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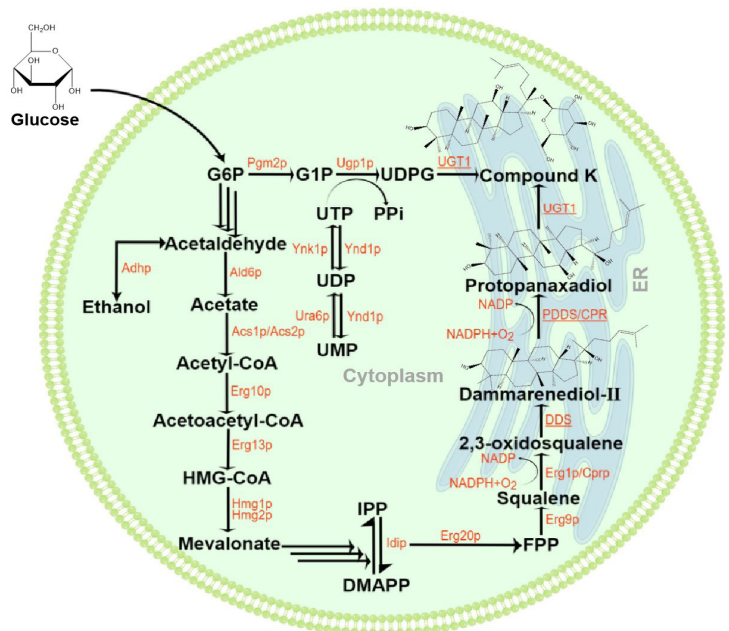

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Synthesis of ginsenoside triterpenoids by engineered *Saccharomyces cerevisiae*

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vorgelegt von

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Shangkun Qiu

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Table of contents

Summary

Ginseng is a traditional plant in Chinese medicine because it includes pharmaceutical ginsenosides that have been used in Asia for thousands of years. Ginsenosides are triterpenoids, a class of natural products mainly present in *Panax* species (e.g., *Panax ginseng*, *P. notoginseng*, and *P. quinquifolium*). However, due to the shortcomings of chemical synthesis and extraction from plants, the synthesis of ginsenosides by microbial cell factories, which has the advantages of environmental friendliness, high efficiency, convenience, and potentially low cost, has received much attention in recent years.

The present study had three goals: 1. Analyze current challenges for the ginsenosides synthesis by yeast. 2. Optimize DoE-based medium and fermentation conditions for improved protopanaxadiol production. 3. Further enhancement of ginsenoside production by in-situ extraction.

In some detail, we designed the DoE experiment by Design Expert 11 (Stat-Ease, Inc.) and analyzed the final result by ordinary least squares (OLS) estimation. It showed that glucose, glutamate, salts, temperature, and oxygen play a crucial role in influencing final protopanaxadiol production. The five parameters were investigated during fermentation to improve the final protopanaxadiol production. Finally, we have got an optimized M3 medium, which vastly increases the final protopanaxadiol production to 1.2 g/L in shake flasks with a membrane lid at 28 °C, 8-fold compared to the original fermentation condition. This M3 medium also works for other engineered strains that produce other triterpenoids (e.g., betulin, and betulinic acid). Because some ethanol evaporated during the fermentation, we tried to replenish the evaporation by feeding ethanol at different times to improve the final protopanaxadiol production. The result showed that the protopanaxadiol production could reach 1.8 g/L when 10 g/L ethanol was fed at 96 h, with a 46 % increase in titer compared to the control without feeding ethanol. To showcase the versatility of the M3 medium, we expanded the triterpenoids to complex ginsenoside, Compound K, for which we integrated the *S. cerevisiae* codon-optimized UGPTg1 glycosyltransferase from *Panax ginseng* into our protopanaxadiol producing strain 102-tm-reerg7-2PP. Comparing CK production by strain CK7 in YPD, WM8+, and our M3 medium revealed respectively a 10-fold and 7.3-fold increase in CK titers in the latter, thus emphasizing the efficacy of the M3 medium for the production of glycosylated ginsenosides. Extracellular production of triterpenoids would be a key strategy to increase the final target triterpenoids production and promote the downstream purification processes. Given this, we added different hydrophobic compounds during the fermentation to carry out in-situ extraction. Different vegetable oils, isopropyl myristate, and dodecane were tested. Finally, almost all the dammarenediol-II and protopanaxadiol produced by the engineered yeast were secreted when vegetable oil was added during the fermentation. In addition, the yeast cells can be repeatedly used for subsequent fermentation rounds after adding isopropyl myristate or vegetable oils to extract all the dammarenediol-II and protopanaxadiol.

In summary, as the cultivation conditions are easily established and transferable to Compound K and betulin producers, the study might facilitate the evaluation of engineered strains in microtiter plates and shake flask cultivations. The amounts of triterpenoid produced enable advanced analytics and even facilitate first ideas for purification of this intriguing class of natural products.

Zusammenfassung

Ginseng wird seit Jahrtausenden in der traditionellen chinesischen Medizin eingesetzt. Die Wurzeln der Pflanze enthalten pharmazeutisch wirksame Ginsenoside. Ginsenoside sind Triterpenoide, eine Klasse von Naturprodukten, die hauptsächlich in *Panax*-Arten vorkommen (z. B. *Panax ginseng*, *P. notoginseng*, *P. quinquifolium*). Aufgrund der Komplxität der chemischen Synthese und der aufwendigen Extraktion aus pflanzlichem Material hat die Produktion von Ginsenosiden durch mikrobielle Zellfabriken in den letzten Jahren viel Aufmerksamkeit erhalten. Die mikrobielle Synthese vereint hohe Effizienz mit potenziell niedrigen Produktionskosten und Umweltfreundlichkeit.

Die vorliegende Studie verfolgt drei Ziele:

1. Analyse der aktuellen Herausforderungen bei der mikrobiellen Synthese von Ginsenosiden durch Hefe

2. Design of Experiments (DoE) basierte Optimierung des Mediums und der Kulturbedingungen für eine verbesserte Produktion von Protopanaxadiol

3. Weiterentwicklung der Ginsenoidproduktion durch in-situ Extraktion

Das DoE-Experiment wurde mit Design Expert 11 (Stat-Ease, Inc.) entworfen und mit Hilfe von OLS-Regression analysiert. Dabei zeigt sich, dass Glukose, Glutamat, Salze, Temperatur und Sauerstoffeintrag maßgeblich für die Protopanaxadiolproduktion sind. In der Folge wurden diese fünf Parameter näher untersucht, um die Protopanaxadiolproduktion weiter zu verbessern. Unter Berücksichtigung der Ergebnisse wurden optimierte Kulturbedingungen entwickelt, die zu einem Protopanaxadioltiter von 1.2 g/L geführt haben. Dies entspricht einer achtfachen Steigerung im Vergleich zu den Ausgangsbedingungen. Die Hauptmerkmale des optimierten Prozesses sind ein verbessertes M3-Medium sowie der Einsatz von Membranverschlüssen, die den Sauerstoffeintrag im Schüttelkolben erhöhen. Das optimierte M3-Medium eignet sich zudem für weitere Produktionsstämme, die andere Triterpenoide (z.B. Betulin und Betulinsäure) produzieren.

Aufgrund der Verdunstung von Ethanol während der Fermentation wurde der Ansatz verfolgt, den Verlust durch Ethanolzugabe auszugleichen. Durch die Zugabe von 10 g/L Ethanol nach 96 Stunden konnte ein Protopanaxadioltiter von 1.8 g/L erzielt werden, was einem Anstieg von 46 % gegenüber der Kontrolle ohne Ethanolzufuhr entspricht.

Um die Vielseitigkeit des optimierten M3-Mediums zu demonstrieren, wurde die Produktion von Compound K, einem komplexen Ginsenosid, untersucht. Zu diesem Zweck wurde die für *S. cerevisiae* codonoptimierte UGPTg1-Glycosyltransferase aus Panax ginseng in den Protopanaxadiol-Produktionsstamm integriert. Die Produktion von Compound K ist im optimierten M3-Medium gegenüber dem YPD-Medium um den Faktor 10 und gegenüber dem WM8+-Medium um den Faktor 7.3 erhöht.

Eine weitere Steigerung der Produktivität kann durch eine in-situ-Extraktion der Triterpenoide erzielt werden, wodurch zudem die Produktaufreinigung erleichtert wird. Neben Isopropylmyristat und Dodecan wurden verschiedene hydrophobe Pflanzenöle als Extraktionsmittel getestet. Durch die Zugabe von Pflanzenölen während der Kultivierung konnte eine nahezu vollständige Sekretion von Dammarenediol-II und Protopanaxdiol beobachtet werden. Isopropylmyristat erlaubt wiederholte Kultivierenden mit demselben Zellmaterial durchzuführen.

Die vorliegende Arbeit präsentiert eine effiziente Methode zur schnellen und effizienten Charakterisierung neuer Triterpenoid-Produktionsstämme in Schüttelkolben und Mikrotiterplatten.

Die erzielten Produktionssteigerungen erleichtern die Analytik von Gensinoiden und ermöglichen zudem erste Schritte zur Aufreinigung dieser faszinierenden natürlichen Produkte.

List of abbreviations

Abbreviations	Full names
ADS	Amorpha-4,11-diene synthase
PPD	Protopanaxadiol
PPT	Protopanaxatriol
IPP	Isopentenyl diphosphate
DMAPP	Dimethylallyl pyrophosphate
Idip	Isopentenyl diphosphate isomerase
Fps/Erg20p	Farnesyl diphosphate synthase
FPP	Farnesyl diphosphate
Sqs/Erg9p	Squalene synthase
Sqe/Erg1p	Squalene epoxidase
DM	Dammarenediol-II
CK	Compound K
ER	Endoplasmic reticulum
G6P	Glucose 6-phosphate
G1P	Glucose-1-phosphate
F6P	Fructose 6-phosphate
GAP	Glyceraldehyde-3P
PP pathway	Pentose-phosphate pathway
EMP pathway	Embden-Meyerhof-Parnas pathway
Ald6p	NADH-generating aldehyde dehydrogenase
Acs1p and Acs2p	Acetyl-CoA synthetase 1 and 2
Erg10p	Acetyl-CoA acetyltransferase
Erg13p	HMG-CoA synthase
HMG-CoA	3-Hydroxy-3-methylglutaryl-CoA
MVA	Mevalonic acid
Hmg1p and Hmg2p	HMG-CoA reductase 1 and 2
Erg12p	Mevalonate kinase
MVAP	Mevalonate 5-phosphate
Erg8p	Phosphomevalonate kinase
MVAPP	Mevalonate 5-pyrophosphate
Erg19p	Diphosphomevalonate decarboxylase
IPP	Isopentenyl pyrophosphate
Erg20p	Farnesyl diphosphate synthase
Erg7p	Lanosterol synthase
DDS	Dammarenediol-II synthase
PPDS	Protopanaxadiol synthase
Cprp	NADPH-cytochrome P450 reductase
CPR	NADPH-cytochrome P450 reductase
UGT1	UDP-glycosyltransferase 1
Ugp1p	UTP-glucose-1-phosphate uridylyltransferase1

UDPG	Uridine diphosphate-glucose
UDP-xylose	Uridine diphosphate-xylose
Pgm2p	Phosphoglucomutase 2
UGTPg45	UDP-glycosyltransferase 45
UGTPg29	UDP-glycosyltransferase 29
PPTS	Protopanaxatriol synthase
UGTPg1	UDP-glycosyltransferase 1
UGTPg100	UDP-glycosyltransferase 100
UGTPg71A54	UDP-glycosyltransferase 71A54
UGTPg94Q13	UDP-glycosyltransferase 94Q13
AMPK	Adenosine 50-monophosphate-activated protein kinase
PBMC	Peripheral blood mononuclear cells
AMPK	Adenosine monophosphate-activated protein kinase
PL	Pancreatic lipase
HUVECs	Human umbilical vein endothelial cells
hERα	Human estrogen receptor α
HUVECs	Human umbilical vein endothelial cells
HBMECs	Human brain microvascular endothelial cells
Xr	Xylose reductase
Xdh	Xylitol dehydrogenase
ATR1	NADPH-cytochrome P450 reductase
ROS	Reactive oxygen species
UPR	Unfolded protein response
Pex34p	Peroxisome population regulated protein 34
PEX11	Peroxisome population regulated protein 11
ATG36	Autophagy-related protein encoding gene
CRISPRi	Clustered regularly interspaced short palindromic repeats interference
LDs	Lipid droplets
PPTS	Protopanaxatriol synthase
NBDs	Nucleotide-binding domains
TMDs	Two transmembrane domains
OLS	Ordinary least squared
DoE	Design of Experiments
UMP	Uridine monophosphate
UDP	Uridine diphosphate
PPi	Pyrophosphate
Ura6p	Uridylate kinase
Ynk1p	Nucleoside diphosphate kinase
Ynd1p	Apyrase
OTR	Oxygen transfer rate
CTR	Carbon dioxide transfer rate
NHEJ	Non-homologous end-joining
ADH1	Alcohol dehydrogenase
ALDH1	Artemisinic aldehyde dehydrogenase

CYP71AV1	Amorphadiene oxidase
CYB5	Cytochrome b5
IPM	Isopropyl myristate
PDR11	Pleiotropic drug resistance exporter
2-HP-β-CD	2-Hydroxypropyl-β-cyclodextrin
ABC	ATP-binding cassette protein
Mrp	Multidrug-resistance-associated protein
Aldp	Adrenoleukodystrophy protein
ORFs	Open reading frames
MEP	2C-Methyl-D-erythritol-4-phosphate
GAs	Ganoderic acids
Yps3p	Aspartic protease
Scw10p	Cell wall glucanase
GA-HLDOA	Ganoderic acid 3-hydroxy-lanosta-8,24-dien-26-oic acid
PYC	Pyruvate carboxylase
MDH	Malate dehydrogenase
SpMae	C4-Dicarboxylate transporter
M-β-CD	Methyl-β-cyclodextrin
DM-β-CD	2,6-Dimethyl-β-cyclodextrin
Cryo-EM	Cryo-electron microscopy
COVID-19	Coronavirus disease 2019
UD	Uniform design
v/v	Volume per volume
PBS	Phosphate buffered saline

Chapter 1

General Introduction

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Shangkun Qiu: Conceptualization, Methodology, Validation, Writing - Original Draft, Visualization. **Lars M. Blank:** Supervision, Project administration, Funding acquisition, Review & Editing.

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1.1 Bioeconomy: A Sustainable Future

The bioeconomy represents a shift from a fossil-based economy to one that uses biological resources, processes, and principles to produce energy, chemicals, and materials sustainably. By harnessing the power of biotechnology, agriculture, and other bio-based sectors, the bioeconomy aims to create an integrated and sustainable approach to economic development.[1]

Central to the bioeconomy is the use of renewable biological resources. Plants, algae, and microorganisms are used to produce biofuels, bioplastics, pharmaceuticals, and other high-value products.[2] This approach reduces reliance on non-renewable resources and minimizes environmental impact by lowering greenhouse gas emissions and reducing waste.[1,2]

One significant aspect of the bioeconomy is the development of biorefineries. These facilities use biomass to produce a range of products, similar to how petroleum refineries produce fuels and chemicals.[3] For example, biorefineries can in future convert agricultural waste into bioethanol, biodegradable plastics, and other value-added products. This provides an additional revenue stream for farmers and promotes waste reduction and sustainable resource use.[3,4]

The bioeconomy also fosters innovation in agriculture through biotechnology to develop crops with improved yields, pest resistance, and nutritional content. These advancements can help meet the growing global food demand while reducing agriculture's environmental footprint. For instance, genetically modified crops that require fewer pesticides and fertilizers can significantly lower agricultural pollution.[3,5] However, such a future would rely on a strict agenda change in the EU.

Furthermore, the bioeconomy supports the transition to a circular economy, where waste products are reused and recycled, creating a closed-loop system. This includes developing biodegradable materials that replace conventional plastics, reducing plastic pollution, and promoting sustainable consumption.[6,7,8] At RWTH Aachen, an open loop recycling is suggested in the project catalaix.

In the context of synthetic biology, the bioeconomy leverages engineered organisms to produce complex molecules, such as pharmaceuticals and specialty chemicals, more efficiently and sustainably than traditional methods.[2] For example, engineered yeast strains can produce high-value natural products like ginsenosides, artemisinin, and other bioactive compounds, offering a renewable and scalable alternative to extraction from natural sources.[9,10]

The bioeconomy also addresses global challenges such as climate change, resource depletion, and environmental degradation.[6] By promoting sustainable practices and technologies, it aims to create a resilient economy that supports both human well-being and ecological health. Investments in bioeconomy-related research and development are crucial for driving innovation and unlocking new economic opportunities.[8]

Overall, the bioeconomy represents a transformative approach to achieving sustainable development, emphasizing the importance of biological resources and processes in creating a sustainable and prosperous future.[11] As we continue to advance in biotechnology and other bio-based sectors, the potential for the bioeconomy to contribute to economic growth, environmental sustainability, and societal well-being becomes increasingly significant.[1,8,11] The challenge is to compete with the petrochemical industry, an industry optimized for seven decades. Examples may be in the realm of natural products, where petrochemistry is less successful, e.g., in the production of terpenoids, like triterpenoids generally isolated from plants.

1.2 Introduction of producing recombinant ginsenosides by yeast

Ginseng (*Panax* spp.), a plant belonging to the *Araliaceae* family, has been a traditional ingredient in medicine throughout East Asia for several millennia.[12,13] The term "ginseng" is a translation of the Chinese words 人 參 (*Renshen*), "the essence of men".[14,13,15] The major pharmacologically relevant constituents of ginseng are ginsenosides, a class of triterpenoids that possess activities on different systems such as the cardiovascular, central nervous, endocrine, and immune systems (Table 1-1). Ginsenosides have shown potential against the coronavirus disease 2019 (COVID-19),[12,16,17,18] making them commercially valuable, with a global market exceeding one billion U.S. dollars annually.[2,17,18] Over 200 ginsenosides have been identified from 17 species in the *Panax* genus, which can be mainly categorized into two groups based on their aglycone number: oleanane-type pentacyclic and dammarane-type tetracyclic ginsenosides.[16,19] Dammarane-type tetracyclic ginsenosides can be further categorized into three groups based on the hydroxylation and carbohydrate moieties at C-3, C-6, and C-20 positions: protopanaxadiol (PPD)-type ginsenosides, protopanaxatriol (PPT)-type ginsenosides, and ocotillol.[20,21]

Table 1-1. Reported functions of PPD and its glycosylated derivatives.[22]

Compounds	Model	Suggested Function	Ref.
PPD	Male C57BL/6 J mice ICR mice Male *ob/ob* mice	Ameliorates metabolic syndrome by gut microbiota remodeling	23
PPD	HepG2 cells	Activates adenosine 50-monophosphate-activated protein kinase (AMPK) and regulates glucose and lipid metabolism	24
PPD	Human oral squamous cell carcinoma cell line KB	Inhibits the overexpression of P-glycoprotein in multidrug-resistant cancer cells	25
PPD	Human ALL cell lines Reh, RS4 Human Burkitt's lymphoma cell line Raji Peripheral blood mononuclear cells (PBMC)	Inhibits growth and cell cycle progression and stimulates differentiation of acute lymphoblastic leukemia	26,27
PPD	Human glioblastoma U251-MG U87-MG cells	Suppresses the viability of human glioblastoma cells by reducing the expression of N-cadherin, integrin β1, and cyclin D1	28
PPD	HCT-116 human colorectal cancer cells	Inhibits growth of HCT-116 colorectal cancer cells, inhibits apoptosis of cancer cells, and reduces the size of tumor cells	29
PPD	Male Kunming mice	Positive effect on the treatment of depressive psychiatric disorders	30
CK	HFD-fed OLETF rats	Enhances glucose metabolism and hepatosteatosis by regulating adenosine monophosphate-activated protein kinase (AMPK)	31
CK	—	Inhibits pancreatic lipase (PL) activity	32
CK	MIN6 pancreatic β-cells mice	Increases the insulin secretion in MIN6 pancreatic β-cells and suppresses ER stress-induced inflammation	33,34
CK	Type 2 diabetes mice HepG2 cells	Suppresses the hepatic gluconeogenesis by activating adenosine-5′monophosphate kinase	35
CK	Human umbilical vein endothelial cells (HUVECs)	Prevents oxidized low-density lipoprotein induced HUVECs inflammation and apoptosis	36
CK	Mouse model Mouse hippocampal HT22 cells	Restores the memory deficit by inducing Nrf2-mediated antioxidant enzymes and leads to cognitive improvement	36,37
CK	Brain disease models of mice	Controls the microglial activation by inhibiting mitogen-activated protein kinases and NF-κB/AP-1 activities, enhancing HO-1/ARE signaling and reactive oxygen species	38

CK	Sprague Dawley rats	Improves spontaneous GABA release by enhancing intradermal Ca^{2+} concentration	37, 39
CK	Human astroglial cells	Inhibits both NF-kB and JNK pathways to exert anti-inflammatory effects	40
CK	Male NIH mice	Exerts antidepressant-like effects by regulating monoamine neurotransmitters NA, adrenocorticotropic hormone, and corticosterone levels	41
CK	Human keratinocyte HaCaT cell line	Increases the production of hyaluronan by increasing the expression of hyaluronan synthase 2	42
CK	Human colon cancer cells	Improves human colon cancer cells convert to TRAIL-induced apoptosis by upregulating cell pro-apoptotic proteins and downregulating cell survival proteins	43
CK	A549 and H1975 cells	Suppresses the proliferation and apoptosis of non-small cell lung cancer by activating AMPK/mTOR and JNK signaling pathways	44
CK	Human breast cancer cell line MCF-7 cells	Inhibits the proliferation and apoptosis of MCF-7 cells	45
CK	SD rats Immortalized rat HSC line HSC-T6 cells	Ameliorates the liver function impairment by the hepatoprotective activity and anti-fibrotic	46
Rh2	Human promyelocytic leukemia cell line HL-60	Induces the differentiation of HL-60 cells by modulating PKC isoform levels	47
Rh2	Human astroglial cells	Inhibits both NF-kB and JNK pathways to exert anti-inflammatory effects	40
Rh2	Human hepatoma SK-HEP-1 cells	Induces apoptosis by Bcl-2-insensitive pathway activation of caspase-3 protease	48
Rh2	Prostate cancer cell line	Inhibition of the proliferation of prostate cancer cell lines	49
Rh2	Mice 3T3-L1 cells	Suppresses the metabolic disorders and anti-obesity by regulating the AMPK signaling pathway	50
Rh2	Male C57BL/6J mice	Exerts antihyperglycemic effect via inducing islet β-cell proliferation	51
Rh2	B16 melanoma cells HEK293 cells	Suppresses the proliferation of B16 melanoma cells and reduces the viability of HEK293 cells	52
Rh2	Mouse melanoma (B16) cell line	Inhibits the growth of the B16 cells line, leads to morphological alterations, and stimulates melanogenesis	53
Rg3	—	Inhibits the pancreatic lipase (PL) activity	32
Rg3	Male NIH mice	Exerts antidepressant-like effects by regulating Neurotransmitters NA, adrenocorticotropic hormone, and corticosterone levels	41
Rg3	Human endothelial cells (ECs)	Inhibits ECs apoptosis by inhibition of the mitochondrial caspase pathway	54
Rg3	Prostate cancer cell line	Inhibition of the proliferation of prostate cancer cells	49
Rg3	C57BL/6 and Balb/c mice Murine B16 melanoma cells	Inhibition of the lung metastasis of tumor cells	55
Rg3	Rat mesothelial cells	Inhibits the invasion of rat ascites hepatoma cells	56
Rg3	Hamster pancreatic b-cell line	Suppresses blood glucose level increase via enhancing insulin secretion	57
PPT	Human breast cancer cell line MCF-7 cells	Binds human estrogen receptor α (hERα) ligand-binding domain and acts as agonists of hERα	58
PPT	Human non-small cell lung cancer cell line A549	Directly binds the DNA-binding pocket of tumor suppressor P53 to involve in the regulation of the antitumor network	59
PPT	Murine RAW 264.7 macrophages	Inhibits the release of NO in RAW264.7 cells by suppressing the expression of the inflammatory enzyme iNOS	60
PPT	Murine RAW 264.7 macrophages Inflammatory model of mouse ear edema	Improves the anti-inflammatory activity by decreasing the production of pro-inflammatory cytokines TNF-α	61, 62
PPT	C57BL/6 male mice	Improves the abundance of gut microbiota, increases the concentration of short-chain fatty acids and their receptor proteins, and decreases colonic inflammation in antibiotic-treated mice	62
PPT	Male mice	Reverses the cognitive deficits induced by	63

		scopolamine, improves cholinergic system reactivity, and inhibits oxidative stress and acetylcholinesterase activity	
PPT	Male Sprague-Dawley rats	Promotes platelet aggregation induced by the P2Y12 receptor and decreases the impairment of cardio-cerebrovascular diseases	64
PPT	H9c2 cardiomyocytes	Inhibits H_2O_2-induced H9c2 cells cardiomyocytes injury by regulating PI3K/Akt pathway and decreases ROS generation and the apoptosis of H9c2 cells	65
PPT	Human EGFR-mutant non-small cell lung cancer cell lines	Inhibits lipid metabolism and cells proliferation by suppressing the expression of lipid metabolism key enzyme stearoyl-CoA desaturase 1	66
Rh1	Mouse melanoma (B16) cell line	Stimulates the melanogenesis of B16 cells line	53
Rh1	Mouse embryo fibroblasts 3T3-L1 cells	Suppresses adipogenesis and ameliorates obesity via inhibiting adipocyte inflammation and differentiation	47
Rh1	SD rats Immortalized rat HSC line HSC-T6 cells	Ameliorates liver function impairment by the hepatoprotective and anti-fibrotic activity	46
F1	Human dermal fibroblasts	Inhibition of collagen degradation and anti-skin aging	67
F1	Natural killer cells	Enhances the innate immune response	68
F1	Mouse 3T3-L1 embryo fibroblast cells	Suppresses lipid accumulation and reactive oxygen species (ROS) production via reducing expression of adipogenesis markers	69
F1	Male C57BL/6 mice ApoE-/- mice	Enhances the viability of ox-LDL-injured endothelial cells and exerts anti-atherosclerosis activities by suppressing the NF-κB signaling	70
F1	Human umbilical vein endothelial cells (HUVECs) Human brain microvascular endothelial cells (HBMECs)	Increases angiogenesis and ameliorate cerebrovascular function by activating the insulin-like growth factor 1 (IGF-1) and insulin-like growth factor 1 receptor (IGF1R) pathway	71
F1	Human HaCaT keratinocytes	Reduces the apoptosis of human HaCaT keratinocytes induced by ultraviolet-B radiation	72

