Bioprocess optimization enables enhanced protopanaxadiol production in yeast

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Abstract: Ginsenosides are the primary active metabolites of ginseng and have been used in traditional herbal medicine in Asia for more than 4,000 years. Protopanaxadiol (PPD) is the precursor of dammarane-type ginsenosides, exhibiting different pharmacological activities. However, extraction of PPD from plant material is cumbersome because of its low concentration. Precision fermentation using recombinant yeast is a promising alternative strategy to produce PPD. For increasing PPD production, the medium and fermentation conditions were optimized by a Design of Experiment (DoE) approach. PPD production increased by 7.5-fold in the final cultivation condition compared to the reference condition. The PPD titer reached 1.2 g/L during simple 500 mL shake flask cultivations, the highest PPD production in shake flasks to date. The protocol presented facilitates parallel evaluation of recombinant yeast, thereby contributing to the much-needed sustainable synthesis of the versatile molecule class of triterpenoids.

Keywords: *Saccharomyces cerevisiae*, Design of Experiment, triterpenoid, ginsenoside, bioprocess optimization

Introduction

Triterpenoids are secondary metabolites ubiquitous in plants with a range of biological properties.[1] 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 *Panax quinquefolius*, *Panax ginseng*, and *Panax notoginseng* are commonly industrially exploited. 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).[2,3,4] More than 180 ginsenosides have been identified in the Panax genus, which can be categorized into two groups based on their aglycones: dammarane-type tetracyclic and oleanane-type pentacyclic ginsenosides.[5,6,7] Dammarane-type tetracyclic ginsenosides can be further divided into three groups, ocotillol, protopanaxadiol (PPD)-type ginsenosides, and protopanaxatriol (PPT)-type ginsenosides, based on the carbohydrate moieties at C3, C6, and C20 positions and the respective hydroxylation.[7]

Ginseng has been used in traditional herbal medicine in Asia for more than 4,000 years.[5,8,3] The term "ginseng" is derived from the Chinese words "人 參 (Renshen)", "the essence of men", indicating its importance.[9] Traditionally, ginsenosides were either consumed using the plant tissue or extracted from the root of ginseng. The root contains a documented total ginsenoside content of 60 mg/g in the dry weight.[3] Changing and low amounts of the single target molecules in plant tissue make commercial production cumbersome.[10,11,8] In addition, Ginseng roots need five to six

88 years of growth until harvesting, with varying influences related to climate, land, soil,
89 and pathogens.[3,5,12] Furthermore, the complex structures of ginsenosides make
90 chemical synthesis economically infeasible.[5,13]

91 Yeasts are simple eukaryotes that can be engineered for the synthesis of high-value
92 metabolites. With their complete organelle structures, including mitochondria,
93 peroxisomes, Golgi apparatus, endoplasmic reticulum (ER), and vacuoles, which
94 allow post-translational modifications of proteins and operation of compartmented
95 biochemical pathways, they are geared for enzymes from plants.[5,11,14,15] Yeasts also
96 possess redox systems that provide similar biological and physical environments
97 encountered by cytochrome P450s and uridine diphosphate glycosyltransferase (UGTs)
98 in plants and mammals.[5,14] Due to these reasons, yeasts are considered excellent cell
99 factories for producing natural plant products, such as ginsenosides, betulinic acid,
100 farnesene, amorpha-4,11-diene, and others.[10,11,17,18,13] For example, the production of
101 α-farnesene was improved by engineered *Yarrowia lipolytica* during fed-batch
102 fermentation via the expression of the acetyl-CoA acetyltransferase (Erg10p) from
103 *Escherichia coli* and HMG-CoA reductase (Hmgp) from *Bordetella petrii*, the final
104 α-farnesene production reached 26 g/L.[11,19] Also, a highly engineered *Saccharomyces*
105 *cerevisiae* expressing the amorpha-4,11-diene synthase (ADS) from *Artemisia annua*
106 and using an unrestricted ethanol feed allowed the final production of 40 g/L
107 amorpha-4,11-diene.[13]

108 Triterpenoids are either synthesized from squalene or 2,3-oxidosqualene, which are
109 both intermediates of sterol synthesis in yeast.[5,16] The synthesis of ginsenosides by

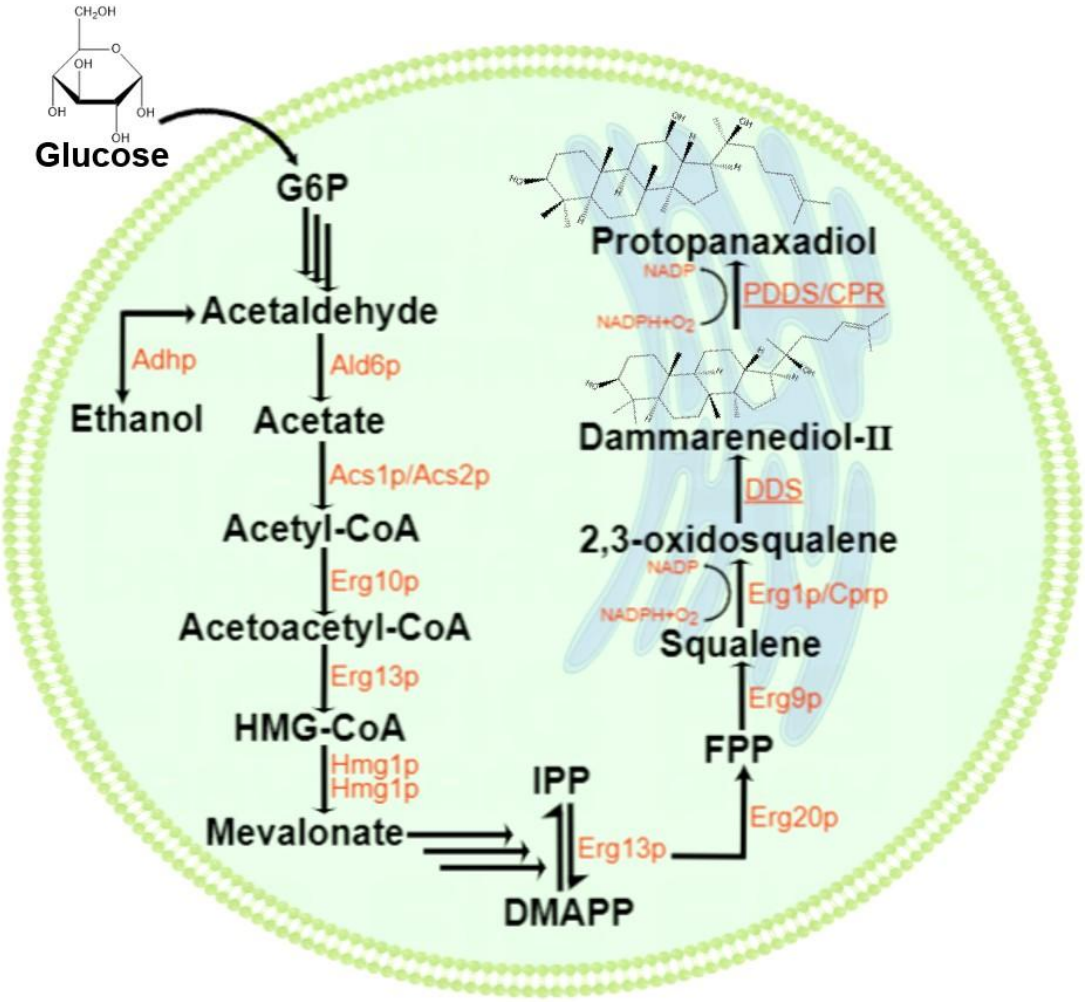
110 engineered yeasts from renewable resources is a promising strategy to address the
111 increasing demand for this interesting class of molecules.[5] The microbial biosynthesis
112 of ginsenosides has several advantages, including rapid growth, controllable culture
113 conditions, space efficiency, sustainable production, environmentally friendly, and
114 well-developed genetic manipulation technology.[5,11,20] At present, only a fraction of
115 ginsenosides are synthesized by engineered yeast, such as dammarenediol-II (DM),
116 PPD, PPT, Compound K (CK), ginsenoside Rh1, ginsenoside Rh2, ginsenoside F1,
117 ginsenoside Rg3, 20S-O-Glc-DM, 3β-O-Glc-DM, ginsenoside Rg1, ginsenoside
118 NgR1, ginsenoside NgR2, ginsenoside Rf, ginsenoside Glc-Rf, and ginsenoside
119 Ro.[5,21] Furthermore, there have been some prominent achievements in synthesizing
120 ginsenosides by yeasts. For example, according to a recent study, researchers
121 constructed a PPD-producing *S. cerevisiae* cell factory via overexpression of
122 mevalonate and PPD synthetic pathways and optimization of P450 expression levels;
123 the final PPD production reached 11 g/L in 10 L fed-batch fermentation.[18] Researchers
124 also introduced the ginsenoside CK synthesis pathway into this strain while
125 decreasing UDP-glucose consumption and improving UDP-glucose biosynthesis by
126 engineering strategies; the final CK yield reached almost 6 g/L in fed-batch
127 fermentations.[12]

