG*1 enzyme that lacks the membrane-binding region, amino acids 1-552, preventing feedback inhibition from ergosterol [28].

tHMG1, ERG9, ERG1 were cloned into pRS413, pRS414 and pRS416 respectively. Each plasmid had auxotroph maker of *HIS3, TRP1 and URA3* respectively. *pRS413GPD-tHMG1, pRS4145GPD-ERG9 and pRS415GPD-ERG1* were constructed. Each of plasmids or combination of plasmids were transformed into *CEN.PK2-1C strain with pRS415GPD-DDS1* plasmid. Then, transformed yeasts were

cultivated in 10 mL SC-His, Trp, Leu , Ura media containing 2% dextrose for 120 hr.

DMD-ii production of *DDS1* expression alone was 15.24 mg/L. When each plasmid of *tHMG1, ERG9, ERG1* was overexpressed with *DDS1* 43.72 mg/L, 39.02 mg/L and 24.53 mg/L of DMD-ii was produced, respectively, which was 2.87 fold, 2.56 fold and 1.61 fold more than *DDS1* expression alone. In the case of combination of plasmids expressed, 49.85 mg/L DMD-ii was produced when *tHMG1 and ERG9* were co-expressed with *DDS1,* and 52.95 mg/L DMD-ii produced when all three *tHMG1, ERG9* and *ERG1* were co-expressed with *DDS1.* These were 3.27 and 3.47 fold increase in DMD-ii production than *DDS1* expressed alone. (Figure 6)

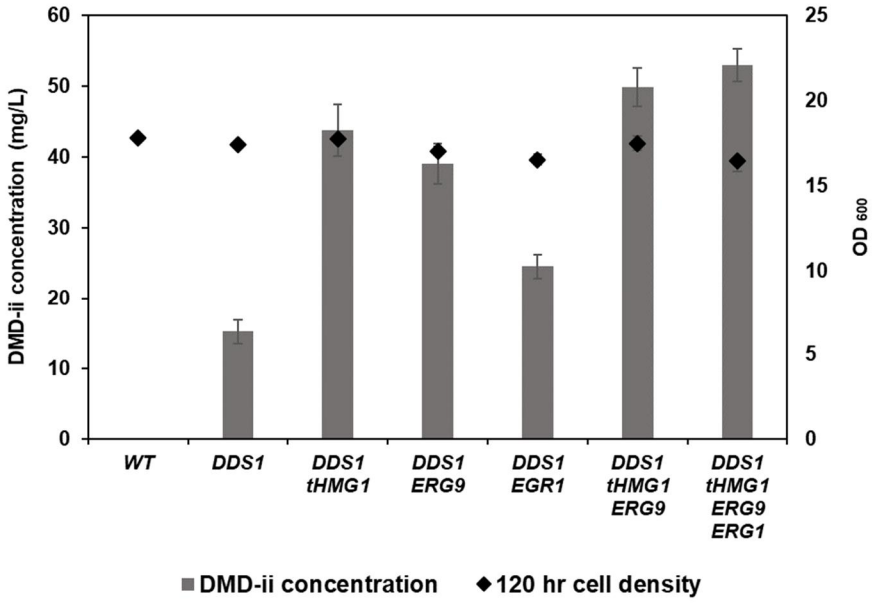


Figure 6. Dammarenediol-ii production by overexpression of innate mevalonate pathway genes of *S. cerevisiae.*

tHMG1: 3-hydroxy-3-methylglutaryl-coenzyme A reductase lack of amino acids 1-552, membrane-binding region

ERG9: Squalene synthase

ERG1: Squalene epoxidase

3. 3. Deletion of competitive pathway gene *DPP1* and *LPP1*

Strategy for strengthening the innate pathway of yeast other than overexpression of related genes was elimination or repression of competitive pathways. To dag metabolic flux toward 2,3-oxidosqualene, with overexpression tactic of latter part of mevalonate pathway genes, FPP using enzymes for other metabolism were attractive target for gene deletion. FPP is not only a precursor of ginsenoside or ergosterol but also a precursor of many metabolic products such as carotenoids, dolichol, heme A, lipid particle and Ubiquinone. FPP is also essential for squalene and 2, 3-oxidosqualene production, and therefore, accumulation of FPP by deletion of FPP-utilizing metabolic pathway that is nonessential to cell phenotypes and growth would help the production of ginsenoside precursors. Transformation of FPP to farnesol contributes to a large loss of essential precursor of ginsenoside production in *S. cerevisiae*. Down-regulation of the farnesol transformation could result in accumulation of FPP. Two genes in *S. cerevisiae*, *DPP1* and *LPP1* accounted for most of the hydrolytic activities FPP and either single or combined disruptions had no effect in growth or phenotypes [29].

DPP1 and *LPP1* were sequentially deleted from *S. cerevisiae* chromosome by using *loxP/Cre* recombination system using pUG72 plasmid and pSH63 plasmids having auxotrophic maker of *URA3* and *TRP1* respectively. *DPP1* and *LPP1* double deletion strain CEN.PK2-1C *dpp1Δlpp1Δ* was constructed. Then, plasmids harboring *DDS1* and genes involved in mevalonate pathway were transformed into the double deletion strain to produce DMD-ii. The transformed yeasts were cultivated in 10 mL SC-His, Trp, Leu, Ura media containing 2% dextrose for 120 hr.