Ginsenosides are synthesized from squalene or 2,3-oxidosqualene via the mevalonate pathway, a pathway present in most eukaryotes and well-studied in yeast. [14,20,73] Acetyl-CoA is first converted to isopentenyl diphosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), the precursors of squalene.[74] From 1.5 moles of glucose, one mol of IPP and three moles of NADPH can be formed in yeast.[20] An improved stoichiometry from glucose using a synthetic pathway was reported, too.[75] Isopentenyl diphosphate isomerase (Idip) converts IPP to DMAPP,[20] followed by condensation of two IPP and one DMAPP by farnesyl diphosphate synthase (Fps/Erg20p) to farnesyl diphosphate (FPP).[20,76] Two FPP are subsequently converted by squalene synthase (Sqs/Erg9p) and squalene epoxidase (Sqe/Erg1p) to one 2,3-oxidosqualene,[20,76] which is the common precursor for the yeast sterol ergosterol and many plant triterpenoids, requiring alternative enzymes (Figure 1-1).[20,76]

Plant natural products are widely used in the food, cosmetic, chemical, and pharmaceutical industries,[77,12,14,78,79] primarily in the form of plant materials or extracts. Two primary methods are used to obtain plant natural products: direct extraction from plant biomass and chemical synthesis.[16] However, plants often contain low amounts of the desired molecule, thus employing a considerable amount of biomass, which in turn requires the intense use of organic solvents during extraction.[80,17,20] Additionally, the supply of derived natural products might easily affect plant protection status, as some plants grow exclusively in a particular environment or at a very slow rate.[77,16] For instance, ginseng roots require approximately six years of growth until harvest, experiencing varying influences related to weather, soil, and the presence of pathogens, while hemp grows quickly and is easily farmed for natural compounds[16,81]. Nonetheless, the number of

natural products obtained directly from plants is still modest when compared to the sheer number of plant natural products reported in the academic literature.[82,83,84] Chemical synthesis is often economically unattractive due to the complex structures of natural products, making microbial synthesis a promising alternative.[85,86,87,88]

With the advent of synthetic biology, designing living systems for the tailored production of bioplastics, functional foods, and natural products has become feasible.[89] In recent years, several natural products with application potential have been synthesized using engineered microbes.[12,90] Three prominent examples are farnesene, artemisinic acid, and amorpha-4,11-diene production by yeast. For example, α-farnesene was produced by engineered *Yarrowia lipolytica*, and its production was improved during fed-batch fermentation via the expression of the HMG-CoA reductase (Hmgp) from *Bordetella petrii* and acetyl-CoA acetyltransferase from *Escherichia coli*, reaching a final titer of 26 g/L.[77,91] Artemisinic acid production in *S. cerevisiae* was increased by overexpressing essential genes involved in the mevalonate pathway as well as optimizing the electron transport chain and yeast cultivation, reaching titers of 25 g/L.[92,9] Amorpha-4,11-diene production by *S. cerevisiae* expressing the amorpha-4,11-diene synthase (ADS) from *Artemisia annua* harboring a fully engineered mevalonate pathway, including three copies of the truncated gene *tHMG* encoding the *Sc*HMG-CoA reductase, and using unrestricted ethanol as carbon source, reached a titer of 40 g/L.[93]

Yeasts have long been domesticated and were used for the production of bread, beer, and wine.[94,95,96] Yeasts are simple eukaryotic cells with complete organelle structures, which allow posttranslational modifications of proteins, thus ensuring the activity of many enzymes found in other eukaryotes, including plants.[80,89] Yeasts also possess redox systems that provide similar biological and physical environments as encountered by cytochrome P450s in mammals and plants.[80,20] Due to these reasons, yeasts are considered excellent cell factories for producing plant natural products, with biosynthetic pathways of varying complexity.[94,97]

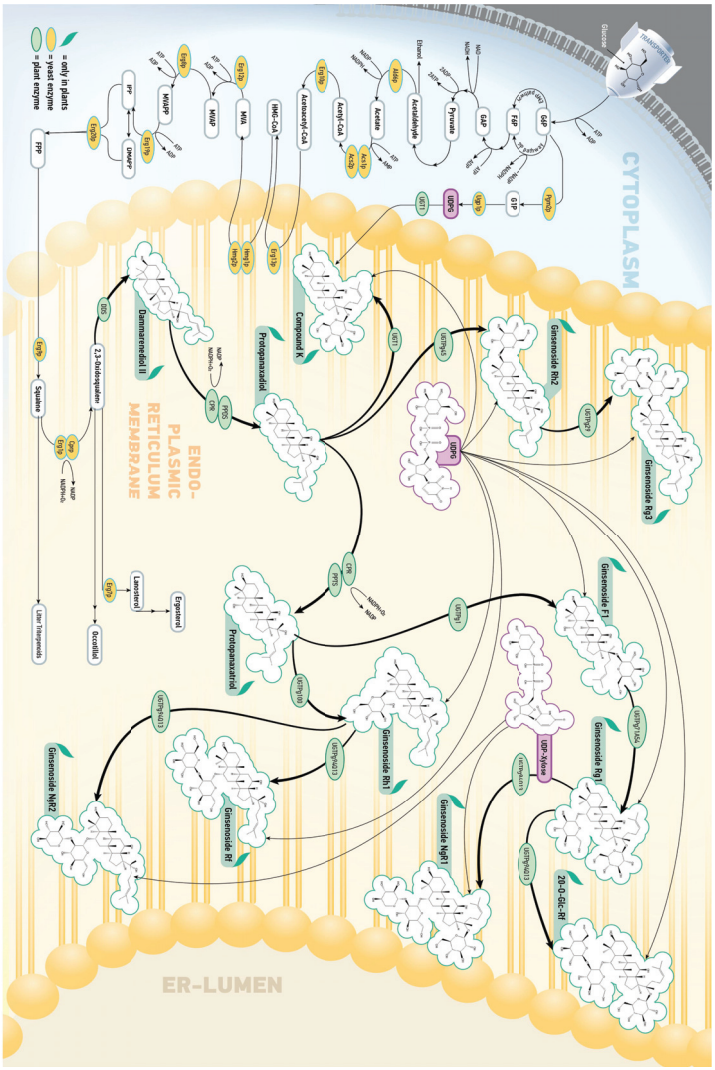

Figure 1-1. Biosynthetic pathway of heterologous ginsenoside triterpenoids in engineered *S. cerevisiae*.[22] Glucose is catabolized mainly through glycolysis, while NADPH may be supplied by the flux through the pentose phosphate pathway and activity of the acetaldehyde dehydrogenase Ald6p. Metabolic reactions involved in glucose phosphorylation to farnesyl diphosphate (FPP) biosynthesis via the mevalonate pathway are carried out in the cytosol, although several enzymes are localized in the endoplasmic reticulum (ER) membrane. Triterpenoids are amphiphilic, with highly hydrophobic residues, explaining their localization and further modification in the ER membrane. The subsequent modifications, including glycosylation of ginsenosides, occur on the cytosolic-oriented

triterpenoid. UDPG or UDP-xylose supplies sugar groups for glycosylation modifications. Also, the active site of the yeast monooxygenase Erg1p and the plant monooxygenases likely face the cytosol, explaining easy access to NADPH. The competitive sterol/ergosterol biosynthetic pathway is also localized in the ER membrane. G6P, glucose 6-phosphate; G1P, glucose-1-phosphate; F6P, fructose 6-phosphate; GAP, glyceraldehyde-3P; PP pathway, pentose-phosphate pathway; EMP pathway, Embden-Meyerhof-Parnas pathway; Ald6p, NADH-generating aldehyde dehydrogenase; Acs1p and Acs2p, acetyl-CoA synthetase 1 and 2; Erg10p, acetyl-CoA acetyltransferase; Erg13p, HMG-CoA synthase; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; MVA, mevalonic acid; Hmg1p and Hmg2p, HMG-CoA reductase 1 and 2; Erg12p, mevalonate kinase; MVAP, mevalonate 5-phosphate; Erg8p, phosphomevalonate kinase; MVAPP, mevalonate 5-pyrophosphate; Erg19p, diphosphomevalonate decarboxylase; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl diphosphate; Erg20p, farnesyl diphosphate synthase; FPP, farnesyl diphosphate; Erg9p, squalene synthase; Erg1p, squalene epoxidase; Erg7p, lanosterol synthase; DDS, dammarenediol-II synthase; PPDS, protopanaxadiol synthase; Cprp, NADPH-cytochrome P450 reductase; CPR, NADPH-cytochrome P450 reductase; UGT1, UDP-glycosyltransferase 1; Ugp1p, UTP-glucose-1-phosphate uridylyltransferase1; UDPG, uridine diphosphate-glucose; UDP-xylose, uridine diphosphate-xylose; Pgm2p, phosphoglucomutase 2; UGTPg45, UDP-glycosyltransferase 45; UGTPg29, UDP-glycosyltransferase 29; PPTS, protopanaxatriol synthase; UGTPg1, UDP-glycosyltransferase 1; UGTPg100, UDP-glycosyltransferase 100; UGTPg71A54, UDP-glycosyltransferase 71A54; UGTPg94Q13, UDP-glycosyltransferase 94Q13. ER, endoplasmatic reticulum.

1.3 Metabolic engineering of yeast for PPD synthesis

PPD, a 30-carbon dammarane II-type tetracyclic triterpene synthesized in the ginseng plant, serves as the precursor for high-value ginsenosides.[20,98,99] The initial dedicated step in PPD synthesis involves the cyclization of 2,3-oxidosqualene by dammarenediol-II synthase (DDS) to DM, which is then converted to PPD by protopanaxadiol synthase (PPDS) (Figure 1-1).[20,97] Research has shown that PPD possesses intrinsic biological properties, including antineoplastic, antidepressant, antioxidant, anti-inflammation, and antitumor effects (Table 1-1).[97,100] PPD production via phytoextraction is expensive and largely non-environmentally friendly.[97,98] Currently, PPD-synthesizing yeasts primarily use glucose as the carbon source for native squalene production via the mevalonate pathway.[12,74,10] PPD production can be vastly improved by combining precursor availability, including coenzymes and enzymatic pathway activity throughout the mevalonate pathway. Although numerous metabolic and process engineering strategies have been reported, studies have yet to combine all the available information for PPD production. This suggests that substantial improvements are still possible, assuming these approaches can complement each other. The state-of-the-art strategies for PPD synthesis by yeast are summarized as follows:

1. The PPD can be produced by yeast through alternative carbon sources. Ethanol, a more reduced substrate than glucose or xylose, directly feeds into the cytosolic acetyl-CoA pool for the mevalonate pathway.[97] As a Crabtree-positive yeast, *S. cerevisiae* consumes glucose and produces biomass, CO_2, and ethanol. Upon glucose depletion, ethanol becomes the next preferred carbon source in the presence of oxygen, leading to the classic diauxic shift (Figure 1-2). Analyses have shown ethanol to be a beneficial carbon source for triterpenoid production.[80,87] For instance, a pure ethanol feed strategy significantly improve amorpha-4,11-diene (a sesquiterpene) production to over 40 g/L, far exceeding productions obtained from glucose or glucose/ethanol mixtures.[97,93] Similarly, using ethanol for PPD production with engineered *S. cerevisiae* achieved high titers of

up to 8 g/L.[97]

Comparable improvements have been reported for squalene production, where continuous ethanol resulted in a superior yield to a pulsed-feed feeding regime.[7] Further optimization of ethanol usage by overexpressing ethanol and acetaldehyde dehydrogenases in *S. cerevisiae*, enhanced squalene synthesis above 9 g/L.[101] These findings indicate that ethanol is an advantageous carbon source for triterpenoids in general and specifically for PPD production, enabling the synthesis of high concentrations of these valuable natural products by *S. cerevisiae.*

Over 100 billion tons of biomass are produced on Earth each year through photosynthesis, driving the development of sustainable bioprocesses like ethanol production by yeast.[74,102,103] In recent research, genes from the xylose assimilation pathway were introduced into *S. cerevisiae* alongside overexpressed genes from the mevalonate pathway. This engineered *S. cerevisiae* could co-ferment glucose and xylose, significantly increasing PPD titer to 150 mg/L, compared to 59 mg/L when grown solely on glucose.[99] In the delta *ku70 Y. lipolytica* strain, which is optimized for homologous recombination, the genes *XYL1* and *XYL2* (encoding xylose reductase and xylitol dehydrogenase from *Scheffersomyces stipitis*) were introduced. In this strain, increased PPD synthesis from xylose was combined with the overexpression of the mevalonate pathway genes *tHMG1/ERG9/ERG20* and the overexpression of the genes encoding transaldolase, transketolase, and the xylose transporter. PPD production reached 300 mg/L, remarkably higher than the content of PPD obtained during growth on glucose alone (170 mg/L), although the metabolic basis for increased PPD production remains elusive.[74]

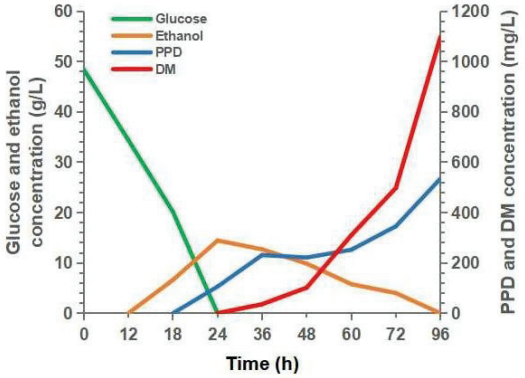

Figure 1-2. Fermentation kinetics of PPD and DM in *S. cerevisiae*.[22] Yeast consumes glucose and produces ethanol. At 24 h, glucose was depleted, and ethanol levels peaked. DM and PPD are synthesized mainly from ethanol. Growth conditions: 50 mL WM8+ medium in 500 mL shake flask; fermentation temperature, 30 °C; engineered yeast strain *S. cerevisiae CEN.PK 102-5B*.[104]

2. Increased electron availability benefits PPD production in *S. cerevisiae*. NADPH, the essential reduced coenzyme for the biosynthesis of 2,3-oxidosqualene, PPD, and PPT, is oxidized to NADP by a P450 reductase, which then transfers electrons to monooxygenases like PPDS.[82,83,101] Various strategies have been reported to enhance electron availability, ranging from ethanol use to pathway and reductase/P450 engineering.[82,83,105] Modification of the transmembrane domain of *P. ginseng* cytochrome P450-type PPDS and construction of a PPDS-*Arabidopsis*

thaliana NADPH-cytochrome P450 reductase (ATR1) fusion enzyme have been shown to boost catalytic activity, resulting in a 70% increase in PPD production (1.4 g/L) compared to co-expression of PPDS and ATR1.[83] Additionally, replacing the NADH-generating acetaldehyde dehydrogenase Ald2p with the NADPH-forming isozyme Ald6p directly improved NADPH regeneration. This approach, combined with strong overexpression of the PPD synthesis pathway using the *ADH2* promoter for DDS and PPDS expression, led to an 11-fold increase in PPD titer.[82] These findings indicate that pathway engineering can foster the availability of precursors and reduced coenzymes to improve triterpenoid production. These strategies should be applied to production strains with high PPD synthesis rates, where metabolic limitations are most significant.

3. PPD production can be further improved by engineering the cell wall, ER size, and peroxisome proliferation. The cell wall is crucial for cellular homeostasis and growth, providing yeast with its morphology during budding, pseudohypha formation, mating, and sporulation, and maintaining osmotic integrity.[106,107] Previous studies have shown that maintaining cell wall integrity can enhance terpene production by *S. cerevisiae*.[83,98] Deleting the *ECM33* gene, a vital regulator of the cell wall integrity pathway, restored membrane rigidity and improved stress tolerance, resulting in increased squalene production.[108] It has also been observed that heterologous expression of cytochrome P450 monooxygenases in microorganisms will result in low coupling between cytochrome P450 monooxygenases and cytochrome P450 reductase (CPR), leading to the release of reactive oxygen species (ROS) as well as reduced growth and terpenoid synthesis.[83] However, overexpressing the *SSD1* gene, which is associated with increased cell wall integrity and cell viability, reduced ROS formation by 25% and significantly boosted PPD production from 1 g/L to 4 g/L.[98]

The ER can be the largest organelle in the cell, functioning in protein modification and secretion, phospholipid synthesis, carbohydrate metabolism, and calcium storage.[109] The unfolded protein response (UPR) triggers ER expansion and enhances its folding capacity.[110] The ER volume impacts many proteins' folding ability, such as cytochrome P450s.[109,110] *INO2* and *INO4* encode two transcription factors involved in phospholipid biosynthesis that can promote the generation of ER sheets and ER expansion.[110,111] In contrast, Opi1p can bind to Ino2p, inhibiting its function.[110] Overexpression of the *INO2* gene in *S. cerevisiae* leads to ER expansion, thereby increasing the ability to synthesize heterologous ER-associated proteins and resulting in a 70-fold increase in squalene production (from 2.5 mg/L to 175 mg/L) and an 8-fold increase in PPD production (from 1.5 mg/L to 12 mg/L).[111] However, overexpression of *INO4* or deletion of *OPI1* did not affect squalene and PPD accumulation, possibly because Ino2p plays a more significant role in phospholipid biosynthesis than Ino4p.[111] Overexpression of *INO2* may lead to an imbalance between *INO2* expression and its repressor Opi1p, ultimately activating the ER's phospholipid biosynthetic pathway.[111]

Additionally, manipulating the peroxisome proliferation by increasing Pex34p (peroxisome population regulated protein 34) production while deleting *PEX11* (peroxisome population regulated protein 11) and *ATG36* (autophagy-related protein encoding gene) improved PPD production by 78% (4 mg/L) compared to the original yeast strain.[112] These results indicate that all the cell wall integrity, ER size, and peroxisome proliferation likely influence PPD production, although the rationale for the latter is rather unclear.

4. Upregulating the PPD synthetic pathway and increasing 2,3-oxidosqualene availability enhances PPD hyperproduction. Seven genes are involved in the synthesis of mevalonate, IPP, and

DMAPP, namely *ERG10, ERG13, ERG12, ERG8, ERG19, IDI,* and *tHMG1*, while three genes (*ERG1, ERG20, ERG9*) are involved in 2,3-oxidosqualene formation, and finally three genes (*PgDDS*, *PgPPDS*, and *PgCPR1*) are engaged in PPD synthesis (Figure 1-1).[20,82,113] Integrating the six genes involved in 2,3-oxidosqualene and PPD synthesis into the *delta DNA* site of *S. cerevisiae* BY4742 increased PPD synthesis.[113] Additionally, seven genes involved in synthesizing mevalonate, IPP, and DMAPP were integrated into the *Yprc-delta15* site, and an additional copy of *PgPPDS* was incorporated into rDNA sites. These modifications resulted in the highest PPD production reported (11 g/L), with a cell dry weight content of 77 mg_{PPD}/g_{CDW}.[10,113] Further modifications, including introducing the ginsenoside CK pathway, improving UDP-glucose biosynthesis, and reducing UDP-glucose consumption, led to a CK production of nearly 6 g/L in fed-batch fermentations.[10] Collectively, these results revealed that a high PPD titer could be achieved by combining engineering strategies.

Many studies have aimed to improve triterpenoid synthesis by reducing sterol synthesis. The strategies include deleting *ERG7*, which encodes 2,3-oxidosqualene-utilizing lanosterol synthase, and using advanced transcriptional and translational control of Erg7p activity.[114] Recently, a degron tag fused to Erg7p decreased the carbon flux into the sterol synthesis pathway, improving the precursor supply for triterpenoid synthesis and resulting in a PPD titer of over 400 mg/L in shake flasks.[104] Similarly, Guo et al. used protein engineering to reduce Erg7p activity by mutating the protein sequence position I705, which is crucial for Erg7p catalysis. The $Erg7^{I705K}$ mutation with reduced sterol synthesis increased the PPD titer to 55 mg/L, 2.2-fold higher than the reference strain.[115] Additionally, CRISPRi (clustered regularly interspaced short palindromic repeats interference)-based customized metabolic flux control was used to regulate *ERG7* expression, achieving a final PPD production of 295 mg/L, 5.7-fold higher than the reference strain in batch-fed fermentation.[114] Since ergosterol is essential for the plasma membrane, a complete deletion of *ERG7* will stop ergosterol synthesis, which is infeasible.[114] These results suggest that reducing, but not abolishing, Erg7p activity can increase carbon flux towards triterpenoid production.

1.4 Synthesis of the PPD-derivative ginsenosides

1.4.1 Biosynthetic pathway for ginsenoside CK production

Ginsenoside CK is a glycosylated PPD derivative with a wide range of potential medical applications (Table 1-1).[77,42,116] CK is mainly found in dried roots of *P. ginseng*, however, accounting for only 0.2% of the biomass.[117] Its precursor, PPD, is the substrate of a UDP-glycosyltransferase (UGT) to yield CK.[77,117] UGTs are commonly found in plants and primarily involved in triterpenoid synthesis, transferring glycosyl moieties to acceptor molecules, including ginsenosides. The activated sugar is derived from glucose-6-phosphate by the activity of Pgm2p (Figure 1-1).[87,117] Engineered PPD-synthesizing yeast can be additionally equipped with *UGT*, resulting in CK production.[87] According to previous research, the cytotoxic effects of the dammarane-type ginsenosides were inversely proportional to the number of hydroxyl groups and sugar moieties linked to the aglycone.[118] Therefore, research has mainly focused on biosynthetic dammarane-type ginsenosides derivatives, such as PPD, CK, and others, rather than DM. This is most likely due to the missing hydroxyl groups that makes this group of ginsenosides more cytotoxic.[118] In general, enzyme and metabolic engineering strategies successful for PPD synthesis

are also crucial for the synthesis of CK.[87,119] The state-of-the-art strategies for CK synthesis by yeast are summarized as follows:

1. Ethanol and glycerol benefit CK production by engineered *S. cerevisiae*. Glycerol is about 17% more reduced than glucose, potentially boosting the synthesis of reduced compounds such as triterpenoids.[120,121] It enhances the stability of cell membrane proteins and improves the generation of NADPH through its metabolism.[122] In a recent study, introducing the *UGT1* gene from *P. ginseng* into a PPD-synthesizing yeast resulted in a CK titer of approximately 150 mg/L after 96 h of cultivation.[87] Further improvements were achieved by introducing the genes *UGP1* and *PGM2*, raising the titer to around 260 mg/L.[87] The optimal CK synthesis was obtained by altering the glycerol/glucose ratio to 20/80.[87] Finally, adding 7 g of glycerol and 15 g of ethanol every 8 h during fermentation resulted in a final CK titer of up to 2 g/L.[87]

2. Engineering yeast subcellular compartments increase CK production. Improving terpenoid synthesis of yeasts through modifying subcellular compartments is attractive, as shown in PPD synthesis.[96] Studies have demonstrated that relocating enzymes from mitochondria to lipid droplets (LDs), which store lipophilic compounds like terpenes, can vastly increase enzyme activity for ester biosynthesis in *S. cerevisiae*.[123,124] It is speculated that DM is located in LDs, while PPDS is usually found in the ER of yeast.[73] Therefore, the different cellular localizations of DM and PPDS may increase the difficulty of converting DM to PPD. Recently, the Pln1p was used to target the ER-localized cytochrome P450 PPDS to LDs, thereby increasing the final CK titer to 5 g/L in a 5 L bioreactor fed-batch mode.[73] This study introduces another metabolic engineering strategy, enzyme localization, to the growing list of methods for increasing triterpenoid production.