128 Research showed that bioprocess optimization, including medium and cultivation
129 conditions, can vastly improve the production of metabolites by cell factories.[22,23] For
130 example, carbon sources (e.g., glucose, glycerol, and glutamate), nitrogen sources,
131 temperature, pH, oxygen, inoculum size, and salts significantly affect the cells' growth

132 and production.[22,24,25] As the parameter space in medium and fermentation parameter
133 optimization is huge, researchers aim for Design of Experiments (DoE) approaches.
134 For example, researchers optimized the concentration of glucose, yeast extract, and
135 peptone via the Box-Behnken design; this optimization increased the geranylgeraniol
136 production by 1.75-fold compared to the traditional YPD medium.[26] Cultured
137 α-farnesene-producing *Y. lipolytica* in a medium optimized by a uniform design (UD)
138 technique reached a final α-farnesene titer of 210 mg/L in a shake flask.[24] In another
139 study, adding citrate (0-10 mM) or acetate (0-10 mM) into the medium improved
140 squalene production. The inhibition of the squalene monooxygenase Erg1p by
141 terbinafine allowed 730 mg/L of squalene titers.[27] Therefore, improving the
142 cultivation conditions of engineered yeast is a promising strategy.

143 This study aims to provide a simple shake flask protocol to evaluate all the different
144 mutants one might have to generate for further improving triterpenoid synthesis in
145 yeast. Here, we use PPD synthesis as the main target, as the synthesis requires the
146 activity of a triterpenoid cyclase and P450 monooxygenase. PPD is one of the
147 30-carbon dammarane II-type tetracyclic triterpenoids found in ginseng and is the
148 precursor of high-value ginsenosides.[10] PPD exhibits valuable biological properties,
149 such as antineoplastic, antioxidant, antidepressant, antitumor, and
150 anti-inflammation.[28,29] For improved PPD production by yeast in shake flasks, the
151 following parameters were considered: temperature, glucose and glutamate
152 concentrations, salts solution, trace element solution, vitamin solution, and oxygen
153 availability. PPD production reached 1.2 g/L using the best combination of shake

154 flask set-up and optimized medium, the highest PPD production in shake flasks to
155 date. This simple set-up facilitates the evaluation of engineered triterpenoid
156 synthesizing yeast, and the amounts of triterpenoid produced enable advanced
157 analytics and even first ideas for purification and use of this intriguing class of natural
158 products.



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160 **Figure 1.** The PPD synthesis pathway in engineered *S. cerevisiae*. The recombinant enzymes are underlined. G6P,
161 glucose 6-phosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; IPP, isopentenyl pyrophosphate; DMAPP,
162 dimethylallyl diphosphate; FPP, farnesyl diphosphate; Erg10p, acetyl-CoA acetyltransferase; Erg13p, HMG-CoA
163 synthase; Hmg1p and Hmg2p, HMG-CoA reductase 1 and 2; Idi1p, isopentenyl-diphosphate delta-isomerase;
164 Erg20p, farnesyl diphosphate synthase; Erg9p, squalene synthase; Erg1p, squalene monooxygenase; DDS,

165 dammarenediol-II synthase; PPDS, protopanaxadiol synthase; ER, endoplasmic reticulum. This image was created
166 by Figdraw.

167 **RESULTS AND DISCUSSION**

168 **Effect of medium composition and Oxygen availability on DM and PPD**
169 **Production.** The DoE experiment included 34 runs designed using Design Expert 11
170 (Stat-Ease, Inc.). The seven significant factors and the high, medium, and low
171 categories are shown in Table 1. The system Duetz microtiter plates were used for
172 cultivation (Enzyscreen). Samples were taken after 72 h, 96 h, and 120 h of
173 cultivation. DM is the precursor of PPD, which is also a triterpenoid and is the
174 substrate of the cytochrome P450 monooxygenase protopanaxadiol synthase (PPDS)
175 for PPD (Figure 1).[30,31] DM and PPD concentrations were highest after 120 h. PPD
176 production reached 0.3 g/L in four experiments (1, 10, 12, and 33) (Figure 2A).
177 Among them, runs 1, run 10, and run 12 were all cultured at 20 °C, and run 33 was
178 cultured at 30 °C. Glucose and glutamate concentrations were three-fold compared to
179 standard WM8+ medium (100 g/L) for runs 1, 12, and 33. Notably, the salt
180 concentration of all four runs was 0.33-fold of standard WM8+ medium. Also, higher
181 oxygen availability was common in these four experiments, using only 1 mL of
182 culture broth. The PPD concentration reached 0.9 g/L in run 1, the highest PPD
183 production among all 34 runs in the DoE experiment.

184 DM production suggests triterpenoid synthesis. However, it is also a potential
185 limitation in the subsequent P450-catalyzed reaction to PPD. Only seven experiments
186 showed DM production (Figure 2B). Among the seven experiments, only runs 21 and

187 27 had DM concentrations above 0.3 g/L. These two runs' glucose, glutamate, and salt
188 concentrations were three-fold that of standard WM8+ medium. The results suggest
189 high glucose and glutamate concentrations support high DM and PPD production. At
190 the same time, low salt concentration, low temperature, and high oxygen
191 concentration might benefit PPD production, indicating that the P450-catalyzed
192 hydroxylation is somewhat challenging, with substrate limitation and a slow rate of
193 catalysis.