Wild type CEN.PK2-1C is marked as WT, *DPP1* and *LPP1* double deletion strain was marked as *ΔΔ* and *ERG9*, *ERG1* and t*HMG*1 overexpression is marked as

MVA. WT and *ΔΔ* did not have much difference in cell density after 120 hr culture. *ΔΔ* expressing *DDS1* produced 20.31 mg/L DMD-ii that showed 33.3 % increased production compare to WT expressing *DDS1*, 15.24 mg/L. *ΔΔ* expressing *DDS1* and *MVA* produced 61.44 mg/L DMD-ii that showed 4.03 fold more production than WT expressing *DDS1* only and 16.0 % increased production compare to WT expressing *DDS1* with *MVA* (Figure 7).

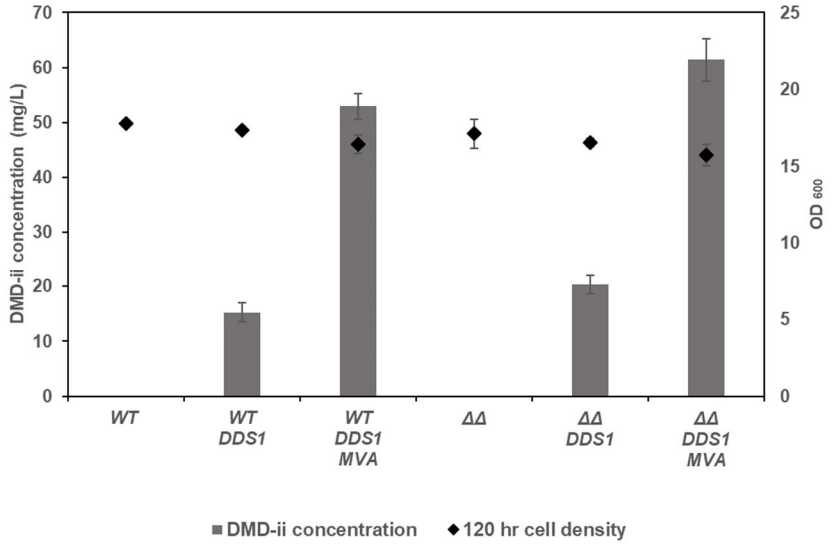


Figure 7. Dammarenediol-ii production of *dpp1Δlpp1Δ* strain compare to WT *S. cerevisiae.*

3. 4. Introduction of protopanaxadiol synthase

The PPD is another the basic skeleton for dammarane family ginsenosides made out of DMD-ii. Formation of PPD is carried out by *PPDS*, which conducts hydroxylation of the 3rd and 20th carbon atom on DMD-ii. Further, PPDS activation requires coupling with NADPH-cytochrome P450 reductase (*CPR*). Therefore, introduction of *DDS*, *PPDS* and *CPR* genes are basic components required for production PPD in *S. cerevisiae.*

Four *PPDS* homologues, which isolated directly from DNA of *P. ginseng* cultivar Chunpoong, have been given by Professor Yang, and codon optimized *PPDS,* original sequence from *P. ginseng* C.A. Meyer, has been given by Professor Kim (Figure 8). Codon optimization method is one way to increase protein expression by switching the codons used in a transgene without changing the amino acid sequence that it encodes for, because it removes and replaces rare codons with abundant codons. Four *PPDS* homologues; *PPDS1, PPDS2, P450-2, P450-3*, and codon optimized *PPDS, PPDSo,* were cloned into p414*GPD* plasmid. For *CPR* pair, *ATR1* was cloned into p416*GPD* plasmid constructing p416*GPD-ATR1*. Constructed p414*GPD-PPDS1*, p414*GPD-PPDS2,* p414*GPD-P450-2,* p414*GPD-P450-3* and p414*GPD-PPDSo* were transformed into CEN.PK2-1C *dpp1Δlpp1Δ* strain with p415*GPD-DDS1*, p416-*ATR1* and p413-MVA. Then, transformed double deletion strains were cultivated in 10 mL SC-His, Trp, Lue, Ura media containing 2% glucose for 120 hr.

For control, *ΔΔ* expressing empty plasmid which marked as control and *ΔΔ* expressing *DDS1* and MVA which marker as *DDS1* were used as comparison of DMD-ii production. All *PPDS*s were expressed with MVA, *DDS1* and *ATR1* in *ΔΔ* and marked as expressing *PPDS* name. Cell density of all *PPDS*s expressing yeasts did not show much difference from control group after 120 hr culture. Control group

showed no ginsenoside production and *DDS1* produced 62.42 mg/L of DMD-ii and no of PPD. Among all *PPDS*s, *PPDSo,* showed best production of ginsenoside, 25.12 mg/L of PPD together with 37.92 mg/L of DMD-ii. *PPDS1* and *PPDS2* showed 4.12 mg/L and 2.67 mg/L of PPD and 54.28 mg/L and 58.57 mg/L of DMD-ii production respectively. Since DMD-ii to PPD conversion was 1:1 ratio, compare *DDS1*, the amount of DMD-ii used to convert to PPD was reduced in case of *PPDSo, PPDS1* and *PPDS2*. On the other hand, *P450-2* and *P450-3* showed 61.75 mg/L and 60.52 mg/L DMD-ii production but only trace amount of PPD produced (Figure 9). As *PPDSo* showed best production performance, *PPDSo* was selected for further experiments.