3. Enhanced metabolic flux for precursor synthesis improves CK production by *Y. lipolytica*. As previously mentioned, *Y. lipolytica* is a promising yeast for terpenoid synthesis due to its ability to accumulate considerable amounts of hydrophobic molecules.[77,122] The overexpression of essential genes involved in the mevalonate pathway in this yeast leads to the increased production of terpenes such as limonene, α-farnesene, lycopene, and betulinic acid.[125,126,127,128] In a previous study, the fusion of *P. ginseng* PPDS and *A. thaliana* reductase ATR1 further improved the catalytic efficiency of these two enzymes.[83] Combining engineering strategies, like overexpression of the critical genes *tHMG1*, *ERG9*, *ERG20*, and *UGT1*, resulted in 160 mg/L CK production by *Y. lipolytica*.[119]

1.4.2 Synthesis of PPD-derivative ginsenosides Rh2 and Rg3

Ginsenosides Rh2 and Rg3, which possess additional sugar residues and valuable pharmacological activities (Table 1-1), have been studied extensively.[48,49,54,55,56] A study showed that *UGTPg45* and *UGTPg29* genes from *P. ginseng* introduced into PPD-synthesizing yeast resulted in trace amounts of Rh2 and Rg3 production (Figure 1-1).[129] In another study, the promiscuous glycosyltransferase UGT51 encoded by the *UGT51* gene from *S. cerevisiae* was shown to synthesize ginsenoside Rh2 effectively. The *EGH1* gene encoding a steryl-beta-glucosidase was deleted, and the supply of UDP-glucose precursor was increased to improve glycosylation. In this strain, expressing PPDS-75tAtr2p as a fusion protein resulted in Rh2 concentrations of up to 300 mg/L in a 5 L fermenter.[130] UGT activity was central for Rh2 synthesis.[113,129] Significant advances were achieved by integrating multi-copy *UGTPg45* into *delta DNA* sites combined with a strong *UAS-TDH* promoter, resulting in a substantial increase in Rh2

synthesis. Further optimization through the error-prone PCR evolution of plant UGTs enabled efficient Rh2 synthesis from PPD in a 10 L fed-batch fermentation, achieving Rh2 concentrations as high as 2 g/L.[113]

1.5 Strategies for the synthesis of PPT and its glycosylated derivatives from *P. ginseng*

PPT, distinguished by an additional α-OH group at the C-6 position compared to PPD, is a prominent triterpenoid of *P. ginseng* known for its reported biological activities, including anti-cancer, antioxidant stress, and anti-inflammatory effects (Table 1-1).[20,60,58,131] Yeast can convert PPD to PPT by heterologously expressing the *protopanaxatriol synthase* (*PPTS*) gene from *P. ginseng* (Figure 1-1).[35] PPT was successfully synthesized in an engineered yeast for PPD synthesis, resulting in up to 16 mg_{PPT}/L.[36] In another study, PPT production reached 5 g/L in fed-batch fermentation through overexpression of codon-optimized *PPTS* from *P. ginseng* and random mutation of amino acid sequences in PPTS.[132] Ginsenosides F1 and Rh1, which have an α-D-glucopyranosyl or α-H group at C-6 or C-20, respectively, are the products of PPT glycosylation reaction catalyzed by UGTs with reported pharmacological activity (Table 1-1).[12] According to a study, two *UTG* genes, namely *UGTPg1* and *UGTPg100*, were identified in *P. ginseng* and introduced in PPT-producing engineered yeast, resulting in the production of F1 and Rh1 levels of 42 mg/L and 93 mg/L, respectively (Figure 1-1).[133]

1.6 DM-type triterpenoids secretion

The metabolic burden caused by natural products accumulation always hinders the efficiency of microbial cell factories. A solution to this issue lies in the extracellular production of triterpenoids, which can serve as a critical performance metric for intensifying product synthesis in fermenters. By adopting this approach, yeast cells can maintain their integrity during product harvest, leading to a higher yield of triterpenoid per cell. This method improves the purity and ease of harvesting triterpenoids and leads to an enhanced yield per substrate, a crucial factor for cost-effective products.[134] In an ideal case, as fermentation time increases, the space-time yield per year should also rise, thereby reducing equipment cleaning downtime, allowing for smaller fermenters, and lowering overall investment costs. Additionally, for intracellular products like triterpenoids, understanding the secretion/transport system of the target molecule is essential. This is also true for other substances, such as storage polymers (e.g., polyhydroxyalkanoates) and recombinant proteins.[134,135] While several studies have identified PPD-type triterpenoids in the extracellular environment (Table 1-2),[97,133,129] the underlying molecular mechanisms involved, whether through active transport, passive diffusion, or cell lysis, are still elusive. Therefore, research is needed to shed light on these mechanisms in the future. Members of the pleiotropic drug resistance family proteins, which are ABC transporters in plant cell membranes with two nucleotide-binding domains (NBDs) and two transmembrane domains (TMDs), have been shown to transport triterpenoids.[136] Initiated by binding the triterpenoid to the TMD, after which a closed NBD dimer is formed, the triterpenoid is transported across the membrane. Subsequently, ATP hydrolysis triggers the dissociation of the closed NBD dimer, the TMD opens, and triterpenoids are secreted to the extracellular space.[136] Whether these transporters can be exploited in yeast or if a yeast transporter can perform a similar function for triterpenoids remains to be determined.

Table 1-2. The secretion of PPD-type triterpenoids by *S. cerevisiae*.[22]

Strains	Triterpenoids	Cultivation set-up	Carbon source	Medium	Titer	Export	Ref.
S. cerevisiae ZD-PPD-010	PPD	Shake flask	Glucose	YPD	53 mg/L	Yes	[16]
S. cerevisiae ZD-PPD-016	PPD	Shake flask	Glucose	YPD	109 mg/L	Yes	[16]
S. cerevisiae ZD-PPD-016	DM	Shake flask	Glucose	YPD	142 mg/L	Yes	[16]
S. cerevisiae ZD-PPD-018	PPD	7.5 L Fermenter	Glucose, lysine	YPD	1.2 g/L	Yes	[16]
S. cerevisiae ZD-PPD-018	DM	7.5 L Fermenter	Glucose, lysine	YPD	1.5 g/L	Yes	[16]
S. cerevisiae W3a-ssPy	PPD	Shake flask	Glucose, ethanol	YPD	2 g/L	Yes	[98]
S. cerevisiae W3a-ssPy	PPD	5 L Fermenter	Glucose, ethanol	YPD	4 g/L	Yes	[98]
S. cerevisiae WLT-MVA5	PPD	5 L Fermenter	Ethanol	YNBE	8 g/L	Yes	[97]
S. cerevisiae W3a-ssPy	PPD	5 L Fermenter	Ethanol	YNBE	4 g/L	Yes	[97]
S. cerevisiae W303-1a GW10	PPD	5 L Fermenter	Glucose, xylose	SD	152 mg/L	Yes	[99]
S. cerevisiae W3a	PPD	Shake flask	Glucose	SD	1.4 g/L	Yes	[83]
S. cerevisiae W3a	PPD	5 L Fermenter	Glucose	SD	1.4 g/L	Yes	[83]
S. cerevisiae ZW04BY-RS	PPD	10 L Fermenter	Ethanol	YPD	11 g/L	Yes	[10]
Y. lipolytica YL-PPDS0	PPD	Shake flask	Glucose	YPD	13 mg/L	Yes	[119]
S. cerevisiae ZW-F1-17	F1	Shake flask	Glucose	YPD	42 mg/L	Yes	[133]
S. cerevisiae ZW-Rh1-20	Rh1	Shake flask	Glucose	YPD	93 mg/L	Yes	[133]
S. cerevisiae BY-V	PPD	Shake flask	Molasses	YPD	1.6 g/L	Yes	[99]
S. cerevisiae BY-V	PPD	5 L Fermenter	Molasses	YPD	16 g/L	Yes	[99]
S. cerevisiae LPPDS	PPD	Shake flask	Glucose	YPD	1.7 g/L	Yes	[137]

Batch fermentations of engineered yeast GW10 in a 5 L bioreactor using a mixed substrate

(glucose and xylose) indicated that a significant portion of PPD was secreted into the extracellular space.[99] Similarly, batch fermentation of engineered yeast ZD-PPD-018 in a 7.5 L fermenter at pH 5.5 produced 810 mg/L of PPD and 780 mg/L of DM, with over half of the triterpenoids found outside the yeast cells.[16] After introducing the *UGTPg100* and *UGTPg1* to PPT-producing yeast, the new strain successfully synthesized ginsenoside Rh1 and F1, with maximum titers of 40 mg/L and 90 mg/L, respectively, and most ginsenosides secreted into the medium.[138]

The explanation for triterpenoids export still needs to be discovered. It is speculated that saponins may co-accumulate with sterols in membranes, potentially affecting membrane integrity and extracellular triterpenoid production.[139] Another hypothesis is that the yeast cytoplasmic membrane swells when DM and PPD accumulate, resulting in unspecific product secretion.[16,83] However, these hypotheses remain unverified. It is known that *Panax* species triterpenoids are natural lipophilic compounds, and previous studies have shown that lipophilic terpenoids can accumulate in microbial membranes, causing membrane damage and affecting membrane function and intracellular substance synthesis.[108,140,141] Further investigation is needed to determine whether triterpenoids interact with or accumulate in the cell membrane..

1.7 Summary and perspectives

In recent years, metabolic engineering strategies that benefit from synthetic biology have been successfully applied to the synthesis of plant natural products, with artemisinic acid and farnesene as notable examples.[91,92] *Panax* species triterpenoids, prominent plant natural products with demonstrated health benefits, have been synthesized using engineered yeast cells (e.g., *S. cerevisiae* and *Y. lipolytica*) (Table 1-1).[20,25,58,131,142] Many patents related to *Panax* species triterpenoid production exist, such as the protein engineering of enzymes involved in triterpenoid synthesis,[143] engineering the ginsenosides synthesis pathway,[144] and optimizing the fermentation of ginseng plant cells.[145,146] Among these patents, 11 are on seven different recombinant ginsenosides produced by yeast (Table 1-3). Biological engineering strategies used in yeast synthesizing ginsenosides include heterologous gene expression, enzyme engineering, redox balancing, increasing precursor supply, compartment engineering, and optimization of fermentation conditions.[80,83,98,147,129,21]

Table 1-3. Patents on recombinant ginsenoside production by yeast.[22]

Ginsenosides	No. of patents	Producer	Country	Publication number
DM	4	*S. cerevisiae*	China, South Korea	CN104498577A, CN104450633A, CN104450769A, KR102207288B1
DM	1	*Pichia pastoris*	China	WO2014169666A1
PPD	3	*S. cerevisiae*	China	CN110616231A, CN110628805A, CN104611303A
PPT	3	Yeast	South Korea	US2019352649A1, US11046990B2, US11186616B2
Ginsenoside Ro	3	Yeast	South Korea	US2019352649A1, US11046990B2, US11186616B2
Ginsenoside	3	Yeast	South Korea	US2019352649A1, US11046990B2,

Rd				US11186616B2
Ginsenoside Rd1	3	Yeast	South Korea	US2019352649A1, US11046990B2, US11186616B2
Ginsenoside Rg1	3	Yeast	South Korea	US2019352649A1, US11046990B2, US11186616B2

Ginsenoside triterpenoids synthesized by metabolically engineered yeast mainly focus on the triterpenoids mentioned above, such as DM, PPD, PPT, Rh1, Rh2, F1, and Rg3.[113,148,133,129] Although *Pichia pastoris* has been explored for ginsenoside synthesis, the final titers are still low (e.g., 0.7 mg/g DCW).[149,150] Notable successes include PPD and its glycosylated derivative CK, achieving high titers of 11 g/L and 6 g/L, respectively.[10,113] However, the titer of amorpha-4,11-diene (sesquiterpene) by engineered yeast reached 40 g/L,[93] far exceeding any previous attempts. Hence, the potential of triterpenoid synthesis improvement in yeast remains to be further explored.

A recent study identified two *P. ginseng* glycosyltransferase genes, *PgUGT8* and *PgUGT18*, which catalyzed the glycosylation reaction of the second sugar moiety of C3 and C28 in the ginsenoside biosynthetic pathway, although resulting in low ginsenosides Ro production (1.4 μg/L).[151] Currently, over 200 ginsenosides have been identified from the 17 species in the genus *Panax*, with only a fraction of these molecules synthesized by engineered yeast (Table 1-4).[20,19,21,151] Although all ginsenosides found in plant species can be synthesized theoretically using yeast, the challenge increases with more modifications, especially those with numerous sugar residues, often due to unknown required enzymes. The prospection of enzymes involved in ginsenosides synthesis and the use of known enzymatic functions from other triterpenoid synthesis pathways will contribute to expanding the product spectrum.

Table 1-4. The highest production of ginsenosides in shake flask or fermenter.[22]

Triterpenoid	Yeast	Prodction	Shake flask or fermenter?	Ref.
DM	*S. cerevisiae*	350 mg/L	shake flask	132
DM	*S. cerevisiae*	7 g/L	1.3 L fermenter	132
20S-O-Glc-DM	*S. cerevisiae*	753 mg/L	shake flask	152
20S-O-Glc-DM	*S. cerevisiae*	5.6 g/L	3 L fermenter	152
3β-O-Glc-DM	*S. cerevisiae*	415 mg/L	shake flask	152
3β-O-Glc-DM	*S. cerevisiae*	2.4 g/L	3 L fermenter	152
PPD	*S. cerevisiae*	1.7 g/L	shake flask	137
PPD	*S. cerevisiae*	11 g/L	10 L fermenter	113
PPT	*S. cerevisiae*	250 mg/L	shake flask	132
PPT	*S. cerevisiae*	5 g/L	1.3 L fermenter	132
CK	*S. cerevisiae*	385 mg/L	shake flask	87
CK	*S. cerevisiae*	6 g/L	1.3 L fermenter	10
Ginsenoside Rg1	*S. cerevisiae*	105 mg/L	shake flask	132
Ginsenoside Rg1	*S. cerevisiae*	2 g/L	1.3 L fermenter	132
Ginsenoside NgR1	*S. cerevisiae*	114 mg/L	shake flask	132
Ginsenoside NgR1	*S. cerevisiae*	1.6 g/L	1.3 L fermenter	132

Ginsenoside NgR2	*S. cerevisiae*	42 mg/L	shake flask	[132]
Ginsenoside NgR2	*S. cerevisiae*	1.3 g/L	1.3 L fermenter	[132]
Ginsenoside F1	*S. cerevisiae*	36 mg/L	shake flask	[132]
Ginsenoside F1	*S. cerevisiae*	1 g/L	1.3 L fermenter	[132]
Ginsenoside Rh1	*S. cerevisiae*	18 mg/L	shake flask	[132]
Ginsenoside Rh1	*S. cerevisiae*	0.1 g/L	1.3 L fermenter	[132]
Ginsenoside Rf	*S. cerevisiae*	3 mg/L	shake flask	[132]
Ginsenoside Rf	*S. cerevisiae*	0.1 g/L	1.3 L fermenter	[132]
Ginsenoside Glc-Rf	*S. cerevisiae*	17 mg/L	shake flask	[132]
Ginsenoside Glc-Rf	*S. cerevisiae*	40 mg/L	1.3 L fermenter	[132]
Ginsenoside Rg3	*S. cerevisiae*	27 mg/L	shake flask	[129]
Ginsenoside Rh2	*S. cerevisiae*	355 mg/L	shake flask	[21]
Ginsenoside Rh2	*S. cerevisiae*	2.3 g/L	10 L fermenter	[113]
Ginsenoside Ro	*S. cerevisiae*	1.4 µg/L	shake flask	[151]

Substrates like ethanol, glycerol, and xylose have been shown to enhance PPD synthesis compared to glucose.[74,97] Depending on the application and the triterpenoid types, the yield on substrate will become of interest. However, increasing the titer is in focus to date, as low concentrations (in the lower gram or even milligram range) indicate high production costs.[153] This is also associated with the intracellular accumulation of the triterpenoids, requiring the destruction of the whole-cell catalyst. However, finding PPD and glycosylated derivatives in the supernatant suggests a potential to overcome this limitation.[16,83] To exploit the phenomenon, one would have to elucidate the transport mechanisms and distinguish them from simple cell lysis. An elusive triterpenoid transporter from plants would open new possibilities for further optimization of natural product synthesis. In addition, the success of in-situ extraction suggests at least a partial accumulation of some triterpenoids in or close to the cytoplasmic membrane.[136] Therefore, it is promising to improve the triterpenoid production of yeast by increasing the triterpenoid secretion outside the cell.

Metabolic network optimization can reduce by-products and increase synthesis pathway flux. Enzymes that supply cytosolic acetyl-CoA, including the acetyl-CoA synthetases, could be prime targets.[74,154] As the redox demand for triterpenoid synthesis is high, the NAD(P)-reducing aldehyde dehydrogenases and the monooxygenases involved in synthesis have to be well arranged for maximal activity.[82,83,101,105] The reductase CPR of the monooxygenases is also crucial for the high catalytic rate. Competing pathways, such as sterol synthesis using 2,3-oxidosqualene, need to be managed to favor triterpenoid synthesis.[20,82,97]

In summary, we envision a future where engineered yeast can produce ginsenosides and other triterpenoids cost-effectively and at high quality, reducing reliance on wild plants and conserving floral diversity. Overcoming the challenges outlined above, namely, lack of enzymatic activity, low enzymatic activity in the recombinant system, and intracellular production of target molecules, will make this future achievable.

1.8 Scope of this thesis

Ginsenosides, triterpenoids of Ginseng, are often not readily available, and hence, in this thesis, I present recombinant production of the ginsenosides DM and PPD using engineered *S. cerevisiae*.

In Chapter 1, I set the scene of ginsenosides and their production using metabolic engineering. The production of natural products is put in the context of the envisaged bioeconomy.

The methods used are presented in Chapter 2.

In Chapter 3, the hypothesis was that an existing DM and PPD producing yeast could be used as an example for high concentration production of triterpenoids in shake flasks. The idea followed was to use statistical experiment planning to explore the parameter space of the medium used and the growth conditions employed. Here, I focused on glucose, the salt mixture, glutamate, oxygen, and temperature. The resulting M3 medium could vastly increase PPD production. Furthermore, the positive results could be transferred to the production of lupeol-based triterpenoids, such as betulin and betulinic acid, and the production of the saponin CK. In summary, the hypothesis could be confirmed that the engineered strains, under appropriate conditions, can produce gram amounts of triterpenoids in simple shake flask experiments.

The challenge of intracellular products is the destruction of the catalyst during product harvest. Hence, I worked on the hypothesis that in situ extraction can overcome intracellular production. Combined with the results from Chapter 3, superior production was envisioned. In-situ extraction was carried out to promote the production and secretion of ginsenosides. The results indicated that almost all DM and PPD were extracted by adding vegetable oil at the end of the production phase. Notably, a white block rich in PPD and DM formed in the presence of 1 % or 2 % peanut oil or olive oil. This agglomeration of the triterpenoids will likely simplify downstream purification. In addition, the “empty yeast” can be reused for the next round of cultivation and production, reducing significantly time and resources for biomass synthesis. The total triterpenoid production was increased again in the second round of cultivation to more than 4 g/L, the highest production of triterpenoids in shake flasks to my knowledge.

The results are discussed in Chapter 5 in the context of natural product synthesis.

Chapter 2

Materials and Methods

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Author contributions:

Shangkun Qiu: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Visualization. **Lars M. Blank:** Conceptualization, Methodology, Supervision, Project administration, Funding acquisition, Review & Editing.

2.1 Materials and methods

2.1.1 Strains, medium, and reagents

Expression vectors were created using the pUCIDT-Amp-Goldengate vector (Integrated DNA Technologies, Iowa, United States) as backbone. Every expression vector consists of two homology arms (respectively 500 bp up and downstream of the locus targeted for genomic integration) that are forming the boundaries of a constitutively active TEF1 promoter, the coding DNA sequence (CDS) and 500 bp terminator of interest. Homology arms, CDS, with exception of UGPTg1, and terminators were amplified via PCR from the *S. cerevisiae* CEN.PK 102-5B genome. The *S. cerevisiae* codon-optimized CDS of UGPTg1 (GenBank: KF377585.1) was ordered as gBlock from (Integrated DNA Technologies, Iowa, United States). By ensuring 20 bp homology with the immediate down and upstream DNA fragment, the opted expression vectors were created via *in vivo* DNA (Gibson) assembly in *Escherichia coli* DH5α (C2987H, New England Biolabs, Massachusetts, United States).[155]

To facilitate genomic integration of the aforementioned expression vectors in the yeast genome, CRISPR plasmids derived from the pV1382 vector, a kind gift from Gerald Fink (Addgene plasmid # 111436; http://n2t.net/addgene:111436; RRID:Addgene_111436), were established.[156] First, the pV1382 vector was linearized through digestion by BsmBI (New England Biolabs, Massachusetts, United States) and dephosphorylated by shrimp alkaline phosphatase (New England Biolabs, Massachusetts, United States). Via ligation of the linearized vector with 5'-phosphorylated hybridized DNA primers, spanning the sgRNA sequence of interest, are the desired CRISPR plasmids obtained. sgRNA sequences were identified via CRISPOR.[157]

Bacterial transformations were done via heat-shock using the chemically competent *E. coli* DH5α strain (C2987H, New England Biolabs, Massachusetts, United States) as recipient of the cloned constructs. Cultivation took place in LB and agar supplemented with 100 µg/ml ampicillin (Carl Roth, Germany). Plasmid purification of transformants was done using the Monarch® Plasmid Miniprep Kit (New England Biolabs, Massachusetts, United States). Confirmation of sequence integrity was done via Sanger sequencing (Eurofins Genomics, Germany).

The previously engineered *S. cerevisiae* CEN.PK 102-5B derivative strain, 102-tm-reerg7-2PP was used for quantifying protopanaxadiol synthesis.[104, 158] Via CRISPR-Cas9 gene editing, the 102-tm-reerg7-2PP strain was further modified towards the production of Compound K. For this, two copies of the constitutively expressed *S. cerevisiae* codon optimized UGTPg1 under control of the TEF1 promoter and ADH1 terminator were integrated respectively near the ARS911 and ARS1309 loci. Compound K biosynthesis was enhanced by addition of a copy of constitutively expressed YNK1, UGP1 and PGM2 (each under control of the TEF1 promoter and respectively PRM9, GAT2 and SPO1 terminators), in respectively the ALG5, near the yprcdelta15, and ARS308 loci. Genomic integration events were verified using colony-PCR and Sanger sequencing (Eurofins Genomics, Germany) (Table S1). All strains and constructs are available on reasonable request.

S. cerevisiae BA6Δ (unpublished) is a *pah1* deletion mutant of BA6, generated through replacement of *PAH1* with a hygromycin resistance cassette (*YMR165CΔ*:hphMX).[159, 160] The engineered yeast BA6 for betulin synthesis was modified from *S. cerevisiae* Simo1575.[104] Yeast 102-tm-reerg7-2LP was modified from *S. cerevisiae* CEN.PK 102-5B for producing B and BA.[158] Standard WM8+ medium includes 50 g/L glucose, salt solution, 10 g/L glutamate, trace elements

solution, vitamins solution, Verduyn-trace elements solution, and Verduyn-vitamins solution. In addition, the salt solution includes 0.25 g/L $(NH_4)H_2PO_4$, 2.8 g/L NH_4Cl, 0.25 g/L $MgCl_2 \cdot 6H_2O$, 0.1 g/L $CaCl_2 \cdot 2H_2O$, 2 g/L KH_2PO_4, 0.55 g/L $MgSO_4 \cdot 7H_2O$, and 0.075 g/L $C_6H_{12}O_6$. The trace elements solution includes 1.75 mg/L $ZnSO_4 \cdot 7H_2O$, 0.5 mg/L $FeSO_4 \cdot 7H_2O$, 0.1 mg/L $CuSO_4 \cdot 5H_2O$, 0.1 mg/L $MnCl_2 \cdot 4H_2O$, 0.1 mg/L $Na_2MoO_4 \cdot 2H_2O$, and EDTA (0.04 mM). The vitamins solution includes 10 mg/L $C_6H_5NO_2$ (nicotinic acid), 25 mg/L $C_8H_{11}NO_3$ (pyridoxine), 10 mg/L $C_{12}H_{17}N_4OS$ (thiamine), 2.5 mg/L $C_{10}H_{16}N_2O_3S$ (biotin), and 50 mg/L $C_9H_{17}NO_5$ (calcium-d-pantothenate). Verduyn-Trace elements solution includes 15 mg/L Na_2EDTA, 4.5 mg/L $CaCl_2 \cdot 2H_2O$, 4.5 mg/L $ZnSO_4 \cdot 7H_2O$, 3 mg/L $FeSO_4 \cdot 7H_2O$, 0.3 mg/L $CuSO_4 \cdot 5H_2O$, 1 mg/L $MnCl_2 \cdot 2H_2O$, 0.4 mg/L $Na_2MoO_4 \cdot 2H_2O$, 0.3 mg/L $CoCl_2 \cdot 6H_2O$, 1 mg/L H_3BO_3, and 0.1 mg/L KI. Verduyn-vitamins solution includes 25 mg/L $C_6H_{12}O_6$ (myo-inositol), 1 mg/L $C_6H_5NO_2$ (nicotinic acid), 1 mg/L $C_8H_{11}NO_3$ (pyridoxine), 1 mg/L $C_{12}H_{18}Cl_2N_4OS$ (thiamin-HCl), 0.05 mg/L $C_{10}H_{16}N_2O_3S$ (biotin), 1 mg/L $C_9H_{17}NO_5$ (calcium-pantothenate), and 0.2 mg/L $C_7H_7NO_2$ (p-aminobenzoic acid).[80] The PPD standard was purchased from PhytoLab. The DM standard was purchased from AOBIOUS. The compound K standard was purchased from MedchemExpress (HY-N0904).

2.1.2 Design of experiment

The eight biological replicates were carried out in the 24-well plate (polypropylene square deep well MTP, re-usable, 24 wells of 11 ml volume, flattened, detoxified, Kuhner shaker, Germany). The fermentation conditions are the following: the total volume is 2 mL; the fermentation time is five days; the temperature is 30 ℃; the original OD of the main culture is 0.1; 300 rpm; the medium is WM8+; 50 mm shaking diameter.

Two temperatures (25 ℃ and 30 ℃) and three different volumes (1 mL, 2 mL, and 3 mL) were selected for evaluating the ethanol evaporation in the 24-well plate. The solvent for ethanol is water. The other fermentation conditions are as follows: the fermentation time is five days, 300 rpm, and 50 mm shaking diameter.

The 34 runs DoE was designed using Design Expert 11 (Stat-Ease, Inc.). The parameters are the following: the Study type is Factorial; the Subtype is Randomized; the Design type is Minimum Run Resolution V; the number of experiments is 34 runs; the Design Model is 2FI; there is no Blocks; the Center Points is 4. The experiments were carried out in 24-well plates.

2.1.3 Ordinary least squared (OLS) estimation

An OLS regression was used with two sets of linear formulas: 1. containing the individual factor ($\sum c_i F_i$, with c_i as the coefficient for each factor F_i=[A, B, C, D, E, F, G]) and 2. the individual factor with first order factor interactions ($\sum c_i F_i + \sum c_{ij} F_i F_j$, with c_{ij} as the coefficient for each two-factor interaction). A regression was performed with the seven factors A-G towards either PPD or DM, resulting in four regressions. All data was centered to have a mean value of 0 and a standard deviation of 1. The evaluation of the OLS models is based on the F-statistics, which reports whether a combination of independent variables (the factors in the OLS model) can explain the variance of the dependent variable (PPD or DM) if the p-value is below 0.05. The p-values of the individual factors in each regression model determine whether the factor significantly contributes to the prediction. The factors are selected by their individual p-values of their contribution to the OLS model. The impact of each factor is then determined by the

respective coefficient. The calculations are performed with the *ols* package in the *statsmodel* (v0.13.2) library in *Python*, and the code is provided as Jupyter Notebook in the Supplementaries.

2.1.4 Cultivation of engineered yeasts in shake flasks

Pre-cultures in the 500 mL shake flasks with an aluminum lid containing 50 mL WM8+ medium and inoculate each pre-culture with 500 µL of a thawed cryogenic stock of the engineered yeast (30 ℃, 200 rpm, and 50 mm shaking diameter). The pre-culture medium with an OD_{600} of 6-10 was used as the inoculum of the main culture. The start OD_{600} of the main culture was set to 0.4, measured with a single-use cuvette (polystyrene, semi-micro, 1.6 mL, Carl Roth, Germany) in a photometer (Biochrom Ltd., United Kingdom). All experiments were carried out in the 500 mL shake flasks with aluminum or membrane lids. The filling volume differed between 25 and 50 mL (see text for details). The cultivation temperature differed between 28 °C and 30 °C (200 rpm and 50 mm shaking diameter). At least duplicates were performed.