194 **Table 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 [°C]	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), middle (yellow), and high (green) value. For factors A-E, the figures in the table represent fold changes in the actual concentration compared to the standard WM8+ medium. For factor F, the figures in the table represent the actual temperature. For factor G, figures in the table represent the volume of the medium in 24-well plates. CT: Control experiment, standard WM8+ medium.

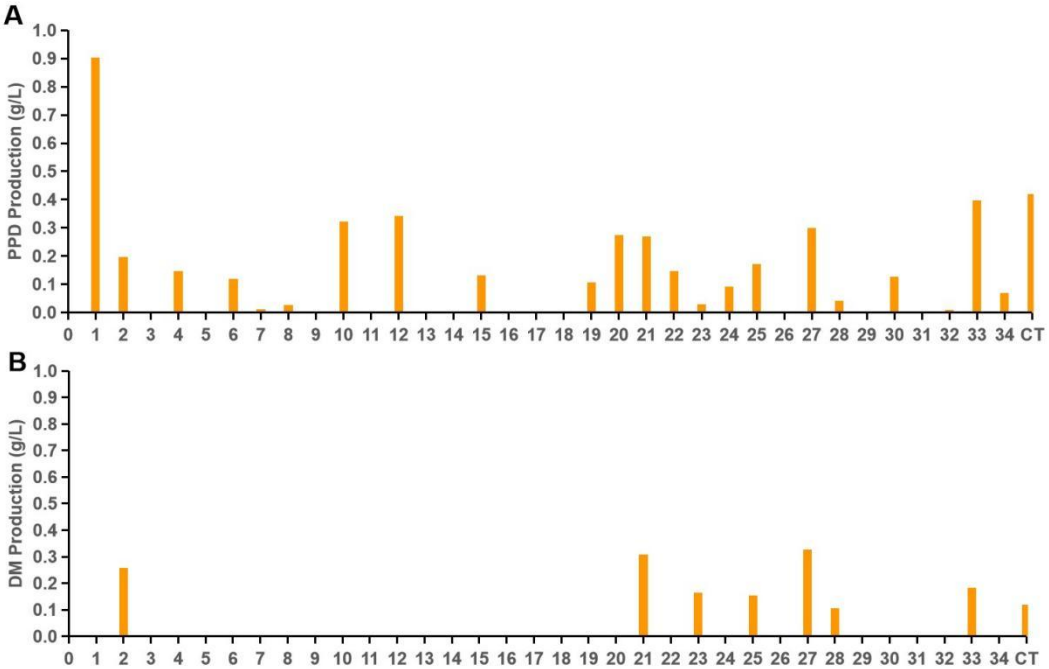


Figure 2. The result of the 34 runs DoE experiment. (A) PPD production by engineered yeast in 24-well plates. (B) DM production at different fermentation conditions in 24-well plates. CT: Control experiment, standard WM8+ medium.

The ordinary least squares (OLS) Estimation of the DoE Data. DoE data was analyzed with OLS regression to PPD and DM to identify the predictive factors. We

205 tested models containing only the individual factor effects (Ind-OLS) as well as
206 individual factors combined with two-factor interactions (Int-OLS) (Table 2). The
207 p-value of the F-Statistics identified models with sufficient power to explain the
208 variance of the observations (PPD) if the p-value is below 0.05. The Ind-OLS and the
209 more complex Int-OLS explain 41% and 83% of the variance in PPD, respectively
210 (adj. R^2(Ind-OLS)=0.41, adj. R^2(Int-OLS)=0.83). The p-values of the individual
211 factors in each regression model determine whether the factor significantly
212 contributes to the prediction. There are two significant factors for Ind-OLS (A:
213 glucose, C: glutamate) and seven for Int-OLS (B: salt, C: glutamate, AxE (vitamins),
214 AxC, B (salt)xF (temperature), CxD (trace element), BxG (oxygen)) (Figure 3). In the
215 simple Ind-OLS model with individual factors, only glucose and glutamate are the
216 most influential predictors that positively contribute to PPD production with
217 correlation values of 0.4 (Figure 3A). Conversely, salt concentration (B), temperature
218 (F), and medium volume (G) correlate negatively to the final PPD production, albeit
219 with low correlation values at -0.2 (Figure 3A). In the more complex Int-OLS model,
220 glucose is only significant via the two-factor interactions with glutamate (A:C) and
221 vitamin (A:E) but no longer as an individual factor. The most significant single factors
222 in the complex Int-OLS model are the positive correlation with glutamate (C) and the
223 negative correlation with salts (B). While the volume level (G, oxygen) individually is
224 just above the significance value (p=0.05), it negatively correlates with PPD and is
225 also negative with the significant interaction with salts (BxG). Thus, less oxygen via
226 higher volumes decreases PPD (Figure 3A). The yeast increases biomass quickly,

while the oxygen concentration decreases fast in the exponential phase.[25] Furthermore, NADPH is oxidized to NADP by a P450 reductase in the presence of oxygen, then transfers electrons to the monooxygenases, such as PPDS.[7] 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 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, Fact. with $p<0.05$, are factors with a p-value below 0.05, along with the estimated coefficient in the OLS model.

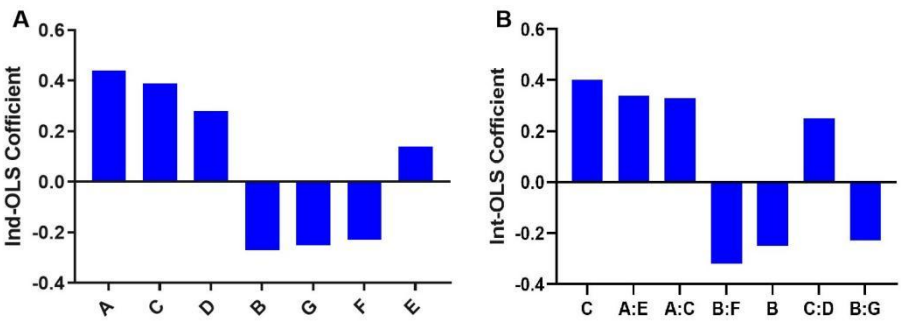


Figure 3. The OLS regression result of the DoE data with prediction of PPD production. (A) The OLS regression result of the Ind-OLS. (B) The OLS regression result of the Int-OLS. The coefficients with decreasing absolute

242 values are shown for factors (Ind-OLS) and factor interactions (Int-OLS), significantly explaining the variance
243 (p-values below 0.05). The larger the coefficient, the greater the influence of this factor or factor interaction on the
244 final PPD production. A: glucose; B: salts; C: glutamate; D: trace element solution; E: vitamin solution; F:
245 temperature; G: medium volume.