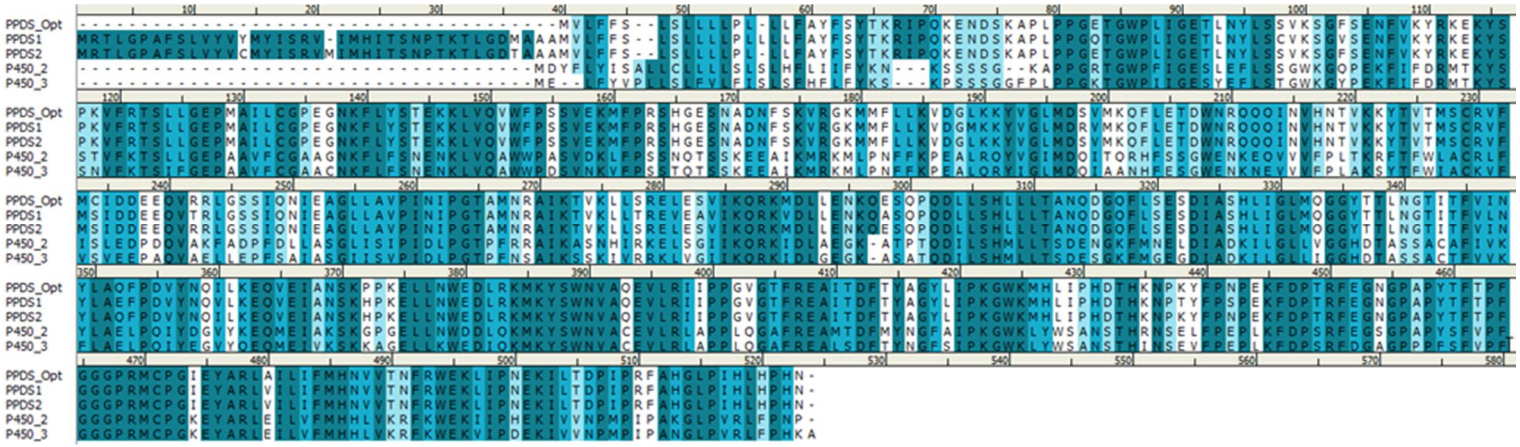


Figure 8. Protein sequence of protopanaxadiol synthase homologues and codon optimized protopanaxadiol synthase

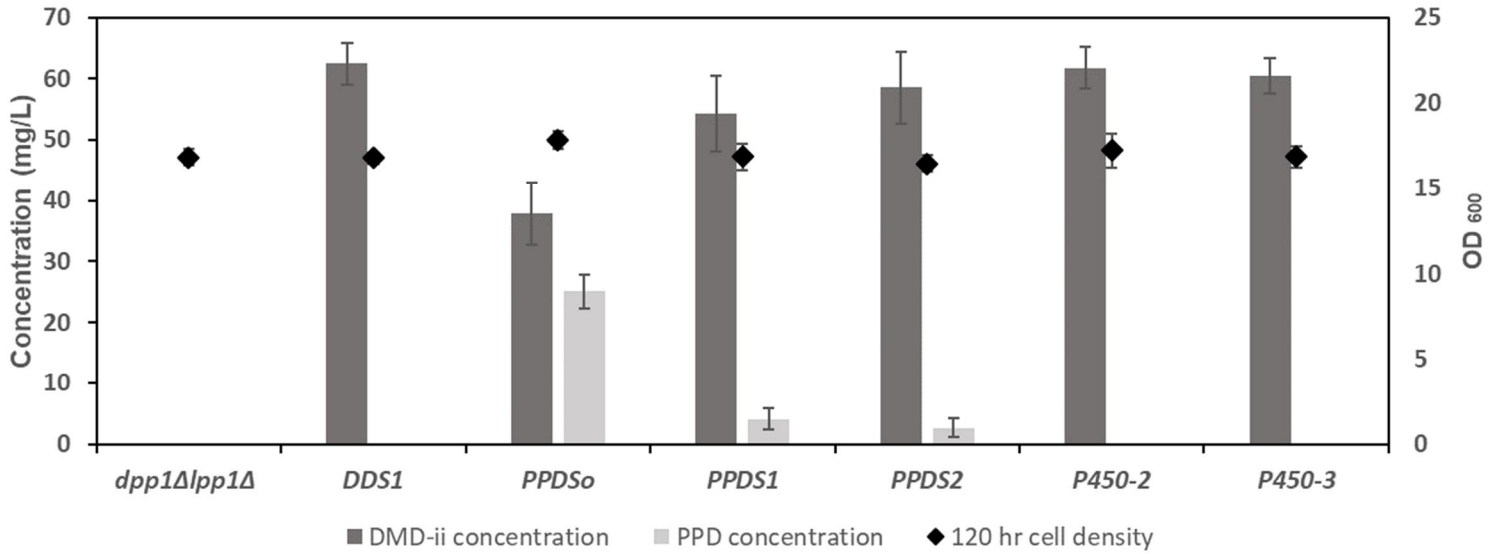


Figure 9. Protopanaxadiol and dammarenediol-ii production by protopanaxadiol synthases homologues in *S. cerevisiae.*

Protopanaxadiol and dammarenediol-ii production of each of *PPDS*s co-expressed with *ATR1*. *ΔΔ* represented empty plasmid expression in *ΔΔ* and DDS1 represented. *DDS1* and MVA expressed in *ΔΔ* as a control. *PPDSo*, *PPDS1*, *PPDS2*, *P-450-3* and *P-450-3* represented expression of each *PPDS*s with MVA, *DDS1*, *ATR1* in *ΔΔ*

3. 5. Comparison of NADPH-cytochrome P450 reductases

PPDS is cytochrome P450 membrane protein that requires coupling with *CPR* for enzymatic activity. *CPR* helps cytochrome P450 activity by donating electrons from the two-electron donor NADPH to the heme of P450. In previous experiment, *ATR1* was used as *CPR* pair, *CPR* of *A. thalinana* is commonly using plant *CPR* many researches due to its excellence in reduction activity for many kinds of plant cytochrome P450 enzymatic activities.