2.1.5 Determination of glucose, ethanol, DM, PPD, CK, and biomass concentrations

800 µL of cell broth were sampled, transferred into 2 mL reaction tubes (Eppendorf, Germany), and frozen at -20 ℃ until further use. For PPD and DM extraction, 250 µL of glass beads (0.5 mm diameter, Carl Roth, Germany) were added, followed by 800 µL chloroform: methanol (80:20 ratio) and 80 µL HCl (1 M). The samples were then placed into the MinibeadBeater (Biospec Products, USA) for 3 minutes twice to disrupt cells. For the yeast cells producing CK, 250 µL of glass beads (0.5 mm diameter, Carl Roth, Germany) were added, followed by 800 µL n-butanol and the samples were then placed into the MinibeadBeater (Biospec Products, USA) for 3 minutes twice to disrupt cells. For phase separation, samples were centrifuged for 10 minutes at 4 ℃, 13,000 rpm (Eppendorf Centrifuge 5418 R, Eppendorf, Germany). 500 µL of the lower organic phase (chloroform) or higher organic phase (n-butanol) containing the DM, PPD, and CK were filtered (4 mm syringe filters RC membrane, pore size 0.2 µm, Phenomenex, Germany) and transferred into HPLC vials (Flat N9 vials, screw neck, 1.5 mL, clear, Macherey-Nagel, Germany) and stored at 4 ℃ until HPLC analysis. DM, PPD, and CK concentration were determined with an HPLC system (UltiMate 3000 HPLC system, Thermo Fisher Scientific, USA) coupled to a Dionex Corona Veo Charged Aerosol Detector. The column was an EC150/4.6 NUCLEODUR C18 gravity column (Macherey-Nagel GmbH and Co. KG, Germany), the mobile phase was Acetonitrile: 0.2 % (v/v) formic acid, the column temperature was set to 40 ℃, the flow rate was programmed to 1.2 mL/min, the injection volume was set to 10 µL, the runtime was 18 min. To measure the cell dry weight (CDW), 2 mL culture was harvested by centrifugation for 5 minutes at 4 ℃, 13,000 rpm (Eppendorf Centrifuge 5418 R, Eppendorf, Germany). The collected yeast cells were washed with deionized water and oven-dried to a constant weight. An OD_{600} to CDW correlation of CDW (g/L)=$0.2\times OD_{600}$+1.3 was estimated (Figure 2-1). All values are reported as the average of three replicates.

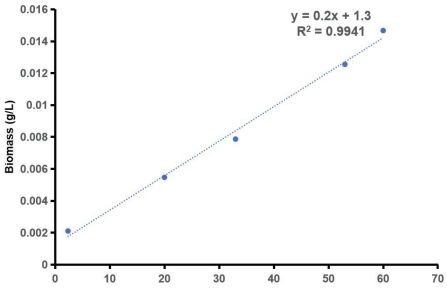

Figure 2-1. The correlation of OD and biomass. This correlation applies to OD values greater than 2.36. The fermentation condition is 30 ℃, WM8+ medium, 250 rpm, and 50 mm shaking diameter.

The supernatant of each sample was taken for analyzing the glucose and ethanol. Then the supernatant was filtered (syringe filters ROTILABO Mini-tip cellulose acetate (CA), pore size 0.2 µm, Carl Roth, Germany) into HPLC vials and stored at 4 ℃ for later HPLC analysis. The HPLC system for glucose and ethanol analysis was UltiMate 3000 HPLC (Thermo Fisher Scientific, USA) coupled to an UltiMate 3000 Variable Wavelength Detector (UV detector) as well as a Shodex RI-101 Refractive Index Detector (RI detector). The column is Metab-AAC 300 x 7.8 mm (P/N A1BF-A1AA0N) (ISERA GmbH, Germany) with a styrene divinylbenzene copolymer as the stationary phase. The mobile phase is 5 mM H_2SO_4. The column temperature is 30 ℃. The flow rate is 0.6 mL/min. The injection volume is 20 µL. The runtime is 30 min.

2.1.6 Water evaporation

Water evaporation was monitored using 500 mL shake flasks with either the aluminum or membrane lid. Different water volumes (25 mL and 50 mL) were incubated in a shaker for 120 h (28 ℃, 200 rpm, and 50 mm shaking diameter).

2.1.7 Online respiration activity measurement (OTR, CTR) in shake flasks

The online respiration activity was monitored in the TOM shaker (Kuhner shaker). Fig 2-2 illustrates the set-up of a TOM shaker with eight measuring 500 mL shake flasks running in parallel for sampling. For detailed information on the general set-up of TOM shakers, refer to the literature presented by Anderlei and Büchs [161]. The media used for this experiment are WM8+ and M3 media; the temperature is ℃, 200 rpm.

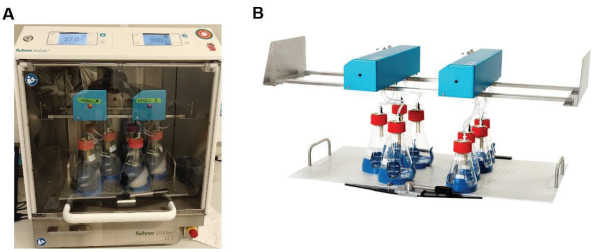

Figure 2-2. Online respiration activity measurement was carried out in the TOM shaker. (A) is the TOM shaker,

and (B) is the inside of the shaker.

2.1.8 Optimization of total triterpenoid production by adding isopropyl myristate (IPM) and vegetable oils

For the in-situ extraction of adding IPM, the experiment was carried out in the 500 mL shake flask with a membrane lid; the 150 % (v/v) IPM was added to 10 mL broth after five days of fermentation and incubated in a shaker for one day. Then, the IPM supernatant was separated from the pellet by centrifuging for 10 mins at 5000 rpm. The pellet was washed using phosphate-buffered saline (PBS). After that, the pellet was again dissolved in 25 mL fresh M3 medium for the second round of fermentation.

The experiment of adding different concentrations of sunflower oil (1 %, 2 %, 4 %, and 6 % v/v) to the medium at different fermentation times (i.e., 48 h, 72 h, 96 h) was carried out in the 500 mL shake flask with a membrane lid. 1 % v/v Tween 80 was also added to the medium with different concentrations of sunflower oil to make an emulsion and avoid cells adhering to the shake flask wall. The total volume of the medium is 25 mL. The fermentation condition is 28 ℃, 200 rpm, M3 medium, and 50 mm shaking diameter. The experiment of adding different concentrations of peanut or olive oil (1 %, 2 %, 4 %, and 6 % v/v) to the medium at different fermentation times (i.e., 24 h, 48 h, 72 h, 96 h) was carried out in the 24-well plate and shake flasks. The total volume of the medium is 2 mL in the 24-well plate and 25 mL in shake flasks. The fermentation condition is 28 ℃, M3 medium, 250 rpm for the cultivation in the 24-well plate and 200 rpm for the cultivation in shake flasks, and 50 mm shaking diameter. The other parameters are the same as those in the above cultivation protocol. The samples were centrifuged for 1 min at 13000 rpm, and the supernatant of each sample was taken to analyze the PPD and DM extracellularly. For in-situ extraction, 10 mL broth was collected after five days of fermentation and centrifuged at 13,000 rpm for 10 mins at room temperature. The pellet was again dissolved in 25 mL fresh M3 medium for the second round of fermentation.

Chapter 3

A simple protocol for increased ginsenoside and universal triterpenoids production by engineered yeast

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Shangkun Qiu: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Visualization. **Mariam Dianat:** Software, Formal analysis, Writing - Review & Editing. **Conrad Müller:** Software, Formal analysis. **Roberto-Michael Zarazua-Navarro:** Methodology. **Ulf Liebal:** Software, Formal analysis, Writing - Review & Editing. Roberto-Michael Zarazua-Navarro: Validation. **Roy Eerlings:** Conceptualization, Validation, Formal analysis, Investigation, Writing - Review & Editing. **Lars M. Blank:** Conceptualization, Methodology, Supervision, Project administration, Funding acquisition, Review & Editing.

3.1 Abstract

Ginseng is a traditional Chinese herbal medicine that has garnered significant attention for its therapeutic potential. At the heart of its pharmacological effects are ginsenosides, the primary active metabolites, many of which are dammarane-type triterpenoids sharing protopanaxadiol as a common precursor. In this Chapter, we optimized the fermentation conditions using a Design of Experiment approach, achieving 1.2 g/L protopanaxadiol in simple shake flask cultivations. Applying this optimized setup to other triterpenoids, such as CK, resulted in a 7.3-fold increase (0.22 g/L) in titers. These cultivation conditions enable the production of natural triterpenoids. We pave the way for the sustainable production of bioactive compounds from plants through synthetic biology, microbial engineering, and cultivation.

3.2 Introduction

Ginseng (*Panax spp.*), part of the Araliaceae family, has been a critical element in Asian traditional herbal medicine for over 4,000 years.[20] Ginseng extracts are valued for their anti-cancer, anti-diabetic, anti-aging, anti-inflammatory, cardiovascular-protective, and COVID-19 inhibiting properties, attributed to triterpenoid ginsenosides.[18, 162, 163,154] Over 200 ginsenosides, categorized into dammarane-type tetracyclic and oleanane-type pentacyclic, have been identified in the *Panax* genus.[20-22, 19] Dammarane-type tetracyclic ginsenosides can be further subdivided into ocotillol, protopanaxadiol (PPD)-type ginsenosides, and protopanaxatriol (PPT)-type ginsenosides, which differ in the carbohydrate moieties at C3, C6, and C20 positions and the respective hydroxylation pattern.[22] However, ginseng roots have a low ginsenoside content (60 mg/g dry weight), and commercial production is complex due to the plant's long cultivation period and environmental susceptibility. Chemical synthesis of ginsenosides is economically impractical.[163, 17, 77, 80]

Recombinant yeast cell factories have emerged as a promising alternative for ginsenoside production. Yeast can naturally synthesize intermediates like squalene and 2,3-oxidosqualene, which are necessary for ginsenoside synthesis, offering advantages such as rapid growth, controlled culture conditions, and sustainable production.[20, 104, 164] Yeast's redox systems and advances in microbial engineering make it suitable for producing various plant-derived metabolites.[89, 127, 165] Taken together, yeasts are considered excellent cell factories for producing natural plant products, including but not limited to ginsenosides, like betulinic acid, farnesene, and amorpha-4,11-diene.[77, 80, 84, 93, 113]

Recent studies have successfully introduced the ginsenoside biosynthetic pathway in *S. cerevisiae*, achieving significant production levels of dammarenediol-II, PPD, PPT, and CK.[20, 137, 151] Via overexpression of the native mevalonate synthesis route as well as through introduction and optimization of the PPD synthetic pathway, a final PPD production of 11 g/L was achieved in 10 L fed-batch fermentation,[113] while introducing and optimizing the CK synthesis pathway achieved almost 6 g/L of CK.[10]

Optimal metabolites production requires both genetically engineered strains and meticulous bioprocess optimization.[15, 166, 167] Parameters like carbon sources (e.g., glucose, glycerol, and glutamate), nitrogen sources, temperature, pH, oxygen, inoculum size, and salts significantly affect the cell's growth and, thus, metabolites production.[166, 168, 169] The DoE experiment can be applied to identify the correlations among different process parameters and their influence on the final productivity by performing a large number of experiments.[170] For example, optimizing the concentration of glucose, yeast extract, and peptone via the Box-Behnken design increased the geranylgeraniol production by 1.75-fold.[171] Therefore, bioprocess optimization is a promising strategy to enhance metabolites production.

In this study, we optimized the cultivation conditions of *S. cerevisiae* to maximize the ginsenosides titers in shake flask cultivation. We focused on PPD and DM (Figure 3-1), the precursors of high-value ginsenosides that exhibit valuable biological properties.[97, 100] By adjusting temperature, glucose, glutamate, salts solution, trace element solution, vitamin solution, and oxygen availability, PPD production reached 1.2 g/L. Applying these optimized conditions to betulin and CK-producing strains further increased product titers, demonstrating the transferability of the optimized conditions towards alternative, more complex ginsenosides and triterpenoids. This simple setup facilitates the evaluation of engineered triterpenoid synthesizing yeast, and the

amounts of triterpenoid produced enable advanced analytics and even facilitate first ideas for purification and the use of this intriguing class of natural products.

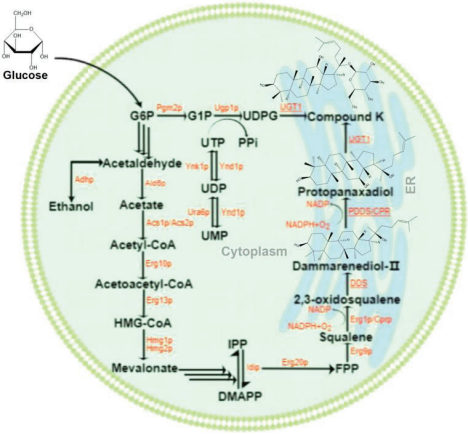

Figure 3-1. The PPD synthesis pathway in engineered *S. cerevisiae*. The conversion of squalene to the final ginsenosides happened in the ER. The recombinant enzymes are underlined. G6P, glucose 6-phosphate; Adhp, alcohol dehydrogenase; Ald6p, NADH-generating aldehyde dehydrogenase; Acs1p and Acs2p, acetyl-CoA synthetase 1 and 2; Erg10p, acetyl-CoA acetyltransferase; Erg13p, HMG-CoA synthase; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; Hmg1p and Hmg2p, HMG-CoA reductase 1 and 2; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; Idip, isopentenyl diphosphate isomerase; Erg20p, farnesyl diphosphate synthase; Erg9p, squalene synthase; Erg1p, squalene monooxygenase; Cprp, NADPH-cytochrome P450 reductase; DDS, dammarenediol-II synthase; PPDS, protopanaxadiol synthase; CPR, NADPH-cytochrome P450 reductase; ER, endoplasmic reticulum; UGT1, UDP-glycosyltransferase 1; Ugp1p, UTP-glucose 1-phosphate uridylyltransferase 1; UDPG, uridine diphosphate-glucose; Pgm2p, phosphoglucomutase 2; G1P, glucose 1-phosphate; UMP, uridine monophosphate; UDP, uridine diphosphate; UTP, uridine triphosphate; PPi, pyrophosphate; Ura6p, uridylate kinase; Ynk1p, Nucleoside diphosphate kinase; Ynd1p, apyrase. This image was created by Figdraw.

3.3 Results and discussion

3.3.1 Effect of medium composition, temperature, and oxygen availability on DM and PPD Production

Eight biological replicates were carried out in the 24-well plate before the formal DoE experiment to test the errors caused by the uncertainties of growing yeast cells in the 24-well plate. Two different times were selected for conducting the eight biological replicates: the first eight were started on Thursday, and the second eight were started on Friday. The samples were taken at 96 h and 120 h, and the total cultivation time was five days. The result showed that the PPD production of both eight biological replicate groups was stable, indicating that minor errors exist (Figure 3-2). Still, apparent errors can be avoided when cultured yeast in a 24-well plate. The DoE experiment can be carried out in the 24-well plate.

Ethanol evaporation also occurs in the 24-well plate, which may influence the carbon flux

toward the target ginsenosides and decrease the final ginsenosides production. Therefore, we also determined the evaporation rate of ethanol in the 24-well plate at different temperatures. Two temperatures were selected for evaluation, 25 ℃, and 30 ℃. Three different medium volumes were chosen to investigate if the medium's volume would influence ethanol evaporation. The results showed that the ethanol evaporation rate generally increased with time, which was higher at 30 ℃ than at 25 ℃. The higher volume can decrease the ethanol evaporation in the 24-well plate (Figure 3-2 C). This indicated that ethanol evaporation in the 24-well plate may influence the final ginsenosides production.

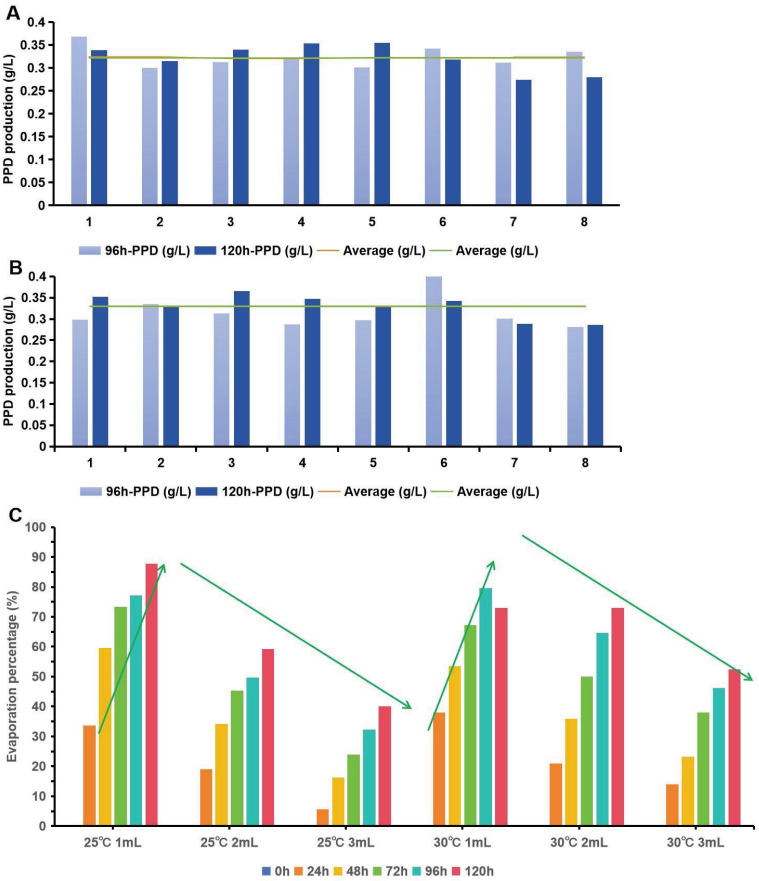

Figure 3-2. The PPD production of both eight groups of biological replicates by the engineered yeast and ethanol evaporation rate in the 24-well plate. (A) The PPD production by the engineered yeast cultured in the 24-well plate on Thursday. (B) The PPD production by the engineered yeast cultured in the 24-well plate on Friday. Cultivation conditions: 30 ℃, 2 mL, WM8+ medium, 200 rpm. (C) The ethanol evaporation rate in the 24-well plate.

The DoE experiment included 34 runs designed using Design Expert 11 (Stat-Ease, Inc.). The seven significant factors and the high, medium, and low categories are shown in Table 3-1. The Duetz microtiter plates system was used for cultivation (Enzyscreen). Samples were taken after 72 h, 96 h, and 120 h of cultivation and subsequently analyzed for the PPD and its immediate precursor, DM. DM and PPD concentrations were highest after 120 h. PPD production reached 0.3 g/L in four runs (1, 10, 12, and 33) (Figure 3-3A). Among them, run 1, run 10, and run 12 were all cultured at 20 ℃, and run 33 was cultured at 30 ℃. Glucose and glutamate concentrations were three-fold compared to standard WM8+ medium (50 and 10 g/L) for runs 1, 12, and 33. Notably, the salt concentration of all four runs was 0.33-fold of standard WM8+ medium. Also, higher oxygen availability was the same in these four runs, using only 1 mL of culture broth. The PPD concentration reached 0.9 g/L in run 1, the highest PPD production among all 34 runs in the DoE experiment.

Table 3-1. DoE for improving the standard WM8+ medium and fermentation conditions.

Run	Factor A: Glucose	Factor B: Salts	Factor C: Glutamate	Factor D: Trace element	Factor E: Vitamin	Factor F: Temperature [℃]	Factor G: Volume [mL]
1	3	0.33	3	3	0.33	20	1
2	1.67	1.67	1.67	1.67	1.67	25	2
3	3	3	0.33	3	0.33	30	3
4	3	3	0.33	0.33	3	30	3
5	0.33	0.33	0.33	3	0.33	20	3
6	3	3	3	3	3	20	1
7	3	3	0.33	3	0.33	20	1
8	0.33	0.33	3	0.33	0.33	30	1
9	0.33	0.33	0.33	0.33	3	30	1
10	0.33	0.33	3	3	3	20	1
11	0.33	3	3	0.33	3	30	1
12	3	0.33	3	0.33	3	20	1
13	0.33	3	3	3	0.33	20	3
14	0.33	3	3	0.33	3	20	3
15	0.33	0.33	0.33	3	0.33	30	1
16	0.33	3	0.33	0.33	0.33	30	3
17	3	3	0.33	0.33	0.33	30	1
18	0.33	0.33	3	0.33	0.33	20	3
19	0.33	3	0.33	3	3	30	1
20	3	0.33	3	3	3	20	3
21	3	3	3	0.33	0.33	30	3
22	3	0.33	0.33	3	3	20	1
23	1.67	1.67	1.67	1.67	1.67	25	2
24	0.33	3	0.33	0.33	3	20	1
25	1.67	1.67	1.67	1.67	1.67	25	2
26	3	0.33	0.33	0.33	0,33	30	3
27	3	3	3	3	3	30	3

28	3	3	0.33	0.33	0.33	20	3
29	0.33	0.33	3	3	0.33	30	3
30	3	0.33	0.33	0.33	3	20	3
31	0.33	0.33	0.33	3	3	30	3
32	0.33	3	3	0.33	0.33	20	1
33	3	0.33	3	3	3	30	1
34	2	1.67	1.67	1.67	1.67	25	2
CT	1	1	1	1	1	30	2

*Every factor has a low (red), medium (yellow), and high (green) level. For factors A-E, the figures in the table represent fold changes in the actual concentration compared to the standard WM8+ medium. The low level means that the concentration is 0.33-fold compared to the WM8+ medium, the medium level means that the concentration is 1.67-fold compared to the WM8+ medium, and the high level means that the concentration is 3-fold compared to the WM8+ medium. For factor F, the figures in the table represent the actual temperature. The low level means that the temperature is 20 ℃, the medium level means that the temperature is 25 ℃, and the high level means that the temperature is 30 ℃. For factor G, figures in the table represent the volume of the medium in 24-well plates. The low level means that the volume is 1 mL, the medium level means that the volume is 2 mL, and the high level means that the volume is 3 mL. CT: Control experiment, standard WM8+ medium.

DM production suggests triterpenoid synthesis. However, it is also a potential limitation in the subsequent P450-catalyzed reaction to PPD. Only seven runs showed DM production (Figure 3-3B). Among the seven runs, only runs 21 and 27 had DM concentrations above 0.3 g/L. These two runs' glucose, glutamate, and salt concentrations were three-fold that of standard WM8+ medium. These results suggest high glucose and glutamate concentrations support high DM and PPD production. At the same time, low salt concentration, low temperature, and high oxygen concentration might benefit PPD production, indicating that the P450-catalyzed hydroxylation is somewhat challenging, with substrate limitation and a slow rate of catalysis.

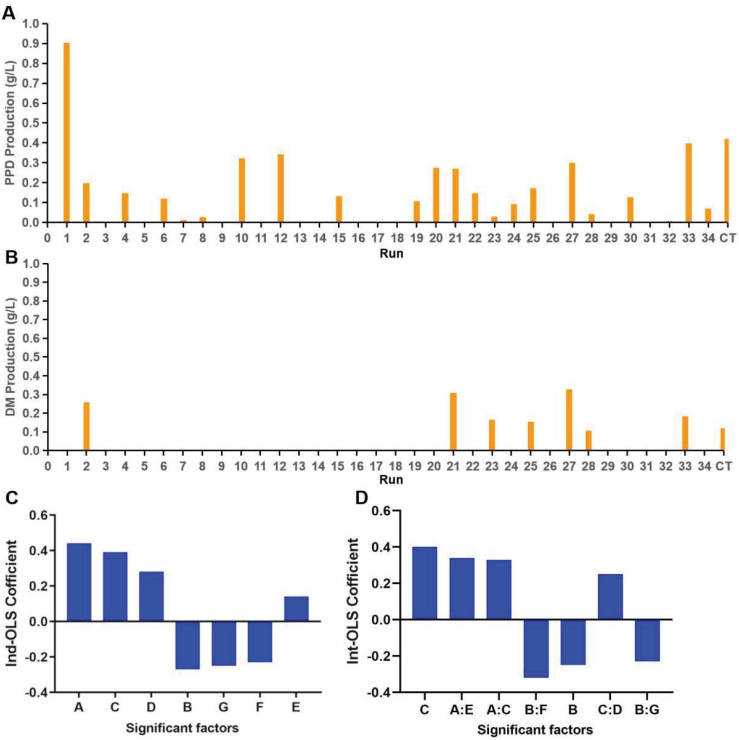

Figure 3-3. The results of the 34 runs of the DoE experiment and the OLS regression of the DoE data with the prediction of PPD production. (A) PPD production by engineered yeast in 24-well plates. (B) DM production at different fermentation conditions in 24-well plates. CT: Control experiment, standard WM8+ medium. (C) The OLS regression result of the Ind-OLS. (D) The OLS regression result of the Int-OLS. The coefficients with decreasing absolute values are shown for factors (Ind-OLS) and factor interactions (Int-OLS), significantly explaining the variance (p-values below 0.05). The larger the absolute values of the coefficients, the greater the influence of this factor or factor interaction on the final PPD production. A: glucose; B: salts; C: glutamate; D: trace element solution; E: vitamin solution; F: temperature; G: medium volume.