246 **Effect of Temperature on DM and PPD Production.** Sesquiterpenoid production
247 by engineered *S. cerevisiae* was reported to be better at 25 °C than at 30 °C.[32] We also
248 investigated temperature as an optimization parameter for PPD production. Here, the
249 highest PPD production was observed at 20 ℃ in the 34 runs DoE experiment (Figure
250 2A). Temperatures above 30 °C were reported to cause stress to baker´s yeast and
251 decrease cell viability.[25] Therefore, the 15 runs of the DoE experiment cultured at
252 20 ℃ were selected to investigate the effect of different temperatures on DM and PPD
253 production in 24-well plates. To explore the optimum temperature, 18 ℃, 20 ℃,
254 22 ℃, 25 ℃, 28 ℃, 30 ℃, and 32 ℃ were selected. The other fermentation and
255 medium conditions remained constant. In general, the production of PPD increased
256 with increasing temperature and reached the highest value at 28°C, then decreased
257 with increasing temperature (Figure 4A, Figure S1). Especially the PPD productions
258 of runs 1 and 12 are the highest among the 15 runs, reached 1.5 g/L at 28 ℃ (Figure
259 4A). But for run 13, run 14, run 18, run 20, run 24, run 30, and run 32, the PPD
260 production reached the highest value at 32 ℃. However, the PPD production for these
261 seven runs is lower than runs 1 and 12 (Figure S1). So, we can speculate that the
262 optimum temperature for PPD production is 28 ℃.

263 The DM production of run 1, run 12, and run 24 reached the highest when cultured

264 at 30 ℃. However, the optimum temperature of the run 6 and run 32 is 25 ℃, run 10,
265 run 20, run 22, and run 28 is 28 ℃, and run 30 is 32℃ (Figure 4B, Figure S1). The
266 first reason why the optimum temperatures of PPD and DM synthesis in all the runs
267 are different may be that the different concentrations of compounds in the medium
268 can affect the DM conversion to PPD at different temperatures. Another reason may
269 be that the different medium compositions affect the enzyme activities involved in
270 PPD and DM synthesis at different temperatures. The DM production of run 28 is the
271 highest when cultured at 28 ℃, which reached 1.1 g/L (Figure S1). The DM
272 production of run 22, run 28, and run 30 are all over 0.8 g/L. The total triterpenoid
273 production of run 1 is the highest among all the runs, cultured at 30 ℃ and reached
274 2.3 g/L (Figure 4C). The total triterpenoid production of run 1, run 12, and run 30 is
275 over 2 g/L. However, the total triterpenoid production of run 1 and run 12 reached 2
276 g/L when cultured at 28 ℃ or 30 ℃, while run 30 reached 2 g/L at 32 ℃ (Figure 4C,
277 Figure S1). Hence, optimum temperature for DM and total triterpenoid production is
278 in the range of 28-32 ℃.

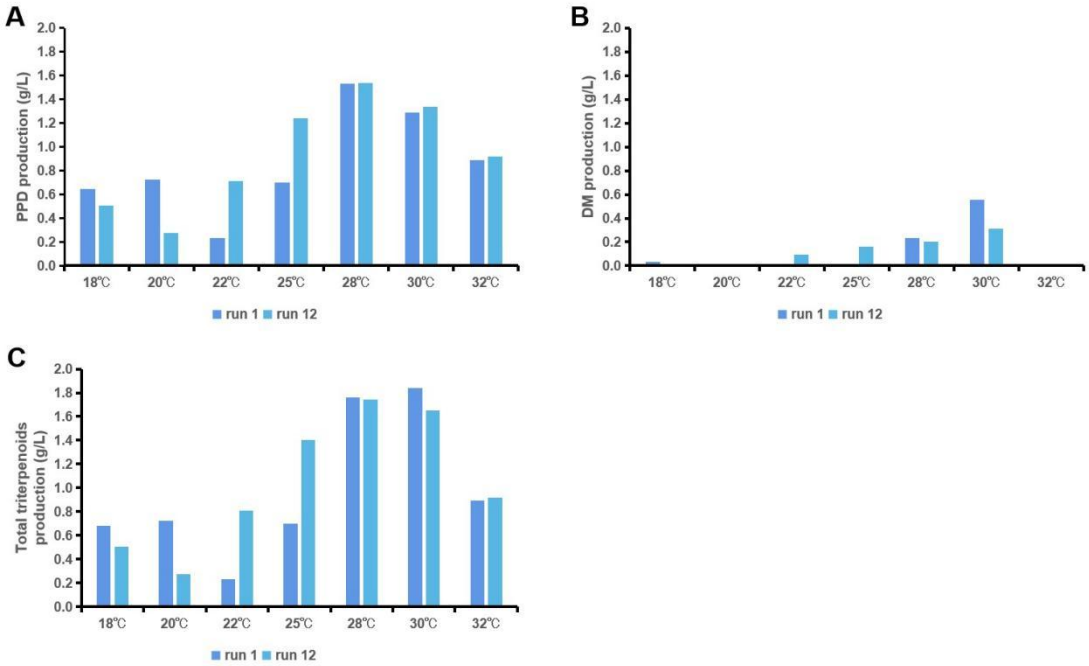
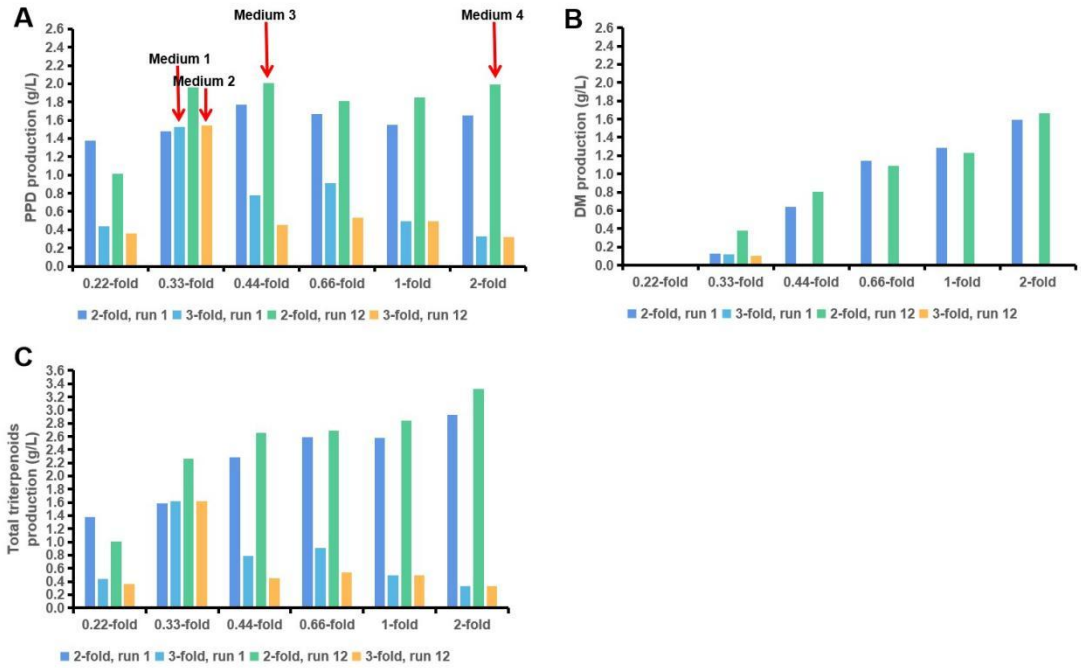


Figure 4. The effect of different temperatures on DM and PPD production. (A) The comparison of PPD production between run 1 and run 12 at 18°C, 20°C, 22°C, 25°C, 28°C, 30°C, and 32°C. (B) The comparison of DM production between run 1 and run 12 at 18°C, 20°C, 22°C, 25°C, 28°C, 30°C, and 32°C. (C) The comparison of total triterpenoid production between run 1 and run 12 at 18°C, 20°C, 22°C, 25°C, 28°C, 30°C, and 32°C. Other runs' data is shown in Figure S1.