To confirm superior activity of *ATR1* paring with *PPDSo* for ginsenoside production, *ATR1* was compared with *CPR*s from *P. ginseng* for PPD production. Four *CPR* homologues, which isolated and amplified directly from whole genome sequenced DNA of *P. ginseng,* were been given by Professor Yang (Figure 10). Four *CPR* homologues; *pgCPR1*, *pgCPR1a*, *pgCPR3* and *pgCPR5* were cloned in p416*GPD* plasmid with *URA3* auxotrophic maker. The plasmid p416*GPD-pgCPR1*, p416*GPD-pgCPR1a,* p414*GPD-pgCPR3 and* p414*GPD-pgCPR5* were constructed. Constructed ginseng *CPR* plasmids and p416*GPD-ATR1,* previously constructed, were transformed into CEN.PK2-1C *dpp1Δlpp1Δ* strain with p415*GPD-DDS1*and p414*GPD-PPDSo* plasmids. Then, transformed yeasts were cultivated in 10 mL SC-Trp, Leu, Ura media containing 2% glucose for 120 hr.

For control group, *ΔΔ* expressing *DDS1*, marker as *DDS1*, was used. All *CPR* were expressed with *DDS1* and *PPDSo* in *ΔΔ* and marked as expressing *CPR* name. Cell density of all *CPR*s expressing yeasts showed not much deference than control group after 120 hr culture. *DDS1* produced 20.19 mg/L of DMD-ii and none of PPD. *ATR1* showed highest production of PPD and lowest DMD-ii production among all *CPR* trials. It produced 7.32 mg/L of PPD and 12.94 mg/L of DMD-ii. Ginseng *CPR*s; *pgCPR* 1, *pgCPR*1a, *pgCPR*3, *pgCPR* 5 produced 4.69 mg/L, 5.85

mg/L, 1.34 mg/L, 0.59 mg/L of PPD and 15.81 mg/L, 14.57 mg/L, 17.79 mg/L, 19.69 mg/L of D MD-ii respectively. Compare to control, *DDS1*, the amount of DMD-ii used to convert to PPD was reduced (Figure 11). *ATR1* was proved better reductase activity toward *PPDSo* than any other *CPR*s from ginseng.

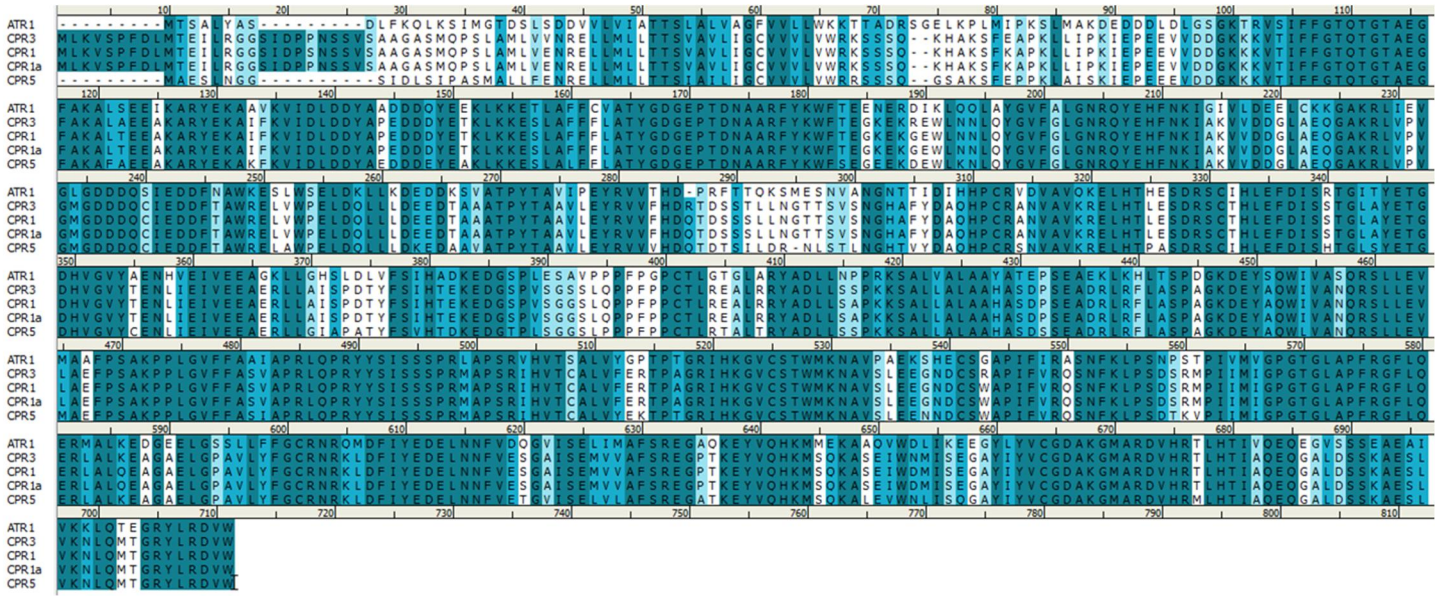


Figure 10. Protein sequence of NADPH-cytochrome P450 reductase homologues from *P. ginseng* and *A. thaliana*