3.3.2 The ordinary least squares (OLS) estimation of the DoE data

Because the Response Surface is inconclusive regarding the relationship between PPD and DM production with the significant factors, the DoE data was analyzed with OLS regression to PPD and DM to identify the predictive factors. For this, we tested models containing only the individual factor effects (Ind-OLS) as well as individual factors combined with two-factor interactions (Int-OLS) (Table 3-2). The Ind-OLS and the more complex Int-OLS explain 41% and 83% of the variance in PPD, respectively (adj. R^2(Ind-OLS)=0.41, adj. R^2(Int-OLS)=0.83) (Table 3-2). There are two significant factors for Ind-OLS (A: glucose, C: glutamate) and seven for Int-OLS (B: salt, C: glutamate, AxE (vitamins), AxC, B (salt)xF (temperature), CxD (trace

element), BxG (oxygen)) (Figure 3-3C and D). In the simple Ind-OLS model with individual factors, glucose and glutamate are the most influential predictors that positively contribute to PPD production, with correlation values of 0.4 (Figure 3-3C). Conversely, salt concentration (B), temperature (F), and medium volume (G) correlate negatively to the final PPD production, albeit with low correlation values at -0.2 (Figure 3-3C). In the more complex Int-OLS model, glucose is only significant via the two-factor interactions with glutamate (A:C) and vitamin (A:E) but no longer as an individual factor. The most significant single factors in the complex Int-OLS model are the positive correlation with glutamate (C) and the negative correlation with salts (B). While the volume level (G, oxygen) is just above the significance value (p=0.05) individually, it negatively correlates with PPD and is also negative with the significant interaction with salts (BxG). Thus, less oxygen via higher volumes decreases PPD (Figure 3-3C). Furthermore, NADPH is oxidized to NADP by a P450 reductase in the presence of oxygen, then transfers electrons to the monooxygenases, such as PPDS.[22] Therefore, controlling the oxygen concentration will also play a critical role in synthesizing the final product. Consequently, in the subsequent investigation, we selected glucose, glutamate, salts, temperature, and oxygen as the main significant factors for further research to check if these factors will affect the final PPD and DM production.

Table 3-2. Regression results for OLS with seven factors alone and first-order interactions of two parameters.

Observable	Equation	F-Statistic (p-value)	Adj. R^2
PPD	Ind-OLS: $\sum c_F F$	0.003	0.41
DM		0.14	0.14
PPD	Int-OLS: $\sum c_F F + \sum c_{Fij} F_i F_j$	0.02	0.83
DM		0.83	-0.53

*The F-Statistic shows the p-value for the hypothesis that the independent variables of the OLS model explain the variability of the dependent variable, R^2 is the percentage of explained variance, Adj. R^2 is a multi-variable corrected correlation coefficient.

3.3.3 Effect of temperature on DM and PPD production

Sesquiterpenoid production by engineered *S. cerevisiae* was reported to be better at 25 °C than at 30 °C.[172] We also investigated temperature as an optimization parameter for PPD production. Here, the highest PPD production was observed at 20 ℃ in the 34 runs DoE experiment (Figure 3-3A). Temperatures above 30 °C were reported to cause stress to baker´s yeast and decrease cell viability.[169] Therefore, the 15 runs of the DoE experiment cultured at 20 ℃ were selected to investigate the effect of different temperatures on DM and PPD production in 24-well plates. To explore the optimum temperature, 18 ℃, 20 ℃, 22 ℃, 25 ℃, 28 ℃, 30 ℃, and 32 ℃ were selected. The other fermentation and medium conditions remained constant. In general, the production of PPD increased with increasing temperature and reached the highest value at 28°C, then decreased with increasing temperature (Figure 3-4A). Especially, the PPD productions of runs 1 and 12 are the highest among the 15 runs, reaching 1.5 g/L at 28 ℃ (Figure 3-4A). But for run 13, run 14, run 18, run 20, run 24, run 30, and run 32, the PPD production reached the

highest value at 32 ℃. However, the PPD production for these seven runs is lower than runs 1 and 12 (Figure 3-4A). So, we can speculate that the optimum temperature for PPD production is 28 ℃.

The DM production of run 1, run 12, and run 24 reached the highest when cultured at 30 ℃. However, the optimum temperature of run 6 and run 32 is 25 ℃, run 10, run 20, run 22, and run 28 is 28 ℃, and run 30 is 32℃ (Figure 3-4B). The first reason why the optimum temperatures of PPD and DM synthesis in all the runs are different may be that the different concentrations of compounds in the medium can affect the DM conversion to PPD at different temperatures. Another reason may be that the different medium compositions affect the enzyme activities involved in PPD and DM synthesis at different temperatures. The DM production of run 28 is the highest when cultured at 28 ℃, which reached 1.1 g/L (Figure 3-4B). The DM production of run 22, run 28, and run 30 are all over 0.8 g/L. The total triterpenoid production of run 1 is the highest among all the runs, cultured at 30 ℃ and reached 2.3 g/L (Figure 3-4C). The total triterpenoid production of run 1, run 12, and run 30 is over 2 g/L. However, the total triterpenoid production of run 1 and run 12 reached 2 g/L when cultured at 28 ℃ or 30 ℃, while run 30 reached 2 g/L at 32 ℃ (Figure 3-4C). Hence, the optimum temperature for DM and total triterpenoid production is 28-32 ℃.

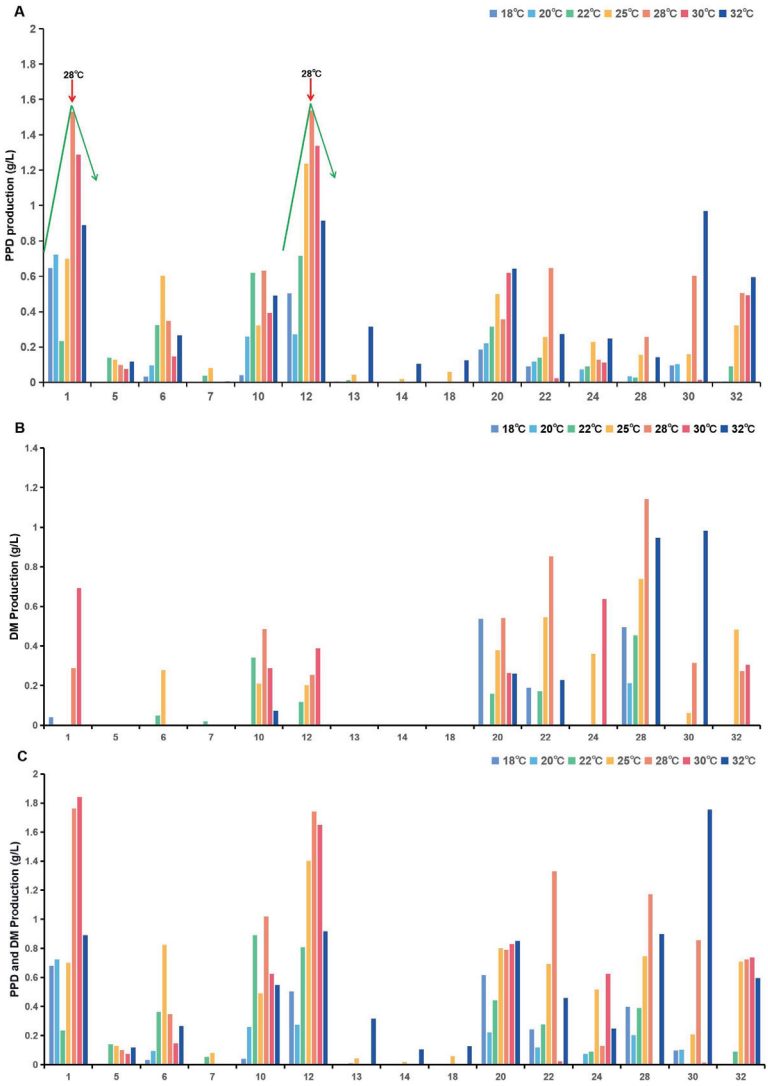

Figure 3-4. The effect of different temperatures on DM and PPD production. (A) The comparison of PPD production at 18℃, 20℃, 22℃, 25℃, 28℃, 30℃, and 32℃. (B) The comparison of DM production at 18℃, 20℃, 22℃, 25℃, 28℃, 30℃, and 32℃. (C) The comparison of total triterpenoid production at 18℃, 20℃, 22℃, 25℃, 28℃, 30℃, and 32℃. The horizontal coordinate is every run's number of the DoE experiment. The medium composition of these runs are same with Table 1.

3.3.4 Effect of glucose, glutamate, and salt concentration on DM and PPD production

According to the results above, runs 1 and 12 have the highest PPD and total triterpenoid production. The conditions of runs 1 and 12 were chosen for further investigation (Figure 3-4). Glucose, glutamate, and salt were selected to be varied in concentration, while all other parameters were kept constant. The optimum salt concentration for PPD production seems to be 0.33, 0.44, or 2 folds the WM8+ medium, with the highest PPD production at 2 g/L (Figure 3-5A). Here, it is indicated that the optimum concentration of glucose and glutamate for PPD synthesis is two times that of the standard WM8+ medium. Higher concentrations might cause osmotic stress and deflect resources from PPD production (Figure 3-5A).

DM production profits from increased salt concentration, and this is different from PPD. Again, three times or even higher concentrations of glucose and glutamate abolish DM production. The highest DM production of all the runs was 1.6 g/L (Figure 3-5B). Indeed, doubling the glucose and glutamate concentrations of WM8+ medium under these conditions, total triterpenoid production exceeded 3 g/L (Figure 3-5C).

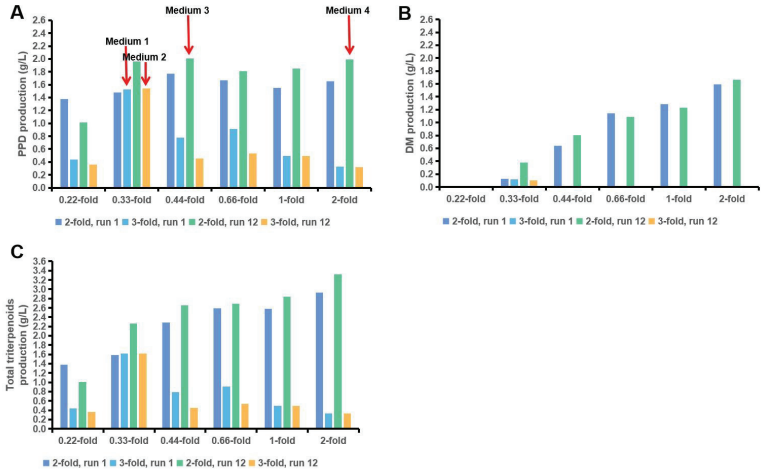

Figure 3-5. The effect of glucose, glutamate, and salt concentration on DM and PPD production. (A) PPD production depends on glucose, glutamate, and salts. (B) DM production is dependent on glucose, glutamate, and salts. (C) The total triterpenoid production is dependent on glucose, glutamate, and salts. The glucose and glutamate concentration is two or three times the standard WM8+ medium. The horizontal coordinate is the salt concentration compared to the standard WM8+ medium. Runs 1 or 12 means the medium is modified from run 1 or run 12.

For the translation of intracellular products, the yield per biomass is central, as is the yield on substrate for any biotechnological process. Here, biomass generally increased with increasing salt concentrations under constant carbon sources (Figure 3-6). The highest biomass yield per gram carbon source is 328 $mg_{CDW}/g_{glucose\ and\ glutamate}$ in two-fold salt concentration (Figure 3-6C). The triterpenoid yield increased with increasing salt concentrations when glucose and glutamate concentration were twice that of the standard WM8+ medium. However, the PPD yield first

increases and then decreases with increasing salt concentrations (Figure 3-6A and C). The highest total triterpenoid yield is 38 mg/$g_{glucose\ and\ glutamate}$ (Figure 3-6C). At the same time, the highest PPD yield is 21 mg/$g_{glucose\ and\ glutamate}$ (Figure 3-6C).

The total triterpenoid content of ripe ginseng root is 0.1-3 %.[163] We here estimated the CDW content of PPD, DM, and total triterpenoids. The CDW content of the total triterpenoid of three runs is above 120 mg/g_{CDW} (Figure 3-6E and G). The carbon source concentration of these three runs is two times that of the standard WM8+ medium. The salt concentration is 0.22, 0.33, or 0.44 times the reference. All the data indicates that low salt concentrations favor high CDW content of PPD. In contrast, high salt concentrations can contribute to increased CDW content of DM (Figure 3-6E and G). The optimum glucose and glutamate concentration for the CDW content of PPD and total triterpenoids is two times of the standard WM8+ medium.

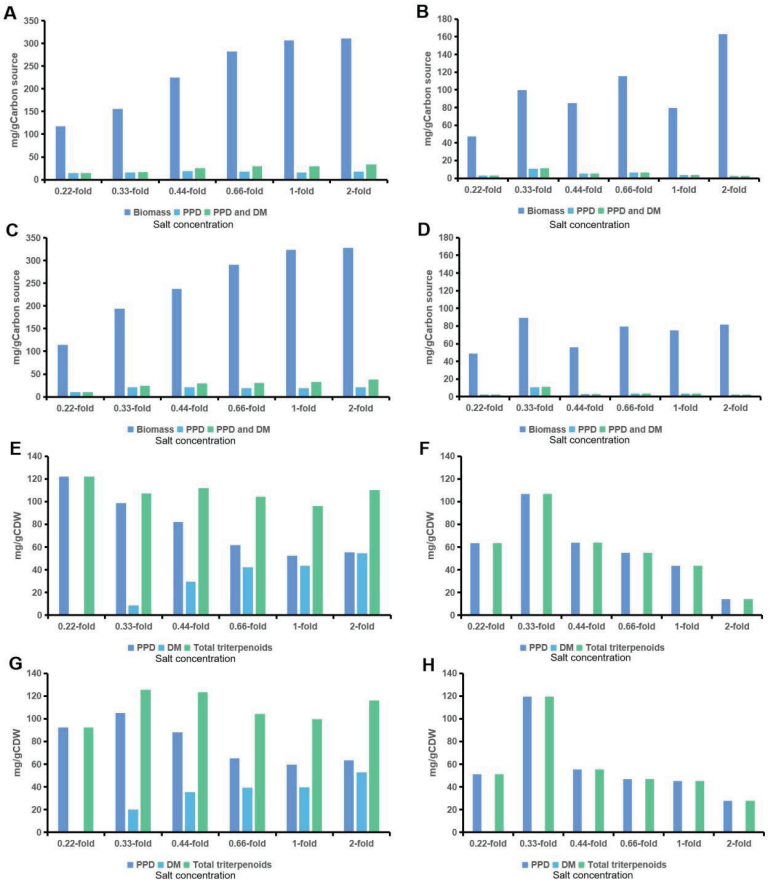

Figure 3-6. The effect of glucose, glutamate, and salt concentration on triterpenoid yield and CDW content of

triterpenoids. (A) and (E) The glucose and glutamate concentration is two times the standard WM8+ medium. These two media are modified from run 1. (B) and (F) The glucose and glutamate concentration is three times the standard WM8+ medium. These two media are modified from run 1. (C) and (G) The glucose and glutamate concentration is twice the standard WM8+ medium. These two media are modified from run 12. (D) and (H) The glucose and glutamate concentration is three times the standard WM8+ medium. These two media are modified from run 12. The horizontal coordinate is the salt concentration compared to the standard WM8+ medium. All other parameters were used as described in Figure 3-5 A-C.

3.3.5 Triterpenoid production in 500 mL shake flasks

While microtiter plate cultivation allows strain comparison, shake flask cultivations can be designed to test product purification ideas and synthesize the first products for use. For this scale increase, we selected four media from the above experiments, which are medium 1 (3-fold, run 1), medium 2 (3-fold, run 12), medium 3 (2-fold, run 12), and medium 4 (2-fold, run 12) (Figure 3-5A). Yeasts were cultivated in two types of 500 mL shake flasks. The shake flask was closed by either an aluminum or a membrane lid (Figure 3-7A). The membrane lid facilitates better aeration when compared to the aluminum lid. Two enzymes in PPD synthesis require oxygen: the conversion of squalene to 2,3-oxidosqualene, catalyzed by Erg1p, and the conversion of DM to PPD, catalyzed by PPDS (Figure 3-1).[101, 173] First, yeasts were cultivated in the shake flask with an aluminum lid. The smaller the filling volume of the shake flask, the better the aeration. Two different filling volumes were used, i.e., 25 mL and 50 mL. The yeast was cultured in the four improved media. The results showed the highest PPD production of 0.8 g/L occurred in medium 3 with 25 mL filling volume. The PPD production decreased with the increase in the medium volume. The PPD production in all four media tested is higher than that in the WM8+ medium, which is 0.16 g/L. It can be speculated that the higher oxygen concentration benefits the synthesis of PPD, which can increase the final PPD production in the shake flask (Figure 3-7B).

The identical experiments were repeated using the membrane lid for improved aeration. Notably, water evaporation is higher using the membrane lid (Figure 3-7C and D), although the shaker was equipped with a moisture controller, keeping water content in the air at 70% saturation. To account for evaporation, we calculated the water evaporation in the 500 mL shake flask with both types of lids at 28 ℃ (Figure 3-7C and D). We measured the volume after 120 h fermentation and calculated the product concentration without evaporation. The corrections were as follows:

$$\frac{a * b}{c} = d \quad (1)$$

where *a* refers to the PPD or DM production (g/L) measured by HPLC; *b* refers to the final volume (mL); *c* refers to initial volume (mL); *d* refers to the actual PPD or DM production (g/L) without evaporation.

The results indicated that the PPD production also decreased with the increase of medium volume. The PPD productions of optimum media 3 and 4 in this membrane lid shake flask are higher than the PPD production of the same media with an aluminum lid, reaching 1.2 g/L and 0.6 g/L when the volume is 25 mL. At the same time, the DM production increased with medium volume (Figure 3-7E). This indicated that the membrane lid and smaller volume allow more oxygen to enter the shake flask, which supplies more oxygen for improving the conversion of squalene to 2,3-oxidosqualene and the conversion of DM to PPD (Figure 3-1). The highest PPD

productions of unoptimized standard WM8+ medium in the shake flask with an aluminum lid or membrane lid are 0.16 g/L or 0.45 g/L, respectively (Figure 3-7E). This indicated that the highest PPD production of the optimum medium 3 is 2.6-fold compared to the unoptimized standard WM8+ medium in the shake flask with a membrane lid (Table 3-3). However, the PPD productions in media 1 and 2 with this membrane lid shake flask are much lower than the PPD production in medium 3 (data not shown). This may be due to the higher glucose and glutamate concentration (three times the standard WM8+ medium) in media 1 and 2, limiting the PPD and DM synthesis in the shake flask, just like the analysis described in Figure 3-5 A-C.

Table 3-3 The composition comparison between WM8+ and M3 media

Medium	Glucose (mL)	Salts (mL)	Glutamate (mL)	Trace element (mL)	Vitamin (mL)	Temperature °C	Oxygen (mL)	Verduyn-Trace elements (100x) (mL)	Verduyn-Vitamins (1000x) (mL)	Water (mL)
M3	5	1,1	2,5	0,034	0,3	28	25	0,25	0,025	15,79
WM8+	2,5	2,5	1,25	0,1	0,1	28	25	0,25	0,025	18,28

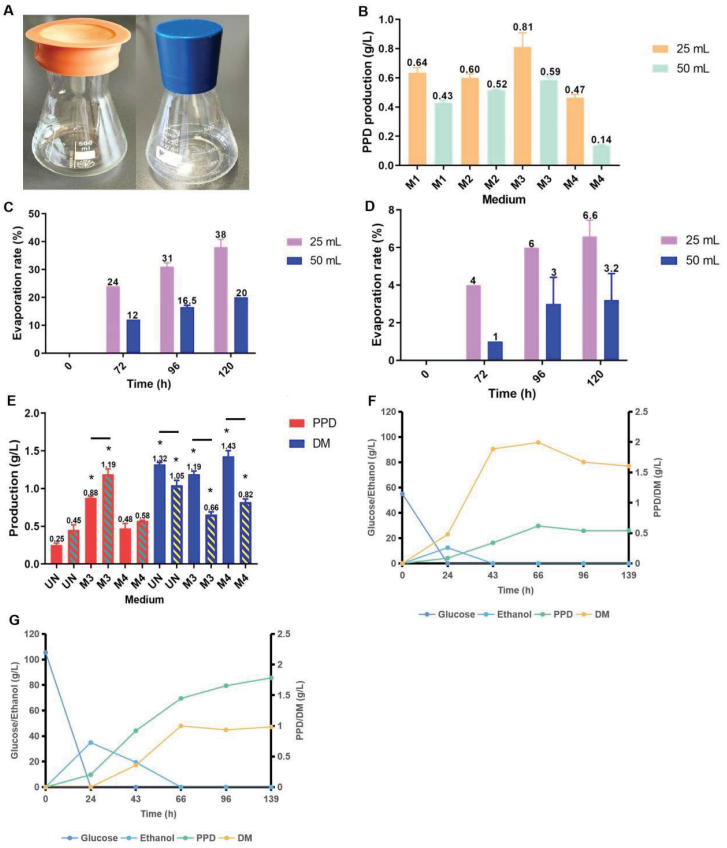

Figure 3-7. Shake flask configuration influences PPD production. (A) Shake flasks with membrane and aluminum lid. (B) PPD production depends on the filling volume using a shake flask with an aluminum lid. (C) The water evaporation rate in the 500 mL shake flask with a membrane lid. (D) The water evaporation rate in the 500 mL shake flask with an aluminum lid. (E) PPD and DM production depends on the filling volume (25 mL, striped bars; 50 mL, filled bars) using a shake flask with a membrane lid. (F) The concentration of glucose, ethanol, PPD, and DM during the fermentation in engineered yeast cultured in the standard WM8+ medium. (G) The concentration of glucose, ethanol, PPD, and DM during the fermentation in engineered yeast cultured in the M3 medium. The asterisk indicates statistically significant differences in PPD and DM production between the 25 mL and 50 mL shake flask based on Student's *t*-tests (*$p < 0.05$). The error bars represent the error of the replicate data; the longer the bars, the greater the error of that data, and vice versa. UN, unoptimized standard WM8+ medium; M1, Optimum medium 1; M2, Optimum medium 2; M3, Optimum medium 3; M4, Optimum medium 4.

3.3.6 The fermentation kinetics of PPD and DM in engineered yeast cultured in the standard WM8+ medium and the M3 medium

Ethanol is a more reduced carbon source than glucose, which feeds directly into the cytosolic acetyl-CoA pool required for the mevalonate pathway.[22] Therefore, we also measured the concentration of the carbon sources and triterpenoid products during the fermentation to investigate the fermentation kinetics of PPD and DM. As a Crabtree-positive yeast, the engineered *S. cerevisiae* uses respiro-fermentative glucose metabolism and produces ethanol and carbon dioxide.[174] After glucose depletion at 24 h, ethanol will be the carbon source in the presence of oxygen, as the classic diauxic shift of *S. cerevisiae*. The ethanol concentration also reached the highest value at 24 h, 12.5 g/L, and 35 g/L in the standard WM8+ and M3 medium (Figure 3-7F and G). Ethanol has been shown to be a beneficial carbon source with its high electron density and reduction for triterpenoid production.[22, 175] Therefore, PPD and DM synthesis was very fast when the carbon source became ethanol. The highest PPD production is 0.6 g/L and 1.8 g/L in the standard WM8+ and M3 medium, respectively, slightly higher than the PPD production above data (0.45 g/L in the standard WM8+ medium and 1.2 g/L in the M3 medium) (Figure 3-7E, F and G). The reason should be due to the several samples taken during the fermentation that reduced the volume and increased the oxygen concentration; this led to higher PPD production. The result indicated that the M3 medium performs better than the standard WM8+ medium for PPD synthesis.

According to previous studies, it is a promising choice to improve the production of the final metabolites in the cell's factories by optimizing the fermentation bioprocess, including medium compounds and fermentation conditions.[166, 167] The PPD production of the M3 medium is nearly 2 g/L in the 24-well plates, which is much more than the PPD production in the shake flask with a membrane lid, 1.2 g/L. The reasons may be due to the cells' sediment, the water evaporation, some cells on the wall, oxygen concentration, and other factors that differ between the two growth formats.

3.3.7 Online respiration activity measurement (OTR, CTR) in shake flasks

Online monitoring of respiration activity (including oxygen transfer rate (OTR) and carbon dioxide transfer rate (CTR)) of microbial cultures in shake flasks has been state-of-the-art for a long time. Together with the respiration activity parameters, the essential cultivating conditions of microorganisms in a fermentation process (respirative, fermentative, respiro-fermentative) can be accurately reflected.[174] Therefore, the respiration activity parameters (OTR and CTR) were online measured by a TOM Shaker when the engineered yeast was cultured in WM8+ and M3 media, which can elucidate the different cultivating conditions caused by two different media. Figure 3-8A indicates the OTR of the engineered yeast cultured in WM8+ and M3 media. During the first 15 h, the OTR of the WM8+ is low. After that, the OTR of WM8+ medium increases to 27 mmol/L/h and remains at this level until hour 45, then it drops. The plateau of the OTR between hours 24 and 45 represents the maximum OTR capacity of the system. This indicates that the yeast fermentation process is oxygen-limited in this phase. The sharp drop in the OTR at hour 45 implies that the carbon source has been completely exhausted. Comparing the OTR values in the M3 medium, the carbon source ethanol of the M3 medium can be maintained until 90 h, twice the time when compared to the WM8+ medium.

Figure 3-8B shows the CTR of the engineered yeast cultured in WM8+ and M3 media. The

CTR of the WM8+ medium rises exponentially during the first 15 h; however, this trend of the CTR of the M3 medium can be maintained until 24 h. As a Crabtree-positive yeast, *S. cerevisiae* performs the respiro-fermentative during this phase, consumes glucose, and produces biomass, ethanol, and CO_2.[22] After this phase, the CTR decreases steeply, which agrees very well with glucose exhaustion. Subsequently, the CTR of the WM8+ medium rises in parallel with the OTR until hour 25 before it levels off and maintains a steady value below the OTR. This trend of the M3 medium will be delayed more than 40 h when compared to the WM8+ medium. This result indicated that the consumption of the carbon source (ethanol), which is more reduced than glucose, happened in this phase; the CTR and OTR are maintained by medium M3 for about twice the time compared to WM8+ medium. The M3 medium is well suited for PPD and DM synthesis.

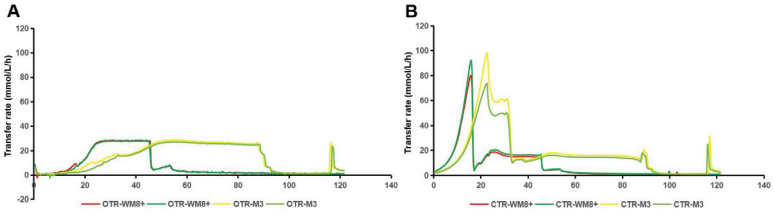

Figure 3-8. The comparison of oxygen and carbon dioxide transfer rates of the engineered yeast cultured in WM8+ and M3 media. (A) The CTR of the engineered yeast cultured in WM8+ and M3 media. (B) The OTR of the engineered yeast cultured in WM8+ and M3 media.