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 4A). 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 times the WM8+ medium, with the highest PPD production is 2 g/L (Figure 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

294 stress and deflect resources from PPD production (Figure 5A).

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



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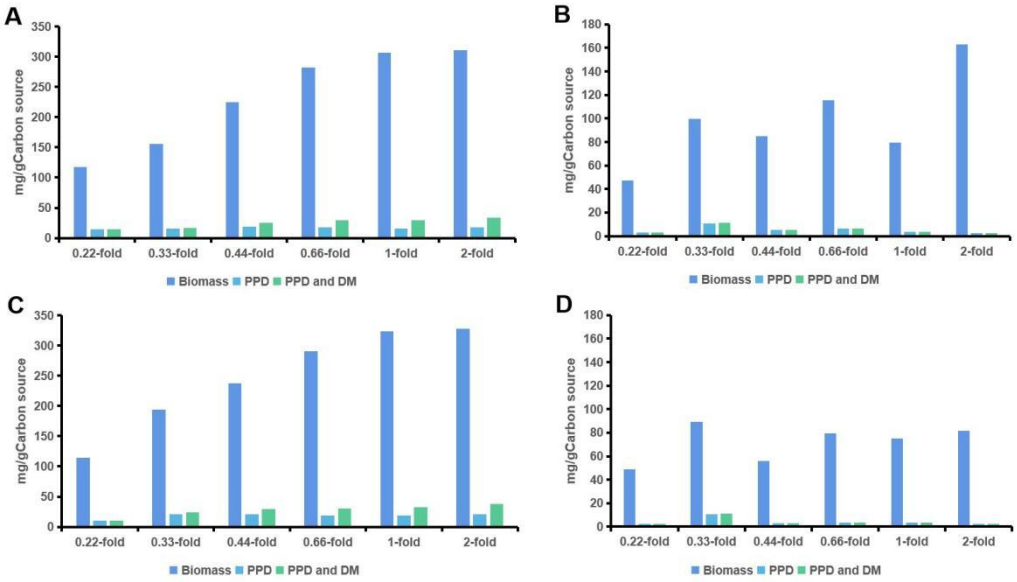
302 **Figure 5.** The effect of glucose, glutamate, and salt concentration on PPD and DM production (A) PPD production
303 depends on glucose, glutamate, and salts. (B) DM production is dependent on glucose, glutamate, and salts. (C)
304 The total triterpenoid production. The glucose and glutamate concentration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

307 For the translation of intracellular products, the yield per biomass is central, as is
308 the yield on substrate for any biotechnological process. Here, biomass generally

309 increased with increasing salt concentrations under constant carbon sources (Figure 6).
310 The highest biomass yield per gram carbon source is 328 $mg_{CDW}/g_{glucose\ and\ glutamate}$ in
311 two-fold salt concentration (Figure 6C). The triterpenoid yield increased with
312 increasing salt concentrations when glucose and glutamate concentration were twice
313 that of the standard WM8+ medium. However, the PPD yield first increases and then
314 decreases with increasing salt concentrations (Figure 6A and C). The highest total
315 triterpenoid yield is 38 $mg/g_{glucose\ and\ glutamate}$ (Figure 6C). At the same time, the highest
316 PPD yield is 21 $mg/g_{glucose\ and\ glutamate}$ (Figure 6C).
317 The total triterpenoid content of ripe ginseng root is 0.1-3 %.[3] We here estimated
318 the CDW content of PPD, DM, and total triterpenoids. The CDW content of the total
319 triterpenoid of three runs is above 120 mg/g_{CDW} (Figure 7A and C). The carbon source
320 concentration of these three runs is two times that of the standard WM8+ medium.
321 The salt concentration is 0.22, 0.33, or 0.44 times the reference. All the data indicates
322 that low salt concentrations favor high CDW content of PPD. In contrast, high salt
323 concentrations can contribute to increased CDW content of DM (Figure 7A and C).
324 The optimum glucose and glutamate concentration for the CDW content of PPD and
325 total triterpenoids is two times of the standard WM8+ medium.



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327 **Figure 6.** The effect of glucose, glutamate, and salt concentration on triterpenoid yield. (A) The glucose and

328 glutamate concentration is two times the standard WM8+ medium. This medium is modified from run 1. (B) The

329 glucose and glutamate concentration is three times the standard WM8+ medium. This medium is modified from

330 run 1. (C) The glucose and glutamate concentration is twice the standard WM8+ medium. This medium is

331 modified from run 12. (D) The glucose and glutamate concentration is three times the standard WM8+ medium.

332 This medium is modified from run 12. The horizontal coordinate is the salt concentration compared to the standard

333 WM8+ medium. All other parameters were used as described in Figure 5.

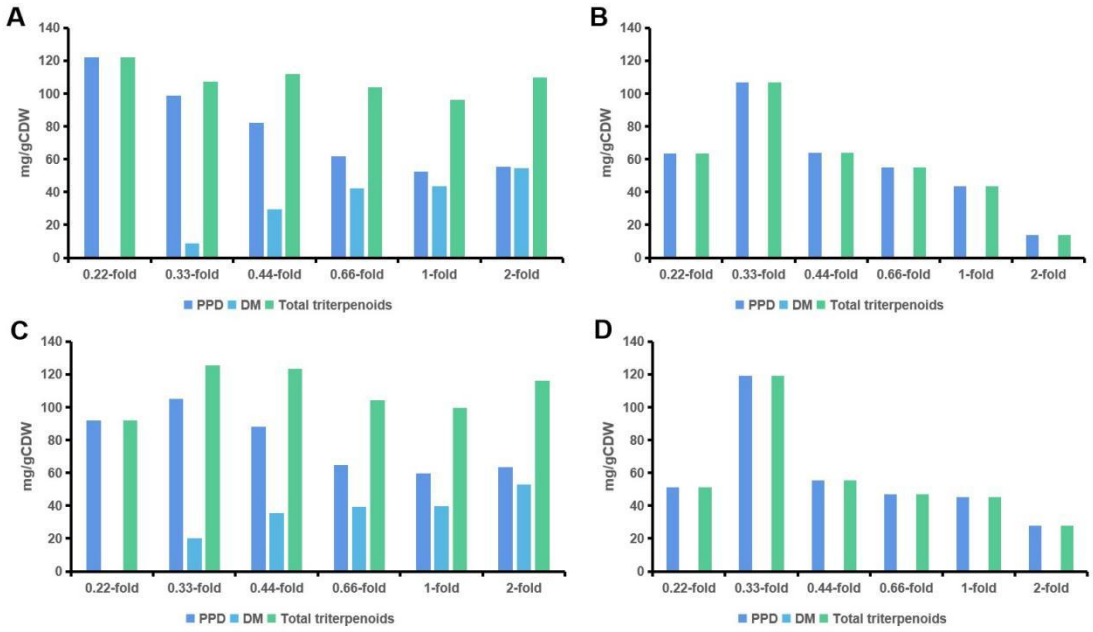


Figure 7. The effect of glucose, glutamate, and salt concentration on CDW content of triterpenoids. (A) The glucose and glutamate concentration is two times the standard WM8+ medium. This medium is modified from run 1. (B) The glucose and glutamate concentration is three times the standard WM8+ medium. This medium is modified from run 1. (C) The glucose and glutamate concentration is twice the standard WM8+ medium. This medium is modified from run 12. (D) The glucose and glutamate concentration is three times the standard WM8+ medium. This medium is 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 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 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 8A). The