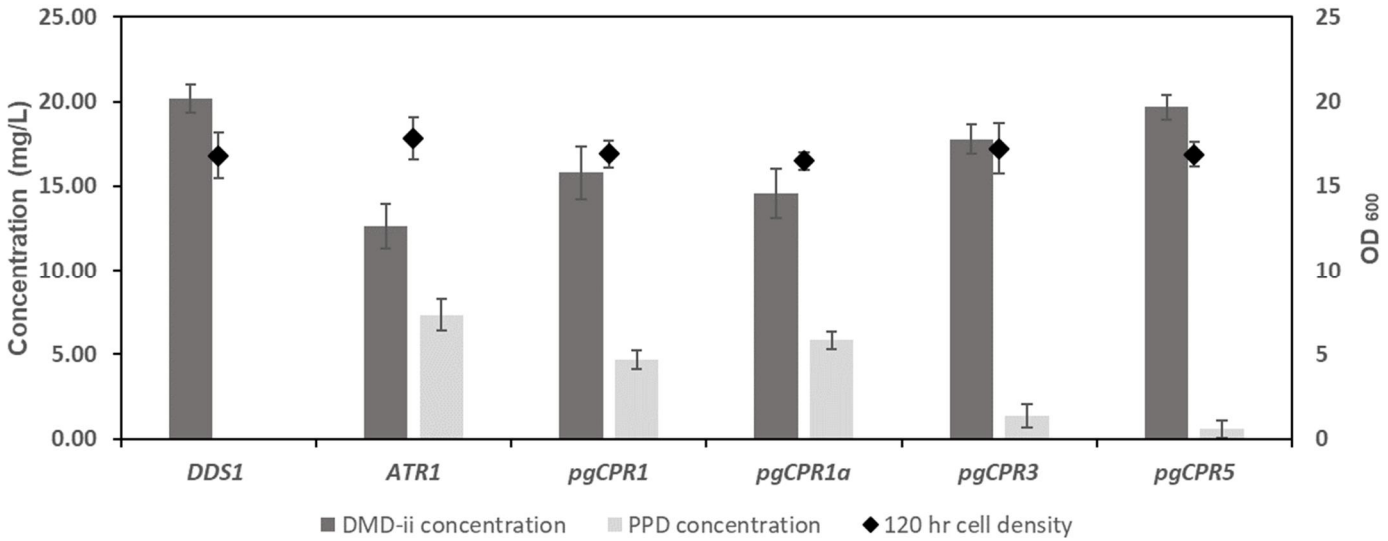


Figure 11. Effects of different CPR homologues in protopanaxadiol and dammarenediol-ii production in *S. cerevisiae.*

Protopanaxadiol and dammarenediol-ii production of each of *CPR*s co-expressed with *PPDSo*. DDS1 represented *DDS1* and MVA expressed in *ΔΔ* as a control. *ATR1, pgCPR1, pgCPR1a, pgCPR3* and *pgCPR5* represented expression of each *CPR*s with DDS1, *PPDSo* in *ΔΔ* (no MVA genes overexpressed)

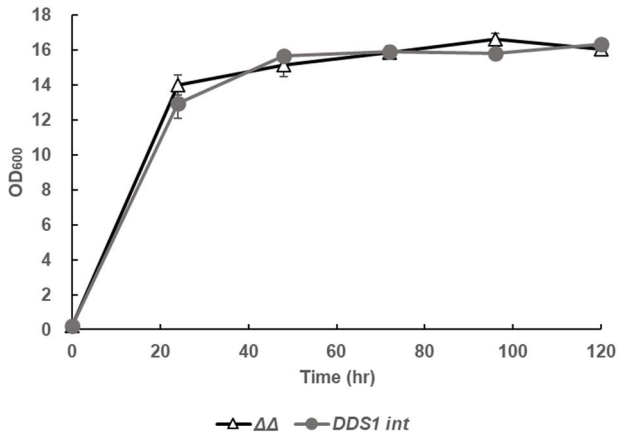
3. 6. Integration of dammarenediol synthase

3. 6 .1. Single-copy integration

For constitutive expression of ginsenoside biosynthesis gene, *DDS1* was integrated into yeast chromosome. P_{TDH3}-*DDS1*-T_{CYC1} was amplified from p415*GPD-DDS1* was cloned to INTpUG72M plasmid with URA3 auxotroph maker. The P_{TDH3}-*DDS1*-T_{CYC}-loxP-*URA3-loxP* cassette amplified by PCR with del_*lpp1*_F and del_*lpp1*_R primer designed to cassette targeting *LPP1* site in yeast chromosome. Then the P_{TDH3}-*DDS1*-T_{CYC}-*loxP-URA3-loxP* cassette integrated into CEN.PK2-1C *dpp1Δlpp1Δ* strain by lithium acetate method with DMSO treatment. Further, *URA3* maker was rescued by expressing pSH63, Cre protein gene containing plasmid. Complete of integration and rescue of maker was confirmed by auxotroph selection and PCR. Therefore, CEN.PK2-1C *dpp1Δ lpp1::DDS1* strain was obtained.

Obtained CEN.PK2-1C *dpp1Δ lpp1::DDS1* strain was cultivated in 10 mL SC media containing 2% glucose for 120 hr. For control group, CEN.PK2-1C *dpp1Δlpp1Δ*, marked as *ΔΔ,* was used and CEN.PK2-1C *dpp1Δ lpp1::DDS1* marked as *DDS1 int.* Cell growth of *DDS1*int during 120 hr showed not much difference with its parental strain. *DDS1*int produced 8.64 mg/L of DMD-ii, which was less than 0.5 fold of DMD-ii production using plasmid, 20.31 mg/L. (Figure 12)

A



B

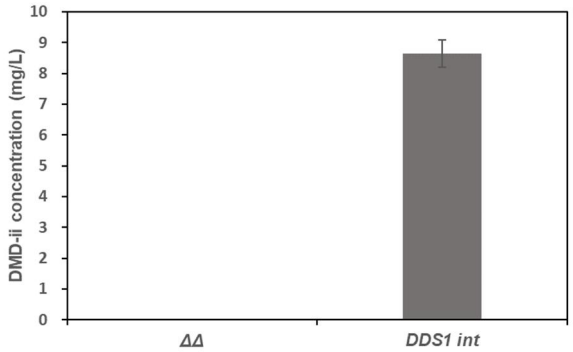


Figure 12. Growth and dammarenediol-ii production of CEN.PK2-1C *dpp1Δ lpp1::DDS1* strain

A: Growth curve of *ΔΔ* (CEN.PK2-1C *dpp1Δlpp1Δ)* and *DDS1 int* (CEN.PK2-1C *dpp1Δ lpp1::DDS1*) cultivated in 10 mL SC media containing 2% glucose for 120 hr.