3.3.8 PPD and DM production can be improved again by feeding ethanol during the fermentation

The above research data showed that the engineered yeast synthesizes the PPD and DM very fast when the carbon source changes to ethanol during the fermentation. This means ethanol is a more favorable carbon source for PPD and DM synthesis than glucose. However, the two different lids with different aeration capacities can allow different evaporation rates of ethanol. Therefore, the amount of ethanol used for PPD and DM synthesis will be significantly reduced due to the ethanol evaporation during the fermentation. For this reason, we measured the changes of the ethanol concentration in the shake flask with the two different lids during the fermentation. The result showed that the ethanol concentration in the shake flask with an aluminum lid decreased to 11 g/L from 34 g/L (0 h) after 144 h fermentation. However, the ethanol concentration in the shake flask with a membrane lid decreased very faster than the shake flask with an aluminum lid, which decreased to 4 g/L from 33 g/L after 48 h fermentation and evaporated entirely after 72 h fermentation (Figure 3-9A). This means there is potential to increase the PPD and DM production.

To reduce the effect caused by ethanol evaporation on the PPD and DM synthesis. The different concentration of ethanol (e.g., 10 g/L, 12 g/L, 15 g/L, 18 g/L, and 20 g/L) was fed at different fermentation times to supply carbon sources for the engineered yeast. The result showed that the PPD and DM production reached 1.8 g/L and 1.3 g/L by feeding 10 g/L ethanol after 96 h fermentation, 38 % and 37 % increased compared to the control. However, the PPD and DM production did not increase clearly when the ethanol was fed after 24 h and 72 h fermentation (Figure 3-9B and C).

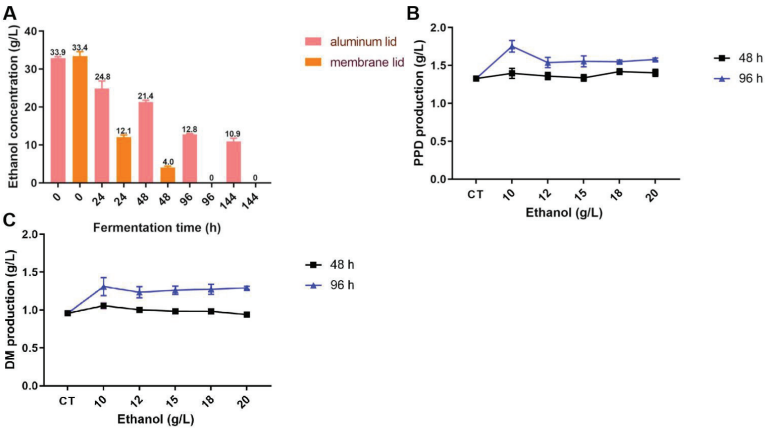

Figure 3-9. Ethanol supplementation influences PPD and DM synthesis. (A) The ethanol evaporation in the shake flask with an aluminum lid or a membrane lid. (B) The PPD production of the engineered strain when the ethanol was fed during the fermentation at 48 h or 96 h. (C) The DM production of the engineered strain when the ethanol was fed during the fermentation at 48 h or 96 h.

3.3.9 Versatility of the M3 medium for improving triterpenoid production

To showcase the versatility of the M3 medium, we expanded the triterpenoids to complex ginsenosides and lupeol-type triterpenoids. For the ginsenosides, we chose compound K (CK), for which we integrated the *S. cerevisiae* codon-optimized UGPTg1 glycosyltransferase from *Panax ginseng* into our PPD producing strain 102-tm-reerg7-2PP, resulting in the CK-producing strain CK1. To improve CK biosynthesis, we increased UDP-glucose bioavailability by introducing constitutively expression cassettes of respectively *PGM2*, *UGP1*, and *YNK1* (Figure 3-1),[87] creating CK2, CK3, and CK4 as well as their respective combinations, realizing CK5 (*UGP1* and *YNK1*) and CK6 (*UGP1*, *YNK1*, and *PGM2*). As illustrated by Nan et al, this approach improved CK production in shake flask cultivation by directing UDP-glucose towards CK biosynthesis [87]. To further increase the flux towards CK, the integration of a second UGPTg1 expression cassette in CK6 was carried out, establishing the CK7 strain (Table 3-4). Using our M3 medium, CK titers of 0.09, 0.19, 0.15, 0.15, 0.05, 0.10, and 0.22 g/L were observed for CK1, CK2, CK3, CK4, CK5, CK6, and CK7 respectively (Figure 3-10A). The CK/PPD ratio of CK 7 strain reached 36 %, the highest among the seven CK producers (Figure 3-11A). Comparing CK production by CK7, in YPD, WM8+, and our M3 medium revealed respectively a 10-fold and 7.3-fold increase in CK titers in the latter, thus emphasizing the efficacy of the M3 medium for the production of glycosylated ginsenosides (Figure 3-11B).

Table 3-4. The list of Compound K producing yeast strains.

Label	Genotype	Source

102-tm-reerg7-2PP	*MATa uraΔ3-52 leuΔ2-3_112 his3Δ1 MAL2-8c SUC2 hmg1Δ::pTEF1-tHMG1 atr2Δ::pPGK1-ATR2 erg7(c.2186delA) Δ::pTEF1-CYP716A49 Δ::pPGK1-PgDDS-tCYC1 Δ::pTEF1-CYP716A49 Δ::pPGK1-PNA*	104, 158
CK1	102-tm-reerg7-2PP *ars911::pTEF1-scUTGPg1-tCYC1*	This study
CK2	CK1 *yprcd15::pTEF1-scUGP1-tGAT2*	This study
CK3	CK1 *ars308::pTEF1-scPGM2-tSPO1*	This study
CK4	CK1 *alg5::pTEF1-scYNK1-tPRM9*	This study
CK5	CK2 *alg5::pTEF1-scYNK1-tPRM9*	This study
CK6	CK5 *ars308::pTEF1-scPGM2-tSPO1*	This study

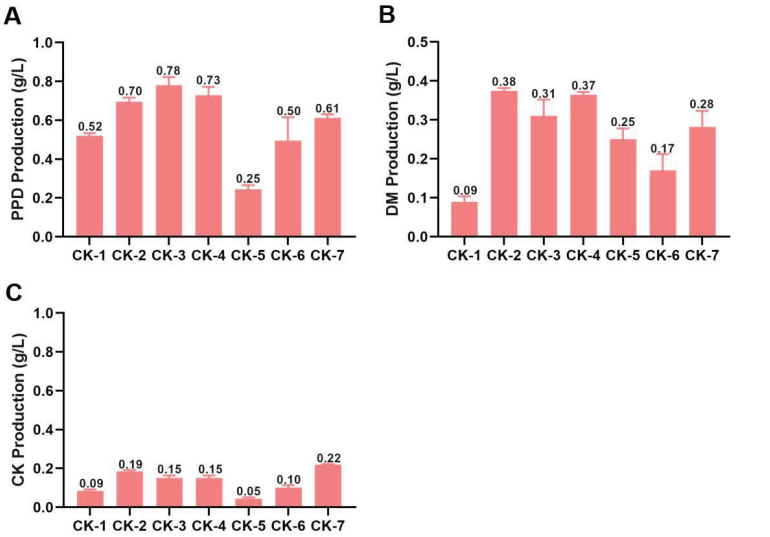

Figure 3-10. The PPD, DM, and CK production of seven different engineered strains. (A) The PPD production in the seven CK-producing strains CK1-7. (B) The DM production in the seven CK-producing strains CK1-7. (C) The CK production in the seven CK-producing strains CK1-7. The error bars represent the error of the replicate data; the longer the bars, the greater the error of that data, and vice versa.

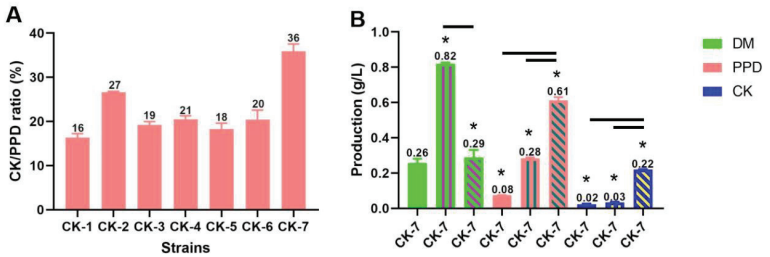

Figure 3-11. The versatility of the M3 medium for the characterization of strains engineered for triterpenoid synthesis. (A) The ratio of CK/PPD in the seven CK-producing strains CK1-7. (B) The PPD, DM, and CK production in the CK-producing strain CK7 on different media, YPD (filled bars), WM8+ (vertical line bars) and M3 (striped bars) were compared. The asterisk indicates statistically significant differences in PPD, DM, and CK production between the YPD and M3 media or the WM8+ and M3 media based on Student's *t*-tests (*$p < 0.05$). The error bars represent the error of the replicate data; the longer the bars, the greater the error of that data, and vice versa.

Ginsenosides belong to the versatile class of high-value molecules, triterpenoids. As microbial triterpenoid cell factories are currently only producing limited quantities during simple shake flask cultivation, transition to our M3 medium can benefit their production, as exemplified for PPD, DM, and CK. To extend the applicability of the M3 medium, we studied the production of the lupeol-type triterpenoids betulin and betulinic acid using our previously published yeast BAΔ and 102-tm-reerg7-2LP strains.[158-160] The result indicates that, indeed, the M3 medium supports generally high triterpenoid production during shake flask cultivations, as the production of betulin and betulinic acid of the yeast BAΔ strain increased 4.3-fold (0.9 g/L) and 4.6-fold (0.41 g/L), respectively when compared to cultivation on WM8+ medium (Figure 3-12). As aimed, the results indicate that the presented M3 medium supports the evaluation of yeasts engineered for triterpenoid synthesis under simple growth conditions.

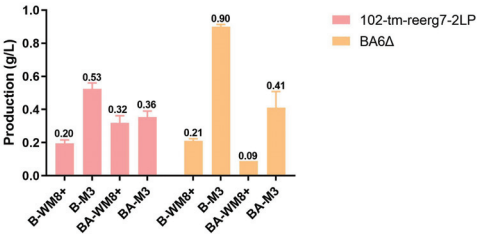

Figure 3-12. Betulin (B) and betulinic acid (BA) produced by engineered yeasts. The aluminum lid was used for shake flask cover and the media WM8+ and M3 were compared. The asterisk indicates statistically significant differences in PPD, DM, and CK production between the WM8+ and M3 media based on Student's *t*-tests (*$p < 0.05$). The error bars represent the error of the replicate data; the longer the bars, the greater the error of that data, and vice versa.

Here, we present a simple cultivation for producing triterpenoids at high titers using engineered *S. cerevisiae*. Combining of the M3 medium with the optimal temperature of 28°C and aeration membrane lid allows simple cultivations to compare triterpenoids synthesis. A remarkable titer of 1.2 g/L PPD was achieved in the 500 mL shake flask, a 7.4-fold (0.16 g/L) and >10-fold improvement (0.08 g/L) compared to the WM8+ and YPD media. As the cultivation conditions are easily established and transferable to Compound K and betulin producers, the study might facilitate the evaluation of engineered strains in microtiter plates and shake flask cultivations.

Chapter 4

Enhancing the production and secretion of ginsenosides by different strategies

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Author contributions:

Shangkun Qiu: Conceptualization, Methodology, Validation, Writing - Original Draft, Visualization. **Lillith-Jade Yoendem:** Validation. **Lars M. Blank:** Supervision, Project administration, Funding acquisition, Review & Editing.

4.1 Abstract

Natural products are seemingly endless bioactive chemicals with remarkable potential for applications. However, extracting them from plants or other natural resources can lead to biodiversity loss, environmental pollution, and high prices. The complex chemical structure of many natural products makes chemical synthesis difficult, if not economically impossible. Synthetic biology allows the synthesis of natural products efficiently and eco-friendly in large quantities using engineered microbial cell factories. However, the metabolic burden caused by natural products accumulation always hinders the efficiency of microbial cell factories. This Chapter revealed that almost all the PPD and DM produced by the engineered yeast were exported when vegetable oil was added after 96 h fermentation. In particular, the “empty cells” can be reused again after the first round of in-situ extraction, and the total triterpenoid production was over 3.4 g/L after five days of cultivation during the second round of in-situ extraction, the highest triterpenoid production until now in the shake flask cultivation.

4.2 Introduction

Natural products are bioactive chemicals with different applications (e.g., food, cosmetics, and medicine) extracted from plants, fungi, and bacteria.[176] Microbial natural products mainly comprise alkaloids, flavonoids, terpenoids, and polyketides.[77,177] Natural products have played an essential role in drug discovery from ancient times to the present day, with prominent examples in cancer, inflammation, and infectious diseases treatment.[22,178,179] Natural products provide special features compared to traditional synthetic molecules, bringing advantages and challenges to the drug discovery process.[180,181] For example, natural products are evolutionarily optimized for serving different biological functions, including binding to specific target proteins or other biomolecules.[182] Moreover, their long-standing use in traditional medicine may shed light on efficacy and safety.[180] Overall, the natural products pool is enriched with countless 'bioactive' compounds covering a wider area of chemical space compared with typical synthetic small-molecule libraries.[183]

Traditionally, two methods are applied to obtain natural products: (1) by directly separating the target chemicals from plant biomass and other natural resources or (2) by chemical synthesis of the target compounds.[16,184] However, plants often contain limited amounts of the target products, thus employing a considerable amount of biomass, which, in turn, requires the intense use of organic solvents for the extraction, such as dichloromethane, methanol, toluene, 2-pentanol, or ethyl acetate.[185,186] The supply of natural products from plants might easily affect plants' protection status because some plants grow exclusively in a particular natural environment or at a prolonged rate.[22,77,176] In addition, natural products obtained from plants by crude extraction methods will result in a relatively low yield of the target products and loss of other valuable metabolites.[77] Furthermore, the complex chemical structures of many natural products make chemical synthesis extremely difficult to achieve.[20] Therefore, a stable source of affordable natural products is required.

Synthetic biology aims to redesign living systems at the genetic level for understanding life or for practical real-world applications.[89] With the development of synthetic biology, designing living systems to optimize target metabolite titers has become feasible.[187] In the past decade, many natural products have been produced via synthetic biology by taking the genes from the host plants and putting them into microorganisms.[89,77,177] Recently, some prominent examples of natural products synthesized by redesigning living systems were reported. For example, the α-farnesene production was up to 26 g/L by *Yarrowia lipolytica* through non-homologous end-joining (NHEJ) mediated integration, culture condition optimization, and fed-batch fermentation in 1L bioreactor.[91] Artemisinic acid production in *Saccharomyces cerevisiae* reached 25 g/L by expressing *Artemisia annua* alcohol dehydrogenase (ADH1) in conjunction with *A. annua* artemisinic aldehyde dehydrogenase (ALDH1), amorphadiene oxidase (CYP71AV1), *A. annua* cytochrome b5 (CYB5) and *A. annua* CPR1 involved in the artemisinic acid synthesis pathway as well as growing the engineered strain Y1284 in the presence of 10 % isopropyl myristate (IPM).[9] The artemisinin precursor amorpha-4,11-diene production by *S. cerevisiae* CEN.PK2 engineered strain overexpressed every enzyme of the mevalonate pathway to Erg20p and, using the unrestricted ethanol pulse feed strategy, reached 40 g/L after 116 h fermentation.[93]

For centuries, yeast *S. cerevisiae* has been used for food production, such as wine, bread, and beer.[188,94] Yeasts are simple eukaryotic cells with complete organelle structures (e.g., mitochondria,

ER, and peroxisomes), which allow many critical cellular processes, such as protein folding and chaperone functions, and post-translational modifications of proteins, thus ensuring the functions of many enzymes found in other eukaryotes, including plants.[94,189] Yeasts also possess redox systems that provide similar physiological environments to those encountered by cytochrome P450s and uridine diphosphate glycosyltransferase (UGTs) in mammals and plants.[20,190] Among yeasts, *S. cerevisiae* is the most commonly used synthetic biology workhorse for the heterologous synthesis of natural products (e.g., carotene, lycopene, triterpenoids) due to its safe status and the availability of in-depth cell biology knowledge.[189,94,191,192, 193,80,159,194,195] Other attractive yeasts, such as *Ogataea polymorpha* (syn. *Hansenula polymorpha*), *Rhodosporidium toruloides*, *Kluyveromyces lactis*, *Yarrowia lipolytica*, and *Komagataella pastoris* (syn. *Pichia pastoris*), have exhibited potential for natural products synthesis, which makes up for the disadvantages of *S. cerevisiae* might have, e.g., being Crabtree-positive.[189]

The metabolic burden caused by natural products accumulation always hinders the efficiency of microbial cell factories, such as inefficiency in cellular energy generation, changes in the cell membrane structure, organelle damage, and the generation of reactive oxygen species (ROS), which can be surmounted by exporting these chemicals outside of the cells.[191,176] In addition, natural products accumulated within the cells could be only collected after cell disruption.[178] Therefore, natural products secretion is an effective approach for improving the final bioproduction.[136] Improving natural products yield and enabling exportation are essential goals to simplify downstream purification procedures and reduce process costs.[178] In such a scenario, the cells would remain intact during product harvest, thereby leading to an increase in natural products yield per cell as well as its purity, and the ease of harvest would improve significantly.[22] The conflict between the hydrophobicity of natural products and the hydrophilic microenvironment in the cells enables the possibility of enhancing the target production by secretion.[178]

There have been many studies about the secretion of chemicals synthesized by yeast to increase the final production, for example, carotene, lycopene,[191,192] and δ-tocotrienol.[178] Strategies for improving natural products secretion are mainly focus on engineering the transporter proteins and organic solvent in-situ extraction (Figure 4-1). Recent research calculated the binding affinity of pleiotropic drug resistance exporter (PDR11) with different terpenoids using batch molecular docking and verified the result in *S. cerevisiae* that can synthesize these terpenoids. Finally, they showed that extracellular β-carotene titer in the PDR11-overexpressed strain (197 mg/L) was 80 % higher than that of the original strain (109 mg/L), and the extracellular lycopene titer was also vastly increased compared to the original strain. Another research showed that overexpressed PDR1 resulted in 20 % higher δ-tocotrienol production (226 mg/L), with the extracellular δ-tocotrienol increased by 35% to 26 mg/L. The addition of 60 mM 2-hydroxypropyl-β-cyclodextrin (2-HP-β-CD) again increased δ-tocotrienol production to 242 mg/L, with 30% (74 mg/L) δ-tocotrienol secreted extracellularly. These research indicated that it is a promising strategy to develop high-production microbial cell factories for efficiently producing the target chemicals by improving the secretion of natural products.

Although the structures of some natural products, such as triterpenoids, are more complex than the chemicals described above, triterpenoids can also be secreted outside the cells, according to some reports.[193] Triterpenoids might exhibit some toxicity to single-cell systems,[118] and intracellular accumulation of triterpenoids might cause inhibition of biosynthetic pathways in

yeast.[193] Therefore, triterpenoid secretion into the culture broth will potentially improve the final triterpenoid titer while simplifying downstream processing.[136] Several studies have revealed that ginsenoside triterpenoids can also be secreted by yeast during fermentation; however, the underlying molecular mechanisms need more in-depth research.[22,97,133] Therefore, improving the final production by researching the export of triterpenoids from cells is promising.

In this chapter, the secretion of ginsenosides was investigated by adding organic solvent during the cultivation. Our engineered yeast strain could improve the secretion of the PPD and DM by adding vegetable oil after 96 h fermentation, indicating that it is possible to expand the production of triterpenoid by in-situ fermentation. In particular, the "empty cells" can be reused again for the subsequent round of cultivation, while the triterpenoid production increased a lot.

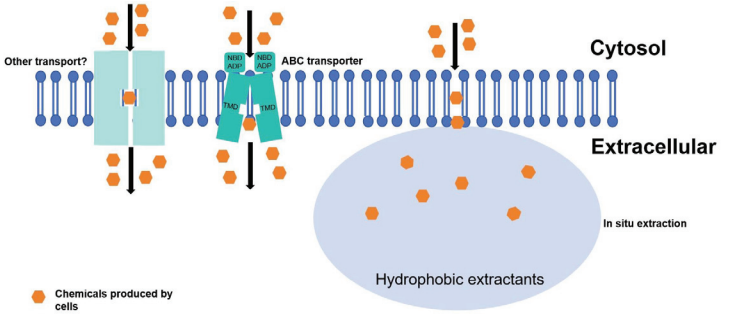

Figure 4-1 The secretion process of recombinant natural products by yeast. The natural products (e.g., carotenoid, tocotrienol, canthaxanthin, triterpenoid) produced by yeast may be secreted outside the cell in three ways: (1) by ABC transporters, (2) by other transport channels or (3) by in-situ fermentation with adding hydrophobic extractants (e.g., dodecane, IPM, sunflower oil, olive oil, and β-Cyclodextrin or its derivatives) in the growth medium. The ABC transport process was initiated by binding the natural product to TMD, after which the NBD was bound by ATP, and a closed NBD dimer was formed. Then, the dissociation of the closed NBD dimer was triggered by the ATP hydrolysis. The TMD opened, and the natural product was pumped out of the extracellular space.[136]

4.3 Investigation of the triterpenoid synthesis kinetics during sequential in-situ extraction

It was reported that adding isopropyl myristate (IPM) to the media during shake flask cultivation resulted in extracellular triterpenoids.[196] In the previous chapters, we optimized the WM8+ medium to the M3 medium, and the production of PPD in the M3 medium reached 1.2 g/L. If we can promote the secretion of PPD and DM extracellularly, we should be able to enhance the efficiency of the engineered yeast in producing PPD and DM. So, we added the IPM to the medium after five days of fermentation, and the result indicated that all the PPD and DM could be extracted from the yeast after one day of extraction. The question was whether the now "empty yeast" could be again used for ginsenoside production. We collected the yeast and used them for a second round of fermentation. The result showed that the yeast cells were able to grow well during the second round fermentation, and the final DM production of the second round fermentation was much higher than the first round fermentation. At the same time, the PPD production of the second round fermentation is lower than the first round fermentation (Figure 4-2). This means that the CYP enzyme activity might be reduced after the first round fermentation. As oxygen is a second

substrate for the CYP enzyme, we investigated two different lids: the membrane lid for increased oxygen supply and the aluminum lid for decreased ethanol evaporation. The influence of oxygen on CYP activity could not be shown, as the cultivation with the membrane lid compared to the control had more PPD, but overall, fewer triterpenoids were produced.

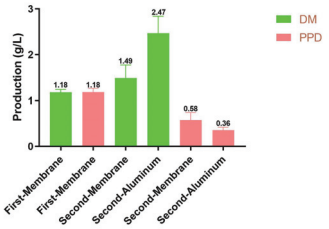

Figure 4-2 Optimization of total triterpenoid production by adding isopropyl myristate (IPM). Two rounds of fermentation were carried out in the 500 mL shake flask. The membrane lid was used in the first round, both lids were used in the second round of fermentation, and the productions were compared.

4.4 Optimization of total triterpenoid production by adding sunflower oil

Much research has shown that triterpenoids can be secreted outside the cells during fermentation, and one hypothesis to explain this process is that the triterpenoids may co-accumulate with sterols in membranes.[22] Because both triterpenoids and vegetable oils are hydrophobic, and it has been shown that the addition of vegetable oils to the culture medium during engineered yeast fermentation can dramatically increase the production and secretion of hydrophobic natural products (e.g., carotenoid, tocotrienol, and canthaxanthin),[197,179,198] the use of plant oils as extractant was tested. So, we decided to add sunflower oil to the medium during yeast fermentation to check if this strategy would increase DM and PPD production and secretion. Because the engineered yeast largely produces PPD and DM after 48 h fermentation, so we added different concentration sunflower oil (1 %, 2 %, 4 %, and 6 % v/v) to the medium at different fermentation times (i.e., 48 h, 72 h, 96 h). 1 % v/v Tween 80 was also added to the medium together with different concentration of sunflower oil to make an emulsion and to avoid that cells adhere to the shake flask wall. Notably, almost all DM and PPD were found outside the cells when sunflower oil was added (Figure 4-3). The PPD and DM production decreased with the increase in sunflower oil concentration, while the PPD and DM production with 1 % v/v sunflower oil was almost the same as the control. The reason may be that the sunflower oils might oxidize during the fermentation, and more lipid peroxides may interfere with cell growth.[199] However, we found some small white blocks in the medium by adding 1 % v/v sunflower oil at 96 h fermentation, and the number of small white blocks is more than six (Figure 4-3 G). We selected one white block and measured the PPD and DM production included in this white block; it showed that 0.8 mg PPD and 0.7 mg DM were contained. This means that the PPD and DM concentration in the medium with adding 1 % v/v sunflower oil at 96 h fermentation will increase by at least 0.2 g/L and 0.17 g/L; this is more than the control (1.1 g/L PPD and 1.1 g/L DM). So, this result indicated that adding 1 % v/v sunflower oil at 96 h fermentation can improve PPD and DM production with the advantage of extracellular triterpenoids.