349 membrane lid facilitates better aeration when compared to the aluminum lid. Two
350 enzymes in PPD synthesis require oxygen: the conversion of squalene to
351 2,3-oxidosqualene, catalyzed by Erg1p, and the conversion of DM to PPD, catalyzed
352 by PPDS (Figure 1).[33,34] First, yeasts were cultivated in the shake flask with an
353 aluminum lid. The smaller the filling volume of the shake flask, the better the aeration.
354 Two different filling volumes were used, i.e., 25 mL and 50 mL. The yeast was
355 cultured in the four improved media. The results showed the highest PPD production
356 of 0.8 g/L occurred in medium 3 with 25 mL filling volume. The PPD production
357 decreased with the increase in the medium. The PPD production in all four media
358 tested is higher than that in WM8+ medium, which is 0.16 g/L. It can be speculated
359 that the higher oxygen concentration benefits the synthesis of PPD, which can
360 increase the final PPD production in the shake flask (Figure 8B).

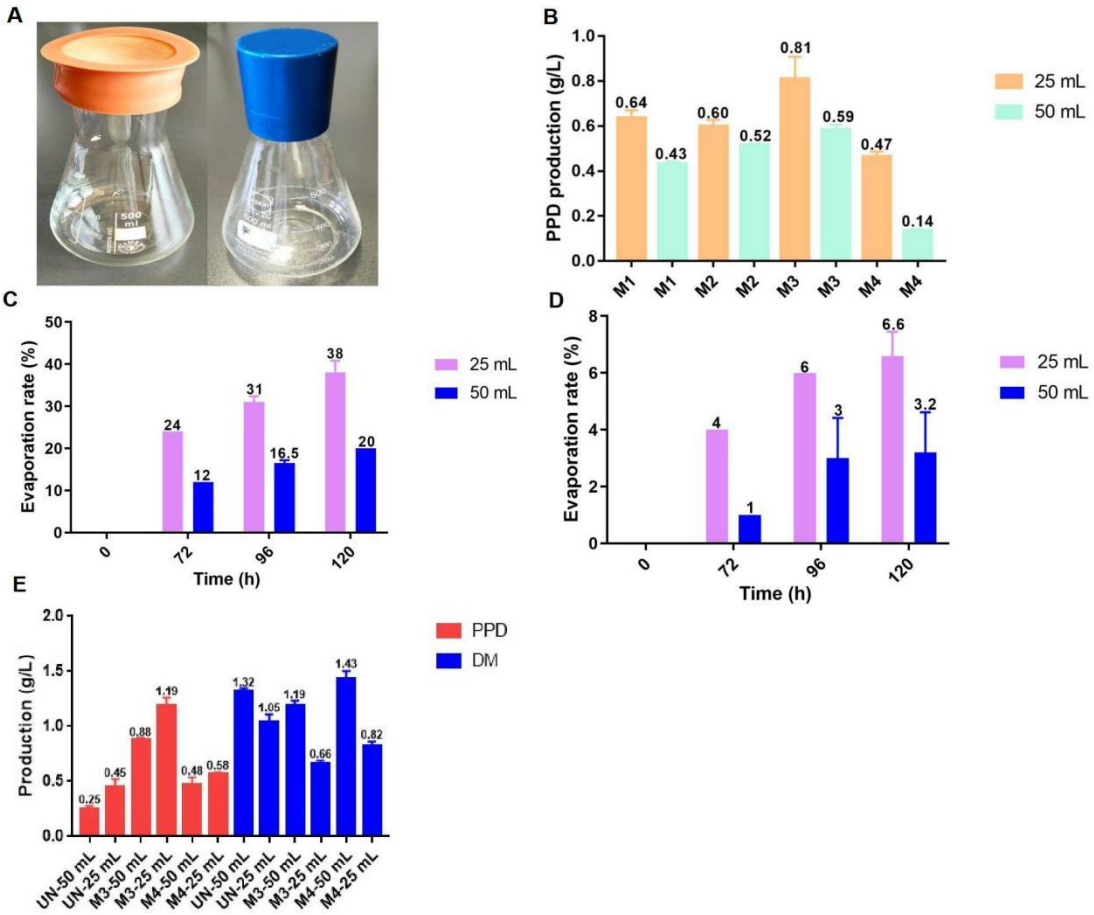


Figure 8. Shake flask configuration influences PPD production. (A) Shake flasks with membrane and aluminum lid. (B) PPD production depends on the filling volume using a shake flask with an aluminum lid. (C) Water evaporation rate in the 500 mL shake flask with a membrane lid. (D) Water evaporation rate in the 500 mL shake flask with an aluminum lid. (E) PPD and DM production depends on the filling volume using a shake flask with a membrane lid. UN, unoptimized standard WM8+ medium; M1, Optimum medium 1; M2, Optimum medium 2; M3, Optimum medium 3; M4, Optimum medium 4.

The identical experiments were repeated using the membrane lid for improved aeration. Notably, water evaporation is higher using the membrane lid (Figure 8C 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 °C (Figure

373 8C and D). We measured the volume after 120 h and calculated the product
374 concentration without evaporation. The corrections were as follows:

$$\frac{a \times b}{c} = d \qquad (1)$$

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

378 The results indicated that the PPD production also decreased with the increase of
379 medium volume. The PPD productions of optimum media 3 and 4 in this membrane
380 lid shake flask are higher than the PPD production of the same media with an
381 aluminum lid, reaching 1.2 g/L and 0.6 g/L when the volume is 25 mL. At the same
382 time, the DM production increased with medium volume (Figure 8E). This indicated
383 that the membrane lid and smaller volume allow more oxygen to enter the shake flask,
384 which supplies more oxygen for improving the conversion of squalene to
385 2,3-oxidosqualene and the conversion of DM to PPD (Figure 1). The highest PPD
386 productions of unoptimized standard WM8+ medium in the shake flask with an
387 aluminum lid or membrane lid are 0.16 g/L or 0.45 g/L, respectively (Figure 8E). This
388 indicated that the highest PPD production of the optimum medium 3 is 2.6-fold
389 compared to the unoptimized standard WM8+ medium in the shake flask with a
390 membrane lid. However, the PPD productions in media 1 and 2 with this membrane
391 lid shake flask are much lower than the PPD production in medium 3 (data not
392 shown). This may be due to the higher glucose and glutamate concentration (three
393 times the standard WM8+ medium) in media 1 and 2, limiting the PPD and DM

394 synthesis in the shake flask, just like the analysis described in Figure 5.