B: Dammarenediol-ii production of *ΔΔ* and *DDS1 int* strain after 120hr cultivation.

3. 6. 2. Delta (δ) integration

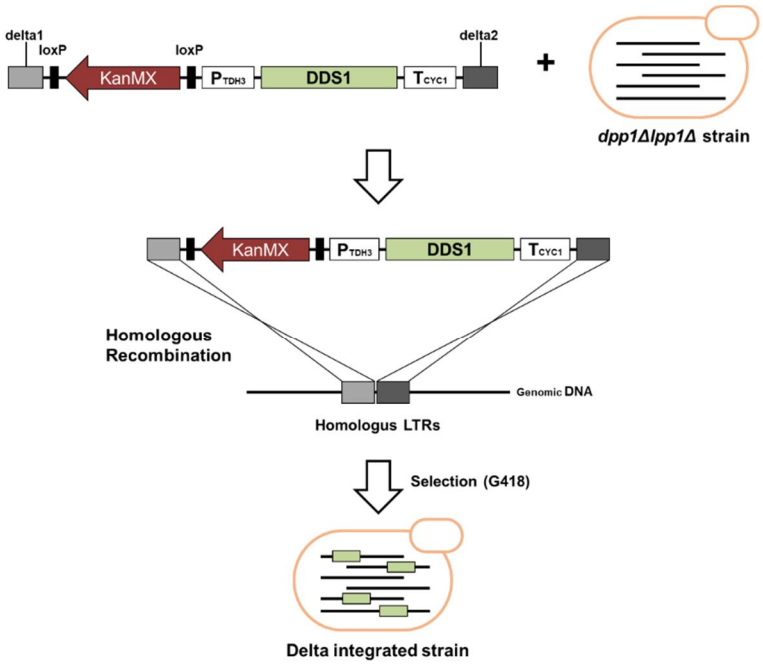
Since *DDS1* single copy integrated strain produced one half amount of DMD-ii compare to *DDS1* expression by plasmid, multi copy integration of *DDS1* became necessary for construction of constitutive DMD-ii producing strain. Therefore, *DDS1* was integrated into delta (δ) site of yeast chromosome. The δ sequence is site of sequences existing on long terminal direct repeat (LTR) sequence of the Ty element on *S. cerevisiae* chromosome. More than 100 copies of the δ sequence were distributed on the *S. cerevisiae* chromosome. Random number of multi copy genes could be introduced into yeast chromosome by using the δ sequence as a recombination site [30].

This time, P_{TDH3}-*DDS1*-T_{CYC1} was cloned to delta6M plasmid with *kanMX* antibiotic maker. The delta-P_{TDH3}-*DDS1*-T_{CYC1}-*loxP-kanMX–loxP*-delta1 cassette was amplified by PCR with delta_int_F and delta_int_R primer designed to targeting δ sequence in yeast chromosome. Then the cassette integrated into CEN.PK2-1C *dpp1Δlpp1Δ* strain by lithium acetate method with DMSO treatment. The completion of integration was confirmed by antibiotic, G418 (2,000 μg/mL) selection (Figure 13 A). Since random copy number of cassette, selection of the most DMD-ii production strain was necessary. The 24 colonies from selection plate were randomly picked and cultivated in 10 mL SC media containing 2% glucose for 120 hr. The 24 colonies produced 0 - 12.34 mg/L of DMD-ii after 120 hr culture (Figure 13 B).

The 5 most DMD-ii production colonies, marked as δ-5, δ-13, δ-17 and δ-22, were selected then repeated 120 hr cultivation experiment for confirmation of DMD-ii production. Cell growth of selected strains were similar to their parental strain, *ΔΔ*. DMD-ii production of δ-5, δ-13, δ-17 and δ-22 were 5.65 mg/L, 14.74 mg/L, 5.58 mg/L and 4.62 mg/L, respectively (Figure 14). Finally, the most DMD-ii

production strain, CEN.PK2-1C *dpp1Δlpp1Δ* δ::*DDS1*_13 (δ-13) was selected.