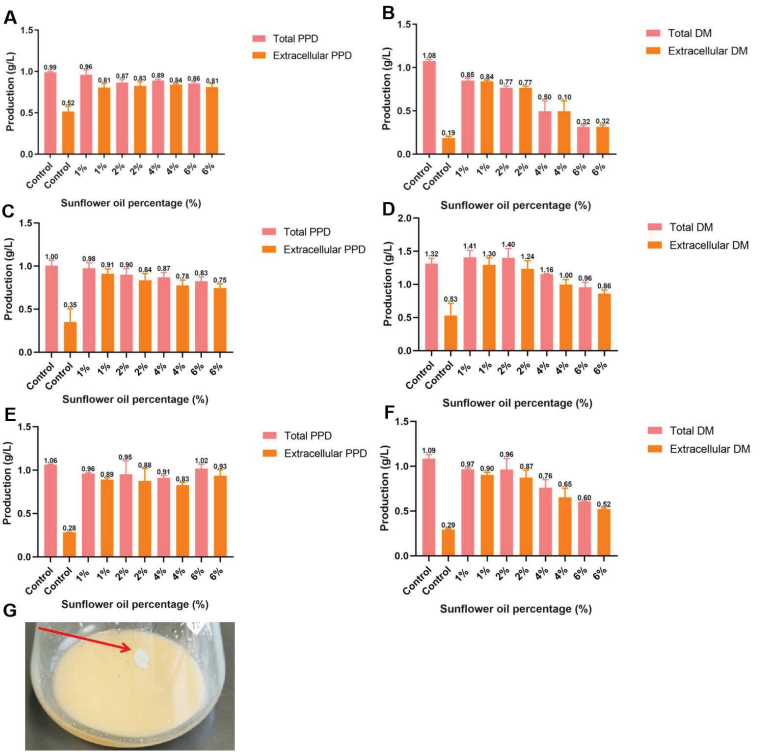

Figure 4-3 Optimization of total triterpenoid production by adding sunflower oil. (A) and (B) PPD and DM production by engineered yeast with the addition of sunflower oil at 48 h. (C) and (D) PPD and DM production by engineered yeast with the addition of sunflower oil at 72 h. (E) and (F) PPD and DM production by engineered yeast with the addition of sunflower oil at 96 h. (G) Some white blocks were formed during the fermentation.

4.5 Optimization of total triterpenoid production by adding other vegetable oils

Research showed that adding 5 % (v/v) olive oil after 24 h fermentation, the engineered *S. cerevisiae strain* YVT17 could increase tocotrienol production by 5.5 %, reaching 77 mg/L. At the same time, the extracellular tocotrienol proportion was also vastly improved, reaching 56 %.[179] Therefore, we investigated whether feeding other vegetable oils can increase PPD and DM production and secretion. We selected peanut and olive oils as the feeding oils, and the peanut or olive oil was fed at different fermentation times (24 h, 48 h, 72 h, and 96 h) with various concentrations (1 %, 2 %, 4 %, and 6 %). The feeding experiment was carried out in the 24-well plate. The result showed that the PPD production could reach 0.9 g/L when fed 1 % peanut oil at 96 h or 4 % olive oil at 96 h, 81 % and 32 % increased compared to the control (Figure 4-4 A and C). The DM production of these two conditions is 1.7 g/L and 2.3 g/L, a 13 % decrease and 14 % increase compared to the control (Figure 4-4 B and D). In addition, some cells adhered to the wall after feeding the vegetable oils. We measured the cells on the wall and found that they contained a

large amount of PPD and DM. Therefore, the real production of PPD and DM should be higher than the above value (Figure 4-4 E).

For the cells' adhesion to the wall after feeding the vegetable oils, which will result in a large amount of PPD and DM accumulating on the wall and decreasing measured concentrations of PPD and DM, we counted the cells' adhesion to the wall for all fermentation conditions. We categorized cells' adhesion into classes I, II, and III. In the case of class I, the fewest cells adhered, and in the case of class III, the most cells adhered. The cells' adhesion of class II was between classes I and III (Figure 4-4 E) (Table 4-1). Adding either peanut or olive oil to the medium after 24h or 48h did not significantly result in cells' adhesion to the wall. With only one exception, adding 6% olive oil at 48h resulted in class II cells' adhesion. However, the PPD and DM measured production was lower than the control when fed peanut or olive oil after 24h or 48h. The reason why adding peanut and olive oil after 24h or 48h will decrease the PPD and DM production needs to be investigated. Adding 4 % olive or 1 % peanut oil after 96 h resulted in a class I cells' adhesion (Table 4-1). This means that the real concentrations of PPD and DM were higher than the measured concentrations (0.9 g/L). Therefore, according to the above analysis, feeding peanut and olive oils after 96 h is a promising strategy to increase the final PPD and DM production by secreting PPD extracellularly.

Table 4-1. The cells' adhesion to the wall of all fermentation conditions.

Classes	Peanut oil	Olive oil
Class I	24 h, 1 % and 2 %; 96 h, 1 % and 2 %	96 h, 1 %, 2 %, 4 %, and 6 %
Class II	72 h, 1 % and 2 %; 96 h, 4 % and 6 %	48 h, 6 %
Class III	72 h, 4 % and 6 %	72 h, 4 % and 6 %

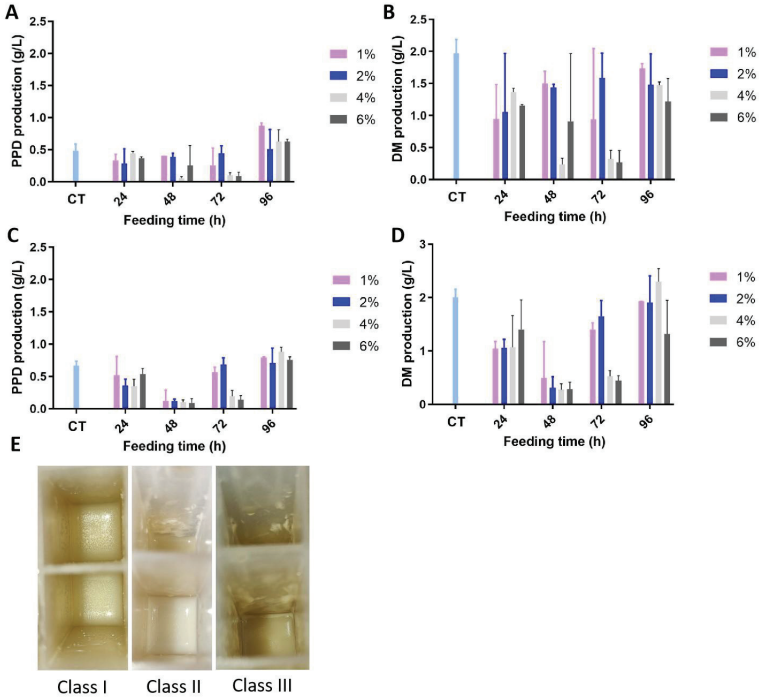

Figure 4-4 Optimization of total triterpenoid production by adding other vegetable oils. (A) and (B) The PPD and DM production of the fermentation with adding peanut oil at different fermentation time. (C) and (D) The PPD and DM production of the fermentation with adding olive oil at different fermentation time. (E) The three classes of cells' adhesion to the wall for all fermentation conditions. CT, control without adding vegetable oil.

The production and secretion of ginsenosides improved by adding peanut or olive oil at hour 96 of the cultivation in a 24-well plate. To verify this result, we used 500 mL shake flasks. We added different concentrations of peanut oil (1 %, 2 %, 4 %, and 6 % v/v) to the medium at 96 h. Also, 1 % v/v Tween 80 was added to the medium for emulsification and to avoid that cells adhere to the shake flask wall. DM and PPD production decreased with increasing concentrations of peanut oil. The literature suggests that lipid peroxides are formed that decrease yeast cell growth in dependence on the added oil amount.[199] Notably, a white block rich in PPD and DM formed in the presence of 1 % or 2 % peanut oil (Figure 4-5 C). The PPD and DM content reached 6.9 mg and 3 mg, and 12.4 mg and 3.9 mg in the white block with 1 % and 2 % peanut oil, respectively. This agglomeration of the triterpenoids will likely simplify downstream purification significantly. Finally, 89 % PPD and 90 % DM were exported during the fermentation with 1 % peanut oil; however, only 22 % PPD and 26 % DM were found extracellularly in the control without an extractant (Figure 4-5 A and B).

The formation of the white block, the triterpenoid agglomeration, was visible independent of the amount of other plant oils added, like olive oil (1 %, 2 %, 4 %, and 6 % v/v, Figure 4-5 D). We

added different concentrations of olive oil (1 %, 2 %, 4 %, and 6 % v/v) to the medium at 96 h. DM and PPD production decreased with increasing concentrations of olive oil (Figure 4-5 E and F). A white block rich in PPD and DM formed in the presence of 1 % or 2 % olive oil (Figure 4-5 D). The PPD and DM content reached 5.6 mg and 2.6 mg, and 8 mg and 4.2 mg in the white block with 1 % and 2 % olive oil, respectively. More than 95% of PPD and DM were exported extracellularly by adding 2 % olive oil; in contrast, almost no PPD and DM were exported.

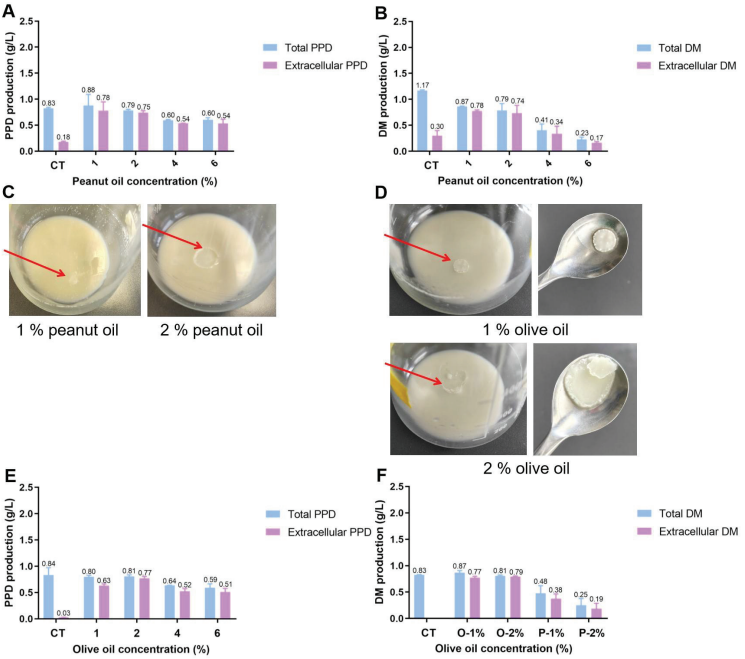

Figure 4-5 In-situ extraction of triterpenoid using other plant oils. Optimization of total triterpenoid production by adding vegetable oils to yeast cultivations in 500 mL shake flasks after hour 96. (A) and (B) PPD and DM production by engineered yeast with the addition of peanut oil. (C) The small white block was formed with 1 % or 2 % peanut oil. (D) The small white block formed when 1 % or 2 % olive oil was added. The extraction run for 24 h. (E) and (F) PPD and DM production by engineered yeast with the addition of olive oil.

4.6 Triterpenoid synthesis kinetics during sequential in-situ extraction

As indicated above, almost all DM and PPD were extracted by adding vegetable oils. So, we decided to investigate whether the “empty yeast” could be used again for ginsenoside production, in accordance with the IPM in-situ extraction. We collected the yeast cells after the first round of in-situ extraction and reused them for a second round of fermentation. The result showed that the yeast cells grew well during the second round of fermentation, and the DM and PPD production of the second round of fermentation increased over time. Notably, the control without adding

vegetable oil displayed great activity for producing DM and PPD during the second round of in-situ extraction. The DM and PPD production at the beginning of the second round of in-situ extraction is 0.4 g/L and 0.3 g/L, which reached 3.2 g/L and 1.1 g/L after five days of fermentation (Figure 4-6 C and D), corresponding to a net production of DM and PPD of the control during the second round of in-situ extraction of 2.8 g/L and 0.8 g/L, respectively. In addition, the DM and PPD production of the yeast (adding 1 % olive oil during the first round of in-situ extraction) was achieved during the second round of in-situ extraction, which was 3 g/L and 0.4 g/L (Figure 4-6 C and D). Adding vegetable oils may decrease the stability of the CYP enzyme and, thus, the conversion of DM to PPD during the second round of in-situ extraction. Generally, except for the control, PPD productions were lower in the second round than in the first, and DM productions were higher in the second round than in the first. Importantly, in the control experiments, the triterpenoids are intracellular, requiring extraction after harvest, while in the experiments with in-situ extraction, above 90% of triterpenoids are extracellular, avoiding extraction after harvest. The results indicate that the strategy of in-situ extraction of triterpenoids in sequential batch culture has excellent potential for repurposing yeast cells.

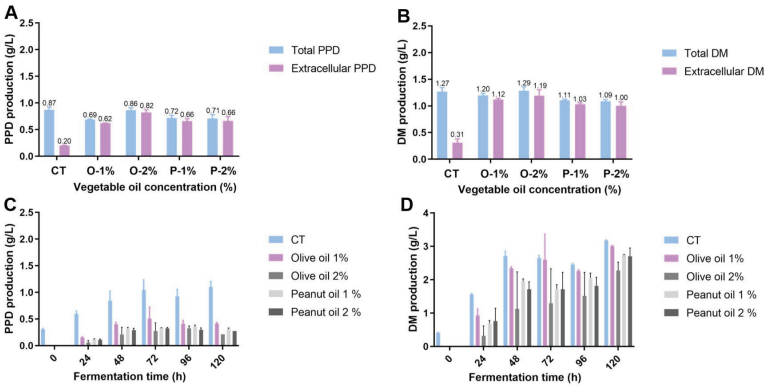

Figure 4-6 Optimization of total triterpenoid production during sequential in-situ extraction by adding vegetable oils. (A) and (B) The DM and PPD production of the first round of in-situ fermentation. (C) and (D) The DM and PPD production of the second round of in-situ fermentation. CT, the control without adding vegetable oil; O, olive oil; P, peanut oil.

According to our results, almost all the PPD and DM were exported outside the cell when vegetable oil was added to the medium after 96 h fermentation. Meanwhile, the PPD and DM productions were improved a lot. However, the effect of vegetable oil on the cell's membrane and the reason why vegetable oil can improve triterpenoid secretion remains to be further explored.

The biodiversity of the natural world is a vast resource for discovering proteins that transport natural products. With the development of synthetic biology, the efficient heterologous expression of transporters is less challenging. In-situ extraction will make the downstream purification process easier while increasing the secretion and yield of natural products.

Chapter 5

General Discussion

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Author contribution:

Shangkun Qiu: Conceptualization, Validation, Writing - Original Draft. **Lars M. Blank:** Supervision, Project administration, Funding acquisition, Review & Editing.

Triterpenoids are essential secondary metabolites ubiquitous in plants with various biological properties.[154] The triterpenoid class ginsenosides are mainly found in Ginseng (*Panax* spp.), a plant belonging to the Araliaceae family, which consists of 17 species, of which *P. quinquefolius*, *P. ginseng*, and *P. notoginseng* are commonly industrially exploited. In addition, ginsenosides are also found in the Brazilian ginseng *Pfaffia glomerata*, which belongs to the Amaranthaceae family.[200] The plant extracts are discussed to possess pharmacological properties, including anti-cancer, anti-diabetic, anti-aging, anti-inflammatory, cardiovascular-protective, and the inhibition of coronavirus disease 2019 (COVID-19).[162,163,18] Ginsenosides can interact with cell membranes, membrane-bound ion channels, and extracellular and intracellular receptors, which alters in transcription levels.[201,202] Since the major bioactive ingredients of ginseng are ginsenosides; ginseng has been widely used as a traditional Chinese herbal medicine for thousands of years in East Asia.[19] In general, South Korea, China, Canada, and the US are the largest producers, with a total production of fresh ginseng of about 79769 tons, according to 2013 data.[203] However, this can't meet the global market, and new synthesis methods need to be developed to improve the production of ginseng and related products to meet the industrial production requirements. Because microorganisms multiply quickly and are easy to cultivate, it is a promising strategy to synthesize heterologous ginsenosides by microbial cell factories.

Research showed that bioprocess optimization, including medium and cultivation conditions, can vastly improve the production of metabolites by cell factories.[166,167] According to some studies, carbon sources (e.g., glucose, glycerol, and glutamate), nitrogen sources, temperature, pH, oxygen, inoculum size, and salts significantly affect the cells' growth and production.[166,168,169] As the parameter space in medium is huge, researchers aim for Design of Experiments (DoE) approaches. For example, researchers optimized the concentration of glucose, yeast extract, and peptone via the Box-Behnken design; this optimization increased the geranylgeraniol production by 1.75-fold compared to the traditional YPD medium.[171] Cultured α-farnesene-producing *Y. lipolytica* in a medium optimized by a uniform design (UD) technique reached a final α-farnesene titer of 210 mg/L in a shake flask.[168] In another study, adding citrate (0-10 mM) or acetate (0-10 mM) into the medium improved squalene production. The inhibition of the squalene monooxygenase Erg1p by terbinafine allowed 730 mg/L of squalene titers.[15] Therefore, improving the cultivation conditions of engineered yeast is a promising strategy.

Because accumulating large amounts of triterpenoids often generates feedback inhibition on normal cell metabolism, triterpenoid secretion is considered an effective strategy to improve the final production.[136] This strategy exports triterpenoids outside the cell by unknown mechanisms, which may be active transport, passive diffusion, or carrier transport, while the cell is not destroyed, thus greatly increasing the production of triterpenoids synthesized by the cell.[193] Currently, research on natural product secretion by yeast mainly focuses on the following two strategies: 1) engineering the ABC transport family proteins and 2) carrying out in-situ extraction (Figure 4-1). Up to now, most studies on improving metabolites production by promoting metabolites secretion have focused on small molecule metabolites, such as terpenes, vitamin E, vitamin A, etc., and very few studies have focused on the secretion of triterpenoids.[176,179,204,205,206] There is one exception, Fang et al. reported that overexpression of the Yps3p and a cell wall glucanase Scw10p in engineered *S. cerevisiae* can increase the secretion of triterpenoid GA-HLDOA by 4-fold (22 mg/L) and 5-fold (27 mg/L) compared to the control strain, respectively, and the total GA-HLDOA production was also increased by 29% in the engineered

strain with overexpression of Scw10p (73 mg/L).[193]

5.1 The ATP-binding cassette (ABC) proteins in the yeast *S. cerevisiae*

ABC transporters appear in all living cells from eukaryotes to prokaryotes, and fulfill many critical biological processes.[207] All ABC transporters share a similar molecular structure, with a hallmark structural domain organization that includes at least one evolutionarily conserved ABC, also known as an NBD (nucleotide-binding structural domain), and several predicted K-helix transmembrane segments (TMS).[208] These proteins mediate membrane translocation of a wide variety of substances, including ions, heavy metals, carbohydrates, amino acids, phospholipids, steroids, glucocorticoids, mycotoxins, antibiotics, pigments, peptides, lipids, and some hydrophobic molecules.[209,207,210] It is worth noting that each cellular compartment except the endoplasmic reticulum (ER) and the nuclear membrane contains ABC proteins.[209] Research revealed that *S. cerevisiae* has about 30 distinct ABC transporters, which can be divided into six subfamilies, including the pleiotropic drug resistance (Pdrp) subfamily, the multidrug-resistance-associated protein (Mrp) subfamily, the mammalian multidrug resistance (Mdrp) subfamily, the adrenoleukodystrophy protein (Aldp) subfamily, the Yef3p and Rlip subfamilies, and the unclassified open reading frames (ORFs) encoding ABC proteins.[209]

5.1.1 Enhanced carotenoids synthesis and secretion by overexpressing ABC transporter

Carotenoids are tetraterpene pigments that exhibit yellow, orange, red, and purple colors and are present in different living cells, including photosynthetic bacteria, archaea, fungi, algae, plants, and animals.[211,212] Carotenoids are categorized into two groups: carotenes (e.g., α-carotene, β-carotene, β,ψ-carotene (γ-carotene), and lycopene) and xanthophylls (e.g., β-cryptoxanthin, lutein, zeaxanthin, astaxanthin, fucoxanthin, and peridinin).[212] Researchers investigated the effect of carotenoids on the lipid bilayer by different experimental methods (e.g., electron paramagnetic resonance, nuclear magnetic resonance, X-ray diffractometry, differential scanning calorimetry, monomolecular layer technique). The results suggest that carotenoids, including polar carotenoids (e.g., zeaxanthin, violaxanthin, lutein) and nonpolar carotenoids (e.g., β-carotene), could influence the fluidity of membranes and the penetration of small molecules (e.g., oxygen) to the hydrophobic membrane.[211] Among all the ABC subfamilies, the Pdrp transporter proteins are the most extensive and best-characterized subfamily in *S. cerevisiae*.[208] Another study revealed that carotenoid synthesis could induce Pdrp family proteins expression in *S. cerevisiae*. Adding sunflower oil in the medium can increase carotenoid secretion; deletion of Pdr10p resulted in the lower carotenoid amount produced.[198] This research suggested the correlation between Pdrp family proteins, carotenoid secretion, and carotenoid production.

β-carotene is a vital human carotenoid and the primary precursor for vitamin A synthesis. β-carotene was always chosen as a model biofuel compound as a type of isoprenoid.[213] A recent study combined three strategies to improve β-carotene production and secretion, including control of the expression level of Pdr5p homologous protein Snq2p by inducible GAL promoter, enhancing intracellular ATP supply in engineered *S. cerevisiae* YBX-20 by supplementation of acetate during the cultivation, and improve cell membrane fluidity by overexpression of the *OLE1* gene encoding stearoyl-CoA 9-desaturase. The final β-carotene secretion and intracellular production increased 5.8-fold and 1.7-fold compared to the parental strain YBX-01.[213]

The ketocarotenoid canthaxanthin is a unique carotenoid characterized by two carbonyl

groups in their β-ionone rings.[214] Canthaxanthin has a significant market demand as a food pigment, human nutraceutical, and cosmetic, with a market of 75 million US dollars in 2017.[214,215] Considering the critical role of the Pdrp proteins in mediating membrane translocation of different substances, a recent study overexpressed Pdr3p in canthaxanthin synthesis *S. cerevisiae* strain Ycmk05, combined in-situ extraction by adding 20 % olive oil in the medium. The final canthaxanthin production in the result yeast strain Ycmk12 increased by 44% (about 165 mg/L) compared to the control strain Ycmk10, which just includes an empty vector. However, only 0.4% of canthaxanthin was exported from yeast; this indicated that Pdrp family proteins might improve canthaxanthin production other than accelerated secretion of canthaxanthin by yet-to-be-explored mechanisms.[197]

5.1.2 Enhanced vitamin E synthesis and secretion by overexpressing ABC transporters

Vitamin E is a collection of molecules consisting of two groups: tocotrienols and tocopherols, each with four different isomers (α, β, γ, and δ).[216] Vitamin E has many biological functions, such as antioxidant properties, which can be used in the food, pharmaceutical, and cosmetics industries.[178,217] Recent research overexpressed Pdr1p in the engineered *S. cerevisiae* YBVT15 that could produce δ-tocotrienol. The result showed that δ-tocotrienol production increased by 20 % compared to the control strain YBVT15, reaching 226 mg/L, and the extracellular δ-tocotrienol also increased by 35 % to 26 mg/L.[178] Another study showed that Pdr11p and Yol075cp were overexpressed in engineered *S. cerevisiae* strain YVT17, respectively; the tocotrienols production was increased by 12% and 5% in the final engineered strains. Meanwhile, the extracellular tocotrienols were also raised 1.3-fold and 1.4-fold, respectively.[179]

5.1.3 Enhanced Terpenes synthesis and secretion by overexpressing ABC transporters

Terpenes are essential groups of natural products with molecular structures containing 5-carbon backbones of 2-methylbuta-1,3-diene (isoprene units), which can be transformed into cyclic structures.[218] Monoterpenes (C10) and sesquiterpenes (C15) are the main types of terpenes, and in addition to that, the other classes are hemiterpenes (C5), diterpenes (C20), triterpenes (C30) and tetraterpenes (C40). Terpenoids (or isoprenoids) are another different type of terpenes constructed via biochemical modifications, which contain oxygen.[219,220] Terpenes are used extensively and are vital in oriental herbal medicines with many crucial pharmaceutical functions, such as antitumor, anticancer, antimicrobial, and antioxidant.[218,22] The synthesis of terpenes and terpenoids by plants is mainly via the mevalonic acid (MVA) pathway that happens in the cytosol, and the 2C-methyl-D-erythritol-4-phosphate (MEP) pathway occurs in plastid for the formation of precursors: isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP).[220]

Rubusoside is a safe and natural sweetener and is about 114-fold sweeter than sucrose.[221,222] Recent research used the Alpha Fold Protein Structure Database (https://alphafold.ebi.ac.uk/) to simulate the docking of rubusoside with the proteins suspected to be rubusoside transporters in the cell membrane, and the result showed that the affinity of rubusoside with an ABC transporter was higher than with other transporters. Then the Pdr11p was overexpressed in the engineered *S. cerevisiae* strain SGN08, and the rubusoside production was increased by 130% to 156 mg/L, which indicated that Pdr11p is the primary transporter to mediate rubusoside secretion.[192] Another research used an automated tool, GROMACS, to simulate the dynamic docking of the Pdr11p transporter to rubusoside and identified 16 key amino acid residues that play a critical role in

mediating rubusoside recruitment. Among these 16 key amino acid residues, the rubusoside production of the engineered yeast with residue F661A single-point Pdr11p mutant increased by 50 % (up to 35 mg/L) compared to the control strain, indicating that F661A residue is essential for rubusoside production and secretion mediated by Pdr11p. This research also introduced Pdr11p into the engineered yeasts for lycopene and β-carotene synthesis; the result showed that the extracellular β-carotene titer improved by 80 % (197 mg/L) and the extracellular lycopene improved from 0 to 7 mg/L in the Pdr11p overexpression strain, indicating that Pdr11p also is a suitable transporter for lycopene and β-carotene secretion.[191]

5.1.4 Enhanced alkane synthesis and secretion by overexpressing ABC transporters

Alkanes are a critical class of hydrocarbons used as liquid transportation fuels, which are found to be produced in nature by microorganisms, insects, and plants.[223] Alkanes with appropriate characteristics can only be obtained economically by cracking crude oil; however, in the envisaged bioeconomy, crude oil use should fade out.[184] Recently, the biological production of alkanes has drawn much attention, and engineered strains have been created.[224] According to some reports, the alkane biofuel exhibits cytotoxicity, potentially affecting the final production.[225,226] In recent research, ABC2 and ABC3 transporters were heterologously expressed in the engineered *S. cerevisiae*, which can increase the cellular tolerance toward alkanes (e.g., decane, undecane). Further data indicated that the yeast cells expressing ABC2 and ABC3 had 5-fold and 30-fold lower intracellular decane and undecane levels than the control when the cells were exposed to growth conditions containing decane and undecane.[227] Therefore, it may be a great strategy to improve the final production by exporting some alkanes to alleviate the cytotoxicity toward the host strains.