395 **The fermentation kinetics of PPD and DM in engineered yeast cultured in the**
396 **standard WM8+ medium and the M3 medium.** Ethanol is a more reduced carbon
397 source than glucose, which feeds directly into the cytosolic acetyl-CoA pool required
398 for the mevalonate pathway.[7] Therefore, we also measured the concentration of the
399 carbon sources and triterpenoid products during the fermentation to investigate the
400 fermentation kinetics of PPD and DM. As a Crabtree-positive yeast, the engineered *S.*
401 *cerevisiae* uses respiro-fermentative glucose metabolism and produces ethanol and
402 carbon dioxide.[35] After glucose depletion at 24 h, ethanol will be the carbon source in
403 the presence of oxygen, as the classic diauxic shift of *S. cerevisiae*. The ethanol
404 concentration also reached the highest value at 24 h, 12.5 g/L, and 35 g/L in the
405 standard WM8+ and M3 medium (Figure 9A and B). Ethanol has been shown to be a
406 beneficial carbon source for triterpenoid production.[7] Therefore, PPD and DM
407 synthesis was very fast when the carbon source became ethanol. The highest PPD
408 production is 0.6 g/L and 1.8 g/L in the standard WM8+ and M3 medium, respectively,
409 slightly higher than the PPD production above data (0.45 g/L in the standard WM8+
410 medium and 1.2 g/L in the M3 medium) (Figure 8E, 9A and B). The reason should be
411 due to the several samples taken during the fermentation that reduced the volume and
412 increased the oxygen concentration; this led to higher PPD production. The result
413 indicated that the M3 medium performs better than the standard WM8+ medium for
414 PPD synthesis.

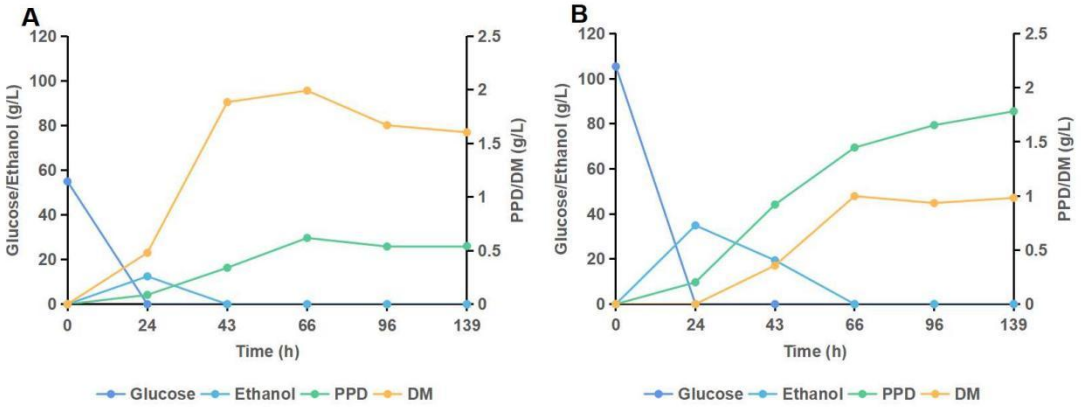
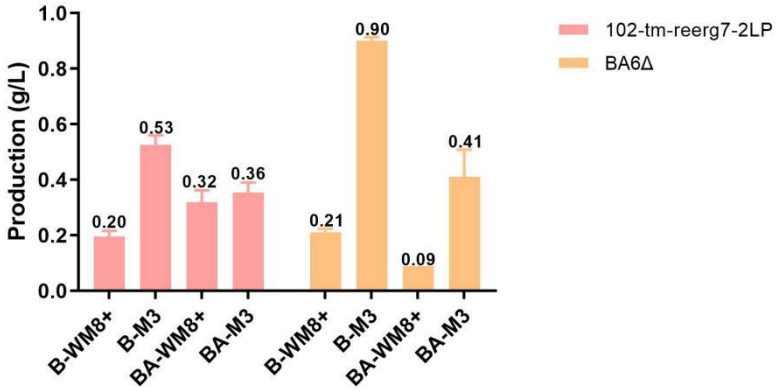


Figure 9. Fermentation kinetics of PPD and DM in engineered yeast. (A) The concentration of glucose, ethanol, PPD, and DM during the fermentation in engineered yeast cultured in the standard WM8+ medium. (B) The concentration of glucose, ethanol, PPD, and DM during the fermentation in engineered yeast cultured in the M3 medium.

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.[22,23] 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 are different between the two growth formats. We also checked other engineered yeasts that can produce other triterpenoids (betulin and betulinic acid) with the M3 medium. The result showed that the betulin and betulinic acid productions of these two strains in the M3 medium are largely increased compared to the productions in the standard WM8+ medium (Figure 10). This means that the M3 medium also works for other engineered strains. As aimed, the results can be generalized to evaluate engineered yeasts under simple

433 growth conditions.



434

435 **Figure 10.** Betulin (B) and betulinic acid (BA) produced by engineered yeasts. The aluminum lid was used for

436 shake flask cover and the media WM8+ and M3 were compared.

437 In conclusion, we identified the optimum temperature for PPD production at 28 °C.

438 The M3 medium presented here for producing PPD by yeast was built by stepwise

439 evaluation and optimization of the WM8+ medium. Furthermore, we used a

440 membrane lid to improve the final PPD production, which facilitated increased

441 aeration. Eventually, a remarkable titer of 1.2 g/L PPD was achieved in the 500 mL

442 shake flask, a 7.5-fold improvement compared to the unoptimized standard WM8+

443 medium with original fermentation conditions (0.16 g/L). As the medium and growth

444 conditions are easily established and transferable to other triterpenoid-producing

445 baker´s yeast, the study might facilitate the simple evaluation of engineered strains in

446 microtiter plates and shake flask cultivations.

447 **MATERIALS AND METHODS**

448 **Strains, Medium, and Reagents.** The engineered yeast 102-tm-reerg7-2PP for PPD

449 synthesis was modified from *S. cerevisiae* CEN.PK 102-5B and originated from a

450 previous study.[16,36] *S. cerevisiae* BA6Δ (unpublished) is a pah1 deletion mutant of