A



B

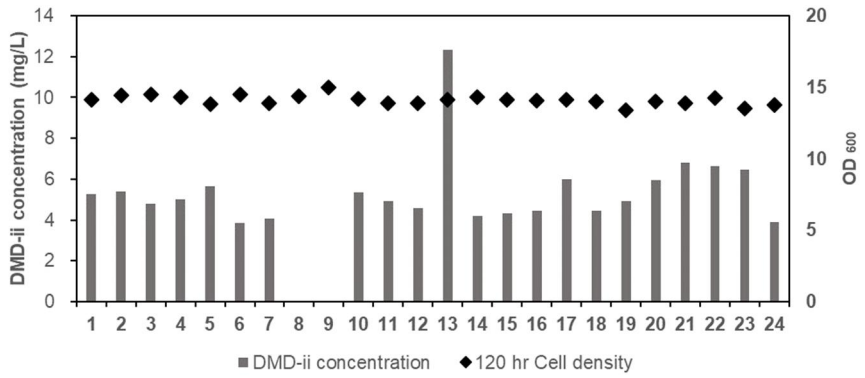
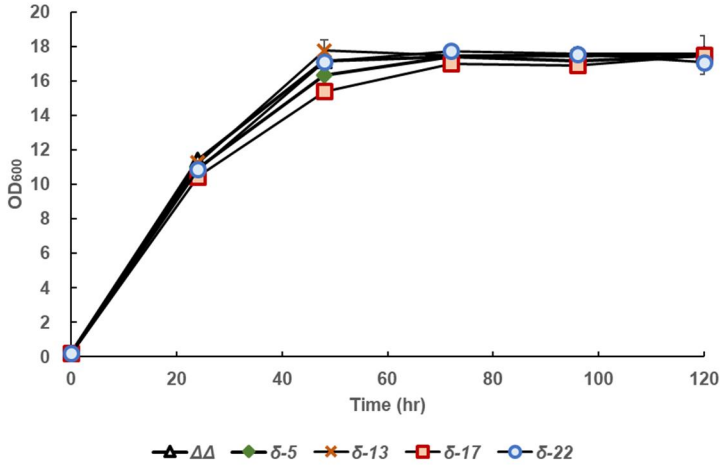


Figure 13. Construction and DMD-ii production of delta (δ) integration strain CEN.PK2-1C *dpp1Δ lpp1Δ* δ::*DDS1*

A: Diagram of delta (δ) integration of *DDS1*

B: Dammarenediol-ii production of 24 CEN.PK2-1C *dpp1Δ lpp1Δ* δ::*DDS1* strains after 120hr cultivation.

A



B

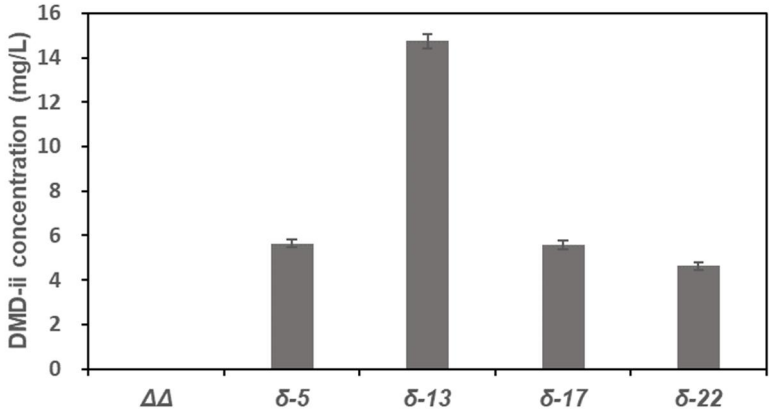


Figure 14. Growth and dammarenediol-ii production in 5 DMD-ii producing CEN.PK2-1C *dpp1Δ lpp1Δ* δ::*DDS1* strains

A. Growth curve of selected 5 DMD-ii producing strains (δ-5, δ-13, δ-17 and δ-22)

B. Dammarenediol-ii production of selected 5 DMD-ii producing strains

Chapter 4. Conclusion

Ginsenoside is the major bioactive component of well-known medicine plant, ginseng. As specific bioactive effects and mechanisms in various parts of human body are revealed, ginsenoside is considered as a strong candidate for modern medicine ingredient for many diseases especially in cancer and tumor treatment. However, due to show growth and labor-intensive circumstances of ginseng farming, alternative methods to produce ginsenoside have been studied. Therefore, the study of producing ginsenoside using fast and easy growing microbial organism is a more practical alternative.

Yeasts do not naturally produce ginsenoside, but have an advantage of having innate mevalonate pathway to produce sterols, which shares many enzymatic processes involved with ginsenoside biosynthesis in ginseng. Compared to *E. coli*, a common industrial micro-organism, yeast has a higher tolerance to mevalonate pathway intermediate such as FPP and IPP. Yeast, as a eukaryotic microorganism, can easily express heterogeneous genes of other eukaryotic organism like plants and has endoplasmic reticulum that can induce enzymatic activity of membrane proteins. Therefore, studies on the production of ginsenoside through yeast as microorganism platform are constantly on the rise. This study also aims to produce ginsenosides efficiently by using metabolic engineering methods in *S. cerevisiae*.

Very basic dammarane family ginsenoside skeleton, dammarenediol-ii is made out of 2, 3-oxidosqualene by the activity of dammarenediol synthase. Four genes assumed as dammarenediol synthase were individually introduced into *S. cerevisiae* strain CEN.PK2-1C. *DDS1* produced 16.47 mg/L dammarenediol-ii which showed the highest production among the four candidates.

In addition, the innate sterol pathway of *S. cerevisiae* was strengthened by overexpressing *ERG9*, *ERG1*, which are involved in the latter part of mevalonate pathway, and *tHMG*1, the truncated form of *HMG*1 to prevent feedback inhibition to increase the production of ginsenoside. Single gene overexpression of these innate genes using strong constitutive promoter was increased 2.56, 1.61 and 2.87 fold of ginsenoside production, respectively. When all three genes were over expressed, 52.95 mg/L of dammarenediol-ii was produced, which was 3.47 fold more production than when *DDS1* was expressed alone.