5.2 Enhanced natural product synthesis and secretion by transport optimization

Ganoderic acids (GAs) are a group of fungal triterpenoids produced by the traditional Chinese medicinal fungus *Ganoderma lucidum*, of which lanosterol is the direct precursor. Research showed that overexpression of aspartic protease (Yps3p) and a cell wall glucanase (Scw10p) in *S. cerevisiae* can increase the secretion of ganoderic acid 3-hydroxy-lanosta-8,24-dien-26-oic acid (GA-HLDOA) by 4-fold (22 mg/L) and 5-fold (27 mg/L) compared to the control strain, respectively, and the total GA-HLDOA production was also increased 29% in the yeast with overexpression of Scw10p (73 mg/L). The overexpression of Yps3p and Scw10p can also vastly increase GA-HLDOA secretion in other engineered strains.[193] This indicated that the enhanced GA-HLDOA secretion via the overexpression of Yps3p and Scw10p can also work for different strains.

C4-dicarboxylic acids, such as itaconic acid, succinic acid, malate, and fumaric acid, are widely used in the food, pharmaceutical, and polymers industries.[228] The transporters have been investigated in the past decades to improve the exportation of C4-dicarboxylic acids by the engineered strains during fermentation. The transporter Mae1 from *Schizosaccharomyces pombe* was introduced into an *S. cerevisiae* strain engineered for producing C4-dicarboxylic acids, and the final extracellular malate, succinate, and fumarate titers were increased by 700 % (4.3 g/L), 200 % (2.6 g/L), and 400 % (0.33 g/L) compared to the control.[229] In another research, the genes encoding the Mae1 transporters were overexpressed from various eukaryotic microorganisms in the succinic acid synthesis engineered *Y. lipolytica* PGC62 strain, and the final succinic acid titer

of all the strains that included SpMae1, YlMae1, AfMae1, ScMae1, and VpMae1 improved above 33%. Introduced SpMae1 into the engineered *Y. lipolytica* strain PGC62-SYF that had been optimized for succinic acid biosynthesis, the final succinic acid titer was increased by 27 % (35 g/L) during shake flask fermentation.[230] Replacing the native FAD-dependent pathway for glycerol catabolism in *S. cerevisiae* by the synthetic NAD-dependent dihydroxyacetone (DHA) pathway, together with the introduction of the heterologous expression of dicarboxylic acid transporter DCT-02 from *Aspergillus niger*, the final succinic acid titer in the engineered strain UBR2CBS-DHA-AnDCT-02 reached 11 g/L during 500 mL shake flask fermentation.[195] The optimization of L-malic acid biosynthesis and the transport system was achieved by combinatorial overexpression of pyruvate carboxylase (*PYC*) and malate dehydrogenase (*MDH*) from *Aspergillus flavus* and *Rhizopus oryzae* and by introduction the C4-dicarboxylate transporter (*Spmae*) from *S. pombe*. The final L-malic acid titer in the resulting engineered *S. cerevisiae* strain W4209 reached 30 g/L.[231] These results indicated that different C4-dicarboxylic acid transporters from different microorganisms can enhance the final C4-dicarboxylic acid production, a promising strategy to increase C4-dicarboxylic acid production vastly.

5.3 Enhanced natural products synthesis and secretion by yeast using in-situ extraction

Studies have shown that using in-situ extraction can improve the production of various hydrophobic compounds by promoting product secretion. This strategy reduces cellular stress caused by product accumulation within cells.[179,232] Hence, biphasic fermentation systems, adding a second organic phase or oil phase, thereby promoting export or true extraction of the target molecule from the cytoplasm membrane, are another promising approach to increase the final target molecules' production in the culture broth.

5.3.1 Enhanced carotenoids synthesis and secretion by in-situ extraction

Rhodosporidium toruloides is a robust oleaginous yeast that produces lipids and carotenoids simultaneously.[233] The Pdr10p gene was transformed from *S. cerevisiae* into *R. toruloides*, and the resulting Pdr10p strain was cultured in the presence of a second organic phase. The total carotenoids (e.g., torularhodin, torulene, and β-carotene) exported and produced by this engineered strain were increased by 500 % (1.8 $\mu g/mg_{CDW}$) and 53 % (2.9 $\mu g/mg_{CDW}$) compared to the control (0.3 $\mu g/mg_{CDW}$) and (1.9 $\mu g/mg_{CDW}$).[234]

5.3.2 Enhanced vitamin A synthesis and secretion by in-situ extraction

Vitamin A is a group of essential micronutrients, including retinol, retinal, and retinoic acid.[235] Only a few research studies on vitamin A produced by microorganisms were conducted using *Escherichia coli* as a host.[204,215,236] However, *S. cerevisiae* has more advantages than *E. coli* in producing natural products, such as including complete organelle structures that allow post-translational modifications of heterologous enzymes from plants, possessing redox systems that provide similar physiological environments as encountered by heterologous enzymes in mammals and plants, and avoiding possible endotoxin contamination in final products because of biologically safety of yeast.[87,22,176] In a recent research, an engineered *S. cerevisiae* was cultured in an emulsion including 500 mL of minimal medium and 500 mL of dodecane in a 3 L bioreactor. Vitamin A titer of the in-situ extraction (3.4 mg/L) was 3.6 folds higher than the control fed-batch fermentation (953 mg/L). Besides overcoming intracellular accumulation, increasing dissolved

oxygen in dodecane may improve the conversion of β-carotene to vitamin A.[204] A retinol production strain CJ2104 was constructed by introducing the retinol dehydrogenase gene, β-carotene biosynthetic gene, geranylgeranyl pyrophosphate synthase gene, flavin adenine dinucleotide synthetase gene, and 11 copies of β-carotene 15,15'-dioxygenase from the marine bacterium 66A03 into *Y. lipolytica.* Also, this engineered strain was cultivated in the presence of a second organic phase, here 10 % Tween 80, resulting in final extracellular and intracellular retinol titers of 4.7 g/L and 0.14 g/L after 104 h fermentation.[205] Secretion of retinol increases product stability as reactive oxygen species (ROS) present inside the cell are absent, increasing the final retinol titer.[237]

5.3.3 Enhanced vitamin E synthesis and secretion by in-situ extraction

Research showed that adding 5 % (v/v) olive oil after 24 h fermentation, the engineered *S. cerevisiae* strain YVT17 could increase tocotrienol production by 5.5 %, reaching 77 mg/L. At the same time, the extracellular tocotrienol proportion was also vastly improved, reaching 56 %.[179]

5.3.4 Enhanced terpenes synthesis

Patchoulol is a tricyclic sesquiterpene and appears in the leaves of *Pogostemon cablin*. Patchoulol is widely used in the perfume and fragrance industry due to its characteristic aroma and flavor.[238] In some research, an in-situ extraction (dodecane-culture) method was used to culture patchoulol producing *S. cerevisiae* REL003-FPTS and HPT15 to increase patchoulol production and reduce volatilization in a 5 L fermenter. The organic solvent dodecane was added during the fermentation; the final patchoulol production reached 467 mg/L and 1632 mg/L, respectively.[206,176]

5.3.5 Enhanced natural products synthesis and secretion by β-cyclodextrin and its derivatives

β-Cyclodextrin and its derivatives have been used as clattering agents. Hence, it supports the emulsion formation of water-insoluble chemicals in the absence of an organic phase. Cyclodextrins have the ability to form stable inclusion complexes with apolar molecules (e.g., cholesterol).[239,240] In addition, adding β-cyclodextrin during fermentation may improve the production and secretion of natural products by enhancing the membrane permeability, which promotes the release of hydrophobic products into the culture medium.[178] Phytosterols and artemisinin production and secretion by *Daucus carota* cell suspension or *Artemisia annua* cell suspension were vastly increased when β-cyclodextrins were added during fermentation.[241,242] Application of different types of cyclodextrins with a concentration of five mM in cultures with the engineered *S. cerevisiae* TM9 that produced the triterpenoid β-amyrin indicated the best performance of methyl-β-cyclodextrin (M-β-CD). It can separate all the β-amyrin from yeast cells, and the total triterpenoid production increased by 5.5-fold compared to the control strain.[240]

Fermentation of engineered *S. cerevisiae* CYP5150L8-r-iGLCPR-r in a 250 mL shake flask with five mM 2-HP-β-CD or 2,6-dimethyl-β-cyclodextrin (DM-β-CD) added at the beginning of the fermentation resulted in over 71% (107 mg/L) and 95 % (155 mg/L) of produced GA-HLDOA outside of the yeast. In comparison, only 1.4 % (2 mg/L) of produced GA-HLDOA was secreted from the untreated control strain.[239] The engineered *S. cerevisiae* YBVT15 increased 90 and 48% (6.5 and 5.1 mg/L) secretion of δ-tocotrienol when five mM of HP-β-CD or DM-β-CD was added to the culture medium. Meanwhile, the intracellular concentrations of δ-tocotrienol also increased

by 46 and 49 % (108 and 111 mg/L) compared to the control. The production and secretion of δ-tocotrienol were increased again when the amount and timing of HP-β-CD addition were adjusted; the final δ-tocotrienol production was 146 % (188 mg/L) higher than the control when 30 mM HP-β-CD was added after 24 h of fermentation.[178] Therefore, β-cyclodextrin and its derivatives can promote the secretion of natural products produced by plants and yeast. Notably, the absolute amount of the natural products is increased in most cases.

5.4 Summary and perspectives

With a growing world population, the increasing demand for natural products has necessitated their efficient bioproduction; therefore, exploring efficient biosynthetic strategies will be a future trend.[5] Cell factories will increase the use of renewable resources for producing natural chemicals, thus effectively reducing the negative impacts of production from fossil fuel or plant biomass, which both come with environmental pollution and waste of resources.[5] One of the challenges for the efficient and eco-friendly production of natural products by microorganisms is exporting them outside the plasma membrane.[243] The cytotoxicity caused by the accumulation of natural products generates a series of adverse effects on cells' normal growth and metabolism.[136,191,176] Therefore, studying the transport and secretion of natural products is a promising strategy to improve the final natural products titer by avoiding the cytotoxicity of natural product's toward the host cells.

Table 5-1. The strategies for improving the secretion of chemicals by yeast

Strain	Strategies	Chemicals	Titer and secretion titer	Shake flask or fermenter	Reference
S.cerevisiae CEN.PK113-7D	20% v/v sunflower oil was added	β-Carotene	——, 0.14 mg/L	2 L bioreactor	[198]
S. cerevisiae BJ5464-r and INVSc1-r	YPS3 and SPW10 were overexpressed	Triterpenoid GA-HLDOA	73 mg/L, 29 mg/L	250 mL shake flask	[193]
S. cerevisiae CYP5150L8-r-iGL CPR-r	5 mM DM-β-CD was added	Triterpenoid GA-HLDOA	118 mg/L, 113 mg/L	10 L bioreactor	[239]
S. cerevisiae BY4741	2-HP-β-CD was added, PDR1 was overexpressed, and olive oil was added	δ- Tocotrienol	211 mg/L, 181 mg/L	shake flask	[178]
S. cerevisiae CEN.PK2-1C	PDR11 was overexpressed	Rubusoside	156 mg/L, ——	250 mL shake flask	[192]
S. cerevisiae SR8A	50% v/v dodecane was added	Vitamin A (retinal and retinol)	3350 mg/L, 2864 mg/L	2 L bioreactor	[204]
S. cerevisiae FY1679-01B	SNQ2 and OLE1 were overexpressed	β-Carotene	160 mg/L, 10 mg/L	250 mL shake flask	[213]
S. cerevisiae BY4741	10% (v/v) dodecane was added	Patchoulol	1632 mg/L, ——	5 L bioreactor	[176]
R. toruloides CBS 5490	Pdr10 was overexpressed and 20 % v/v grape seed oil was added	Carotenoids (torularhodin,	2.9 mg/g, 1.8 mg/L	shake flask	[234]

		torulene and β-Carotene)			
S. cerevisiae Ycarot-02	PDR3 was overexpressed and 20% olive oil was added	Canthaxanthin	168 mg/L, 0.7 mg/L	shake flask	[197]
S. cerevisiae BY4742	5 mM M-β-CD was added	16α-Hydroxy-β-amyrin	199 mg/L, 199 mg/L	shake flask	[240]
S. cerevisiae BY4741	10% v/v dodecane was added	Patchoulol	467 mg/L, ——	5 L bioreactor	[206]
Y. lipolytica CJ0125	10% Tween 80 was added	Retinol	4.9 g/L, 4.7 g/L	5 L bioreactor	[205]
Y. lipolytica CJ0125	10% Tween 80 was added	Retinal	0.26 g/L, 0.25 g/L	5 L bioreactor	[205]
S. cerevisiae CEN.PK TAM	SpMae1 was overexpressed	Malate	4.3 g/L, 0.5 g/L	250 mL baffled shake flask	[229]
S. cerevisiae CEN.PK TAM	AcDct was overexpressed	Malate	17 g/L, ——	250 mL baffled shake flask	[229]
S. cerevisiae CEN.PK TAM	SpMae1 was overexpressed	Succinate	2.6 g/L, 0.8 g/L	250 mL baffled shake flask	[229]
S. cerevisiae CEN.PK113-1A	AnDCT-02 was overexpressed	Succinate	10.3 g/L, ——	2 L bioreactor	[195]
S. cerevisiae RWB837	SpMae was overexpressed	L-Malic acid	30.3 g/L, ——	500 mL shake flask	[231]
S. cerevisiae	20% v/v sunflower oil was added	β-Carotene	0.9 mg/gCDW, 0.5 mg/L	300 mL baffled shake flask	[244]
S. cerevisiae	20% v/v dodecane containing 50% linoleic acid	β-Carotene	0, 0.6 mg/L	300 mL baffled shake flask	[244]
S. cerevisiae YSM5	Pdr11p and Yol075cp were co-overexpressed	Tocotrienol	83 mg/L, 61 mg/L	shake flask	[179]
S. cerevisiae YSM5	5% v/v olive oil was added	Tocotrienol	74 mg/L, 41 mg/L	shake flask	[179]
S. cerevisiae CEN.PK2-1C	PDR11 was overexpressed	β-Carotene	870 mg/L, 197 mg/L	250 mL shake flask	[191]
S. cerevisiae CEN.PK2-1C	PDR11 was overexpressed	Lycopene	6.7 mg/L, 600 mg/L	250 mL shake flask	[191]

Two strategies were mainly used to improve the secretion of natural products by yeasts: (1) by engineering the transporter proteins that can transport natural products from intracellular to extracellular or (2) by using appropriate extractants (e.g., dodecane, IPM, sunflower oil, olive oil, and β-Cyclodextrin or its derivatives) to carry out in-situ extraction (Figure 4-1, Table 5-1).[191,192,179,232,9] However, some challenges for these two strategies also need to be solved in the future. New techniques need to be developed to discover optimal transport proteins for the output of natural products because the transporters from different organisms will perform differently when introduced into the host strains, and it is essential to match the specific transport proteins to particular substrates.[230] The secretion of natural products by host strains is very complex; it is difficult to capture this process by cryo-electron microscopy (cryo-EM), which presents a significant challenge for the structural modification of transport proteins.[191] Some natural products

(e.g., Vitamin E, carotenoids) can be easily extracted by the proper extractants in the medium because they are stored in the cell membranes due to their hydrophobic structures.[179] Meanwhile, the promotion of natural products secretion by plant oils may be explained by the improved cell membrane fluidity by linoleic acid or the corresponding triglyceride as the main components of plant oils (Table 5-2).[244] However, more plant oils in the medium will produce lipid peroxides that decrease yeast cell growth; a report mentioned that 0.1 mM linoleic acid in the medium for four hours increases yeast apoptosis induction.[199] Some research reported that microencapsulation of natural products using various encapsulating agents, such as β-Cyclodextrin and its derivatives, can improve the dispersibility and stability of natural products.[178,239] However, the price of β-Cyclodextrin and its derivatives is not cheaper, and it will save the cost to use the naturally secreting strains to produce natural products.[239] Furthermore, separating natural products from extractants is also a challenge because different extractants have different chemical properties.

Table 5-2. Fatty acid composition of vegetable oils used in in-situ extraction [245,246,247,248]

Vegetable oil	Fatty acid	Carbon number	Amount (%)
Sunflower oil	Myristic	C14:0	0.183
	Palmitic	C16:0	7
	Stearic	C18:0	4.2
	Arachidic	C20:0	0.33
	Behenic acid	C22:0	0.96
	Lignoceric acid	C24:0	0.35
	Palmitoleic	C16:1	0.154
	Oleic	C18:1	34.5
	Linoleic	C18:2	52.6
	Linoleic	C18:3	0.42
Grape seed oil	Caprylic acid	C8:0	0.018
	Capric acid	C10:0	0.03
	Lauric acid	C12:0	0.018
	Myristic acid	C14:0	0.076
	Pentadecanoic acid	C15:0	0.014
	Palmitic acid	C16:0	9
	Palmitoleic acid	C16:1	0.159
	Heptadecanoic acid	C17:0	0.048
	Heptadecenoic acid	C17:1	0.02
	Stearic acid	C18:0	4.9
	Oleic acid	C18:1	16.9
	Linoleic acid	C18:2	68.9
	Linolenic acid	C18:3	0.314
	Arachidic acid	C20:0	0.14
	Eicosenoic acid	C20:1	0.121
	Docosanoic acid	C22:0	0.03
	Lignoceric acid	C24:0	0.028
Olive oil	Palmitic acid	C16:0	12.7
	Palmitoleic acid	C16:1	0.9

Stearic acid	C18:0	2.5
Oleic acid	C18:1	71.5
Linoleic acid	C18:2	10.1
α-Linolenic acid	C18:3	0.685
Arachidic acid	C20:0	0.45
Gadoleic acid	C20:1	0.288
Arachidonic acid	C20:4	0.665
Behenic acid	C22:0	0.128
Lignoceric acid	C24:0	0.063

The fatty acids proportion of grape seed oil is the average of the fatty acids proportion in eight different cultivars (Italian Riesling, Cabernet Franc, Pinot Noir, Sauvignon Blanc, Királyleányka, Rhine Riesling, Merlot, and Lemberger) of grape seed oil. The fatty acid proportion of olive oil is the average in four different cultivars (Koroneiki, Megaritiki, Amfissis, and Manaki) of olive oil.

In general, the research on the secretion of natural products is still focused on compounds with simple molecular structures (e.g., vitamin E, carotenoids, alkanes, and C4-dicarboxylic acids). The research on complex chemicals, such as triterpenoids, is still limited (Table 5-1) (Figure 5-1).[197,179,227,195,231] Some research indicated that triterpenoid ginsenosides can be exported outside the yeast cells; however, the underlying molecular mechanisms need to be explored.[80,97,129] For example, recent research indicated that engineered *S. cerevisiae* WLT-MVA5 was cultured in a 5 L bioreactor for 120 h using ethanol as the single feed carbon source, and almost all produced protopanaxadiol (PPD) was secreted outside the yeast cells that attached on the inside wall of the bioreactor.[97] Another study showed that the ginsenosides PPD, Rh2, and Rg3 could also be secreted outside the yeast cells during the fermentation by engineered *S. cerevisiae* transformants D20RG1 and D20RH18.[129] The betulin and betulinic acid engineered *S. cerevisiae* strains WAT11 and CEN.PK were constructed by introducing a lupeol C-28 oxidase (LO) from *Betula platyphylla*. The result showed that betulin and betulinic acid were mainly accumulated in the medium. The WAT11 strain accumulated about 3-fold higher betulinic acid and relatively lower betulin in the medium than the CEN.PK strain.[187] Therefore, all these studies suggest that a transport pathway for triterpenoids also exists in the cell membrane of yeast. But how the triterpenoids are secreted outside the cell remains elusive.[22] Transporter proteins for triterpenoids may exist in the cell membrane, but whether these transporter proteins are the same as those for simple structural natural products needs to be further explored.

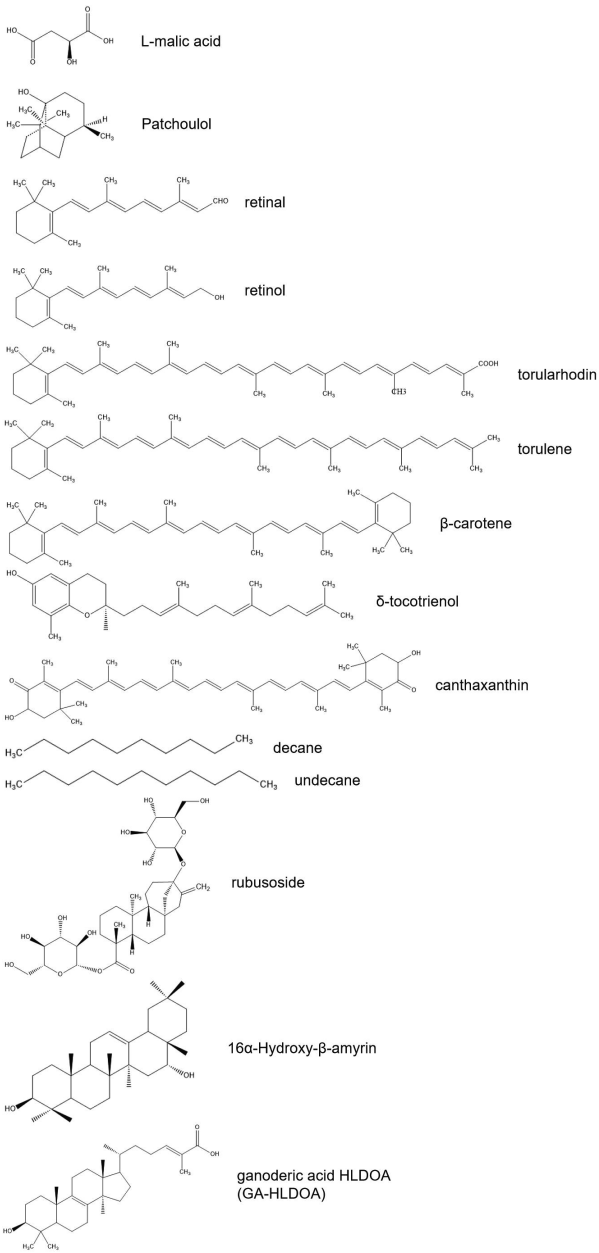

Figure 5-1. The chemical structures of the natural products that can be exported extracellularly by yeast.

In this thesis, we optimized an M3 medium to improve the final ginsenosides production, and the PPD titer reached 1.2 g/L after five days of fermentation. Protopanaxadiol, a 30-carbon dammarane II-type tetracyclic triterpene, is the precursor of high-value ginsenosides. Much research focuses on improving protopanaxadiol production by metabolic engineering approaches, while cultivation is somewhat overlooked, achieving less than 1 g/L in a 500 mL shake flask. There are two exceptions: 1. As reported by Zhu et al., the final protopanaxadiol production reached 1.6 /L after seven days of fermentation by metabolic engineering approaches with sugarcane molasses as the carbon source. 2. Lin et al. improved protopanaxadiol production to 1.7 g/L after five days of fermentation in a 500 mL shake flask by overexpressing *PgPPDS* and optimizing the promoter.[137,249] The above two exceptions indicate that there also exists a lot of potential to increase the final production. The M3 medium can be used for different triterpenoids producers. For example, to vastly improve the lupeol-type triterpenoids (betulin and betulinic acid) and ginsenoside Compound K production, which indicated the versatility of this M3 medium. The mechanism by which the M3 medium can increase the production of triterpenoids remains to be further explored, but the main argument is the very high carbon source use. Intuitively, one would expect oxygen limitation; however, this seemed less of an issue in the long cultivation time and was partially compensated by the lid used.

Our result showed that vegetable oils can improve the final PPD and DM production when added at 96 h during the fermentation. Importantly, almost all the PPD and DM were accumulated in the medium after five days of fermentation. Notably, the “empty yeast” can be reused for the second round of fermentation, and the DM titer was over 3 g/L, the highest production in the shake flask until now. Studies indicated that the natural products (e.g., tocotrienols, β-carotene) secretion promoted by plant oils may be explained by the improved cell membrane fluidity by linoleic acid or the corresponding triglyceride as the main components of plant oils.[179,244] However, there is no research on whether vegetable oils can improve triterpenoid secretion. Therefore, the reason why vegetable oils can promote PPD and DM secretion need to be further investigated. Research showed that IPM and dodecane can improve terpenoid and triterpenoid secretion when these organic solvents are added during fermentation.[196,250] We tried these two organic solvents, but no improvement in total triterpenoid produced was observed (data not shown). Adding large volumes of organic phase (IPM), up to 150 % (v/v) of the medium, was also challenging for keeping the protocol as simple as possible.

In general, this thesis provides a strategy to improve the production and secretion of ginsenosides, which will be a reference for synthesizing natural products by microbial cell factories and lays a foundation for the industrial production of natural products.

Chapter 6

Conclusion and Outlook

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6.1 Conclusion and outlook

In this work, we present a simple cultivation of engineered yeast for superior triterpenoid production. Using the M3 medium with an optimal temperature of 28 ° C and strong aeration by employing a membrane lid increased triterpenoid synthesis, vastly simplifying metabolic engineering and laboratory production, including analytics of this interesting group of natural products. A remarkable titer of 1.2 g/L PPD was achieved in the 500 mL shake flask, a 7.4-fold (0.16 g/L) and >10-fold improvement (0.08 g/L) compared to the WM8+ and YPD media. As the cultivation conditions can be transferred to other triterpenoid producing yeast like Compound K and betulin producers, the study paves the way for further research and commercialization of these fascinating compounds, including the industrial production of triterpenoids.

Currently, most studies on promoting natural product secretion by yeast mainly focus on chemicals with simple structures, and few studies can promote the secretion of triterpenoids through modifying fermentation processes. In-situ extraction improved the PPD and DM synthesis and secretion by adding small amounts of vegetable oils during the fermentation. Meanwhile, the "empty yeast" can be reused for the next round of fermentation. This makes it possible to simplify the downstream purification process in industrial production.

In conclusion, this study provides a simple strategy to improve the production and export of valuable triterpenoids, bringing us one step closer to the efficient industrial production of these valuable natural products.

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Curriculum vitae

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Published Papers During Ph.D. Study:

1. Qiu, S., Dianat, M., Müller, C., Zarazua-Navarro, R., Liebal, U., Roy, E* and Blank, L*. Cultivation optimization promotes ginsenoside and universal triterpenoids production by engineered yeast, New Biotechnology, IF: 4.5, 08. 2024

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