451 BA6, generated through replacement of PAH1 with a hygromycin resistance cassette
452 (YMR165CΔ:hphMX).[37,38] The engineered yeast BA6Δ for betulin synthesis was
453 modified from *S. cerevisiae* Simo1575.[16] Standard WM8+ medium included 50 g/L
454 glucose, salt solution, 10 g/L glutamate, trace elements solution, vitamins solution,
455 verduyn-trace elements solution, and verduyun-vitamins solution. In addition, the salt
456 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
457 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
458 trace elements includes 1.75 mg/L $ZnSO_4 \cdot 7H_2O$, 0.5 mg/L $FeSO_4 \cdot 7H_2O$, 0.1 mg/L
459 $CuSO_4 \cdot 5H_2O$, 0.1 mg/L $MnCl_2 \cdot 4H_2O$, 0.1 mg/L $Na_2MoO_4 \cdot 2H_2O$, and EDTA (0.04
460 mM). The vitamins solution includes 10 mg/L $C_6H_5NO_2$ (nicotinic acid), 25 mg/L
461 $C_8H_{11}NO_3$ (pyridoxine), 10 mg/L $C_{12}H_{17}N_4OS$ (thiamine), 2.5 mg/L $C_{10}H_{16}N_2O_3S$
462 (biotin), and 50 mg/L $C_9H_{17}NO_5$ (calcium-d-pantothenate). Verduyn-Trace elements
463 solution includes 15 mg/L Na_2EDTA, 4.5 mg/L $CaCl_2 \cdot 2H_2O$, 4.5 mg/L $ZnSO_4 \cdot 7H_2O$,
464 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
465 $Na_2MoO_4 \cdot 2H_2O$, 0.3 mg/L $CoCl_2 \cdot 6H_2O$, 1 mg/L H_3BO_3, and 0.1 mg/L KI.
466 Verduyn-vitamins solution includes 25 mg/L $C_6H_{12}O_6$ (myo-inositol), 1 mg/L
467 $C_6H_5NO_2$ (nicotinic acid), 1 mg/L $C_8H_{11}NO_3$ (pyridoxine), 1 mg/L $C_{12}H_{18}Cl_2N_4OS$
468 (thiamin-HCl), 0.05 mg/L $C_{10}H_{16}N_2O_3S$ (biotin), 1 mg/L $C_9H_{17}NO_5$
469 (calcium-pantothenate), and 0.2 mg/L $C_7H_7NO_2$ (p-aminobenzoic acid).[10] The PPD
470 standard was purchased from PhytoLab. The DM standard was purchased from
471 AOBIOUS.

472 **Design of Experiment.** The 34 runs DoE was designed using Design Expert 11

473 (Stat-Ease, Inc.). Study type: Factorial; Subtype: Randomized; Design type: Minimum
474 Run Resolution V; 34 runs; Design Model: 2FI; Blocks: No Blocks; Center Points: 4.
475 The experiments were carried out in 24-well plates (polypropylene square deep well
476 MTP, re-usable, 24 wells of 11 ml volume, flattened, detoxified, Kuhner shaker,
477 Germany).

478 **Ordinary least squared (OLS) Estimation.** An OLS regression was used with two
479 sets of linear formulas: 1. containing the individual factor ($\sum c_iF_i$, with c_i as the
480 coefficient for each factor F_i=[A, B, C, D, E, F, G]) and 2. the individual factor with
481 first order factor interactions ($\sum c_iF_i + \sum c_{ij}F_iF_j$, with c_{ij} as the coefficient for each
482 two-factor interaction). A regression was performed with the seven factors A-G
483 towards either PPD or DM, resulting in four regressions. All data was centered to
484 have a mean value of 0 and a standard deviation of 1. The evaluation of the OLS
485 models is based on the F-statistics, which reports whether a combination of
486 independent variables (the factors in the OLS model) can explain the variance of the
487 dependent variable (PPD or DM) if the p-value is below 0.05. The factors are selected
488 by their individual p-values of their contribution to the OLS model. The impact of
489 each factor is then determined by the respective coefficient. The calculations are
490 performed with the *ols* package in the *statsmodel* (v0.13.2) library in *Python*, and the
491 code is provided as Jupyter Notebook in the Supplementaries.

492 **Cultivation of Engineered Yeasts in Shake Flasks.** Pre-cultures in the 500 mL shake
493 flasks with an aluminum lid containing 50 mL WM8+ medium and inoculate each
494 pre-culture with 500 µL of a thawed cryogenic stock of the engineered yeast (30 °C,

495 200 rpm, and 50 mm shaking diameter). The pre-culture medium with an OD_{600} of
496 6-10 was used as the inoculum of the main culture. The start OD_{600} of the main
497 culture was set to 0.4, measured with a single-use cuvette (polystyrene, semi-micro,
498 1.6 mL, Carl Roth, Germany) in a photometer (Biochrom Ltd., United Kingdom). All
499 experiments were carried out in the 500 mL shake flasks with aluminum or membrane
500 lids. The filling volume differed between 25 and 50 mL (see text for details). The
501 cultivation temperature differed between 28 °C and 30 °C (200 rpm and 50 mm
502 shaking diameter). At least duplicates were performed.

503 **Determination of PPD, DM, and Biomass concentrations.** 800 µL of cell broth
504 were sampled, transferred into 2 mL reaction tubes (Eppendorf, Germany), and frozen
505 at -20 °C until further use. For cell disruption, 250 µL of glass beads (0.5 mm
506 diameter, Carl Roth, Germany) were added, followed by 800 µL chloroform:
507 methanol (80:20 ratio) and 80 µL HCl (1 M). The samples were then placed into the
508 MinibeadBeater (Biospec Products, USA) for 2 minutes twice. For phase separation,
509 samples were centrifuged for 10 minutes at 4 °C, 13,000 rpm (Eppendorf Centrifuge
510 5418 R, Eppendorf, Germany). 500 µL of the lower organic phase containing the PPD
511 and DM were filtered (4 mm syringe filters RC membrane, pore size 0.2 µm,
512 Phenomenex, Germany) and transferred into HPLC vials (Flat N9 vials, screw neck,
513 1.5 mL, clear, Macherey-Nagel, Germany) and stored at 4 °C until HPLC analysis.
514 PPD and DM concentration were determined with an HPLC system (UltiMate 3000
515 HPLC system, Thermo Fisher Scientific, USA) coupled to a Dionex Corona Veo
516 Charged Aerosol Detector. The column was an EC150/4.6 NUCLEODUR C18

517 gravity column (Macherey-Nagel GmbH and Co. KG, Germany), the mobile phase
518 was Acetonitrile:0.2 % (v/v) formic acid, the column temperature was set to 40 °C, the
519 flow rate was programmed to 1.2 mL/min, the injection volume was set to 5 μL, the
520 runtime was 23 min. To measure the CDW, 2 mL culture was harvested by
521 centrifugation for 5 minutes at 4 °C, 13,000 rpm (Eppendorf Centrifuge 5418 R,
522 Eppendorf, Germany). The collected yeast cells were washed with deionized water
523 and oven-dried to a constant weight. An OD_{600} to CDW correlation of CDW (g/L)=0.2
524 $\times OD_{600}$+1.3 was estimated. All values are reported as the average of three replicates.
525 **Water evaporation.** Water evaporation was monitored using 500 mL shake flasks
526 with either the aluminum or membrane lid. Different water volumes (25 mL and 50
527 mL) were incubated in a shaker for 120 h (28 °C, 200 rpm, and 50 mm shaking
528 diameter).

529 **Author Contributions**

530 Shangkun Qiu completed all the experiments, including the DoE design, OLS
531 estimation analysis, triterpenoids measurement, and data analysis. Shangkun Qiu also
532 wrote the first draft of the manuscript. Mariam Dianat Sabet Gilani helped with the
533 yeast fermentation and triterpenoid measurements. Conrad Müller advised on the DoE
534 experiment design and DoE data analysis. Ulf Liebal advised the OLS estimation
535 analysis. Lars M. Blank supervised the study, discussed the results, and revised the
536 manuscript.

537 **Declaration of Competing Interest**

538 The authors declare that they have no known competing financial interests or personal

relationships that could have appeared to influence the work reported in this paper.

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