Deletion of competitive metabolic pathway also increased the ginsenoside productivity. The pathway involved in converting FPP to farnesol was repressed by deletion of *DPP1* and *LPP1* gene, which are known as major responsible oxidizers of isoprenoid intermediates with phosphate. Expression of *DDS1* in *DPP1* and *LPP1* mutant strain produced 20.31 mg/L of dammarenediol-ii which was a 33.3 % increase in production compare to WT. When *ERG9, ERG1* and *tHMG*1 were overexpressed in double deletion strain 61.44 mg/L of dammarenediol-ii was produced, which was 4.03 fold increase than WT expressing *DDS1* only.

Another form of ginsenoside skeletal terpenoid, protopanaxadiol was produced in yeast by introducing protopanaxadiol synthase, NADPH-cytochrome P450 reductase and *DDS1*. Five protopanaxadiol synthase gene candidates were singly introduced into *S. cerevisiae DPP1* and *LPP1* deletion strain with *DDS1*, *ERG9, ERG1*, *tHMG1* and *ATR1* the NADPH-cytochrome P450 reductase from *A. thaliana.* Among the five protopanaxadiol synthase candidates, codon optimized protopanaxadiol synthase produced the highest protopanaxadiol production of 25.12 mg/L. Further, *ATR1* confirmed the best reductase activity among all other reductases from *P. ginseng.*

Finally, ginsenoside production gene was integrated into the chromosome. First, single copy of *DDS1* with strong constitutive promoter and terminator combination was integrated into *LPP1* site. However, this strain produced 8.64 mg/L of dammarenediol-ii, which was less than 0.5 fold of DMD-ii production using plasmid expression. Therefore, random numbered multi-copy integration of *DDS1* was performed by the delta-integration method. Final obtained strain CEN.PK2-1C *dpp1Δlpp1Δδ*::*DDS1*_13 produced 14.74 mg/L without further gene manipulation

In this study, few types of engineering tactics were used to produce unique substances of ginseng by *S. cerevisiae*. This study also demonstrates the viability of producing ginsenosides by methods besides traditional agriculture-based method. This study also demonstrates that production of ginsenoside in yeast is fast and efficient. Also, the best production amount of ginsenoside of this study was 25.12 mg/L PPD together with 37.92 mg/L DMD-ii, which could converted into 6.78 mg/g DCW of PPD together with 10.23 mg/g DCW of DMD-ii. Compare to world record of Zheng group, 8.40 mg/g DCW (1189 mg/L) of PPD together with 10.94 mg/g DCW (1548 mg/L) of DMD-ii [26], the production of PPD and DMD-ii from this study was not very below in ginsenoside per dry cell weight wise.

To increase the efficiency even further, additional metabolic engineering strategies such as downregulation of the another main competitive pathway such as *ERG7*, overexpression of genes related to acetyl-CoA formation, gene manipulation for co-factor balancing or transcriptional factors and introduction of UGT genes can be applied to expand the range of ginsenoside production in *S. cerevisiae*.

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국문 초록

인삼속 식물에서 주로 많이 발견되는 진세노사이드 (ginsenoside) 는 인삼 고유의 이차대사물질로서 세 개의 이소프레노이드 (isoprenoid)가 합쳐진 트리터펜 (triterpene)의 구조를 가지고있다. 인삼의 다양한 생리활성과 약학적 효과는 진세노사이드로부터 비롯된다.

진세노사이드를 생산하기 위해서는 6년 이상 키운 인삼의 뿌리를 가공하여 정제하는 방법이 가장 일반적이다. 농업을 기반한 기존의 방법은 노동 집약적이고 오랜 작물 재배 기간이 필요하거나 원하는 종류의 진세노사이드를 정제하기 어렵다는 단점이 있다. 이러한 단점을 극복하기 위해 산업용 미생물 생산 균주인 *S. cerevisiae*를 사용하여 진세노사이드를 생산하고자 하였다.

본 연구에서는 천풍삼 유래의 dammarenediol synthase의 유전자, *DDS* 를 효모 내부로 도입하여 dammarenediol-ii를 생산하는 균주를 제작하였고, 추가적으로 효모의 codon usage 로 optimize된 고려인삼 유래의 protopanaxadiol synthase 유전자, *PPDSopt* 와 *A. thaliana* 유래의 NADPH-cytochrome P450 reductase의 유전자, *ATR1*을 도입하여 protopanaxadiol도 생산할 수 있는 균주를 제작하였다. 또한 효모 본래의 squalene synthase를 암호화 하는 *ERG9* 유전자, squalene epoxidase를 암호화 하는 *ERG1* 유전자, 그리고 피드백 저해를 받지 않도록 막 통과 부분이 잘려 나간 형태의 3-hydroxy-3-methylglutaryl-CoA reductase를 암호화 하는 *tHMG1* 유전자를 과발현 하였고, 추가적으로 경쟁 경로의 유전자인 *DPP1* 과 *LPP1*을 제거하여 진세노사이드의 전구 물

질을 생산하는 메발산 경로의 강화를 시도 하였다

형질 전환된 효모를 2% 포도당을 포함한 배지에서 배양한 결과 61.44 mg/L 의 dammarenediol-ii를 생산 하였고, 다른 한편으로는 25.12 mg/L (6.78 mg/g DCW) 의 protopanaxadiol 와 37.92 mg/L (10.23 mg/g DCW) 의 dammarenediol-ii 을 함께 생산할 수 있었다.

더 나아가, dammarenediol synthase 를 효모 유전자의 delta site에 삽입한 균주를 제작, 2% 포도당을 포함한 배지에서 배양하여 14.74 mg/L dammarenediol-ii 을 생산하였다.

주요어: Saccharomyces cerevisiae, Metabolic engineering, Ginsenoside, Dammarenediol, Protopanaxadiol

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