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.

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

Yeasts are simple eukaryotes that can be engineered for the synthesis of high-value metabolites. With their complete organelle structures, including mitochondria, peroxisomes, Golgi apparatus, endoplasmic reticulum (ER), and vacuoles, which allow post-translational modifications of proteins and operation of compartmented biochemical pathways, they are geared for enzymes from plants.[5,11,14,15] Yeasts also possess redox systems that provide similar biological and physical environments

88 encountered by cytochrome P450s and uridine diphosphate glycosyltransferase (UGTs)
89 in plants and mammals.[5,14] Due to these reasons, yeasts are considered excellent cell
90 factories for producing natural plant products, such as ginsenosides, betulinic acid,
91 farnesene, amorpha-4,11-diene, and others.[10,11,17,18,13] For example, the production of
92 α-farnesene was improved by engineered *Yarrowia lipolytica* during fed-batch
93 fermentation via the expression of the acetyl-CoA acetyltransferase (Erg10p) from
94 *Escherichia coli* and HMG-CoA reductase (Hmgp) from *Bordetella petrii*, the final
95 α-farnesene production reached 26 g/L.[11,19] Also, a highly engineered *Saccharomyces*
96 *cerevisiae* expressing the amorpha-4,11-diene synthase (ADS) from *Artemisia annua*
97 and using an unrestricted ethanol feed allowed the final production of 40 g/L
98 amorpha-4,11-diene.[13]

99 Triterpenoids are either synthesized from squalene or 2,3-oxidosqualene, which are
100 both intermediates of sterol synthesis in yeast.[5,16] The synthesis of ginsenosides by
101 engineered yeasts from renewable resources is a promising strategy to address the
102 increasing demand for this interesting class of molecules.[5] The microbial biosynthesis
103 of ginsenosides has several advantages, including rapid growth, controllable culture
104 conditions, space efficiency, sustainable production, environmentally friendly, and
105 well-developed genetic manipulation technology.[5,11,20] At present, only a fraction of
106 ginsenosides are synthesized by engineered yeast, such as dammarenediol-II (DM),
107 PPD, PPT, Compound K (CK), ginsenoside Rh1, ginsenoside Rh2, ginsenoside F1,
108 ginsenoside Rg3, 20S-O-Glc-DM, 3β-O-Glc-DM, ginsenoside Rg1, ginsenoside
109 NgR1, ginsenoside NgR2, ginsenoside Rf, ginsenoside Glc-Rf, and ginsenoside

110 Ro.[5,21] Furthermore, there have been some prominent achievements in synthesizing
111 ginsenosides by yeasts. For example, according to a recent study, researchers
112 constructed a PPD-producing *S. cerevisiae* cell factory via overexpression of
113 mevalonate and PPD synthetic pathways and optimization of P450 expression levels;
114 the final PPD production reached 11 g/L in 10 L fed-batch fermentation.[18] Researchers
115 also introduced the ginsenoside CK synthesis pathway into this strain while
116 decreasing UDP-glucose consumption and improving UDP-glucose biosynthesis by
117 engineering strategies; the final CK yield reached almost 6 g/L in fed-batch
118 fermentations.[12]

119 Research showed that bioprocess optimization, including medium and cultivation
120 conditions, can vastly improve the production of metabolites by cell factories.[22,23] For
121 example, carbon sources (e.g., glucose, glycerol, and glutamate), nitrogen sources,
122 temperature, pH, oxygen, inoculum size, and salts significantly affect the cells' growth
123 and production.[22,24,25] As the parameter space in medium and fermentation parameter
124 optimization is huge, researchers aim for Design of Experiments (DoE) approaches.
125 For example, researchers optimized the concentration of glucose, yeast extract, and
126 peptone via the Box-Behnken design; this optimization increased the geranylgeraniol
127 production by 1.75-fold compared to the traditional YPD medium.[26] Cultured
128 α-farnesene-producing *Y. lipolytica* in a medium optimized by a uniform design (UD)
129 technique reached a final α-farnesene titer of 210 mg/L in a shake flask.[24] In another
130 study, adding citrate (0-10 mM) or acetate (0-10 mM) into the medium improved
131 squalene production. The inhibition of the squalene monooxygenase Erg1p by

132 terbinafine allowed 730 mg/L of squalene titers.[27] Therefore, improving the
133 cultivation conditions of engineered yeast is a promising strategy.

134 This study aims to provide a simple shake flask protocol to evaluate all the different
135 mutants one might have to generate for further improving triterpenoid synthesis in
136 yeast. Here, we use PPD synthesis as the main target, as the synthesis requires the
137 activity of a triterpenoid cyclase and P450 monooxygenase. PPD is one of the
138 30-carbon dammarane II-type tetracyclic triterpenoids found in ginseng and is the
139 precursor of high-value ginsenosides.[10] PPD exhibits valuable biological properties,
140 such as antineoplastic, antioxidant, antidepressant, antitumor, and
141 anti-inflammation.[28,29] For improved PPD production by yeast in shake flasks, the
142 following parameters were considered: temperature, glucose and glutamate
143 concentrations, salts solution, trace element solution, vitamin solution, and oxygen
144 availability. PPD production reached 1.2 g/L using the best combination of shake
145 flask set-up and optimized medium, the highest PPD production in shake flasks to
146 date. This simple set-up facilitates the evaluation of engineered triterpenoid
147 synthesizing yeast, and the amounts of triterpenoid produced enable advanced
148 analytics and even first ideas for purification and use of this intriguing class of natural
149 products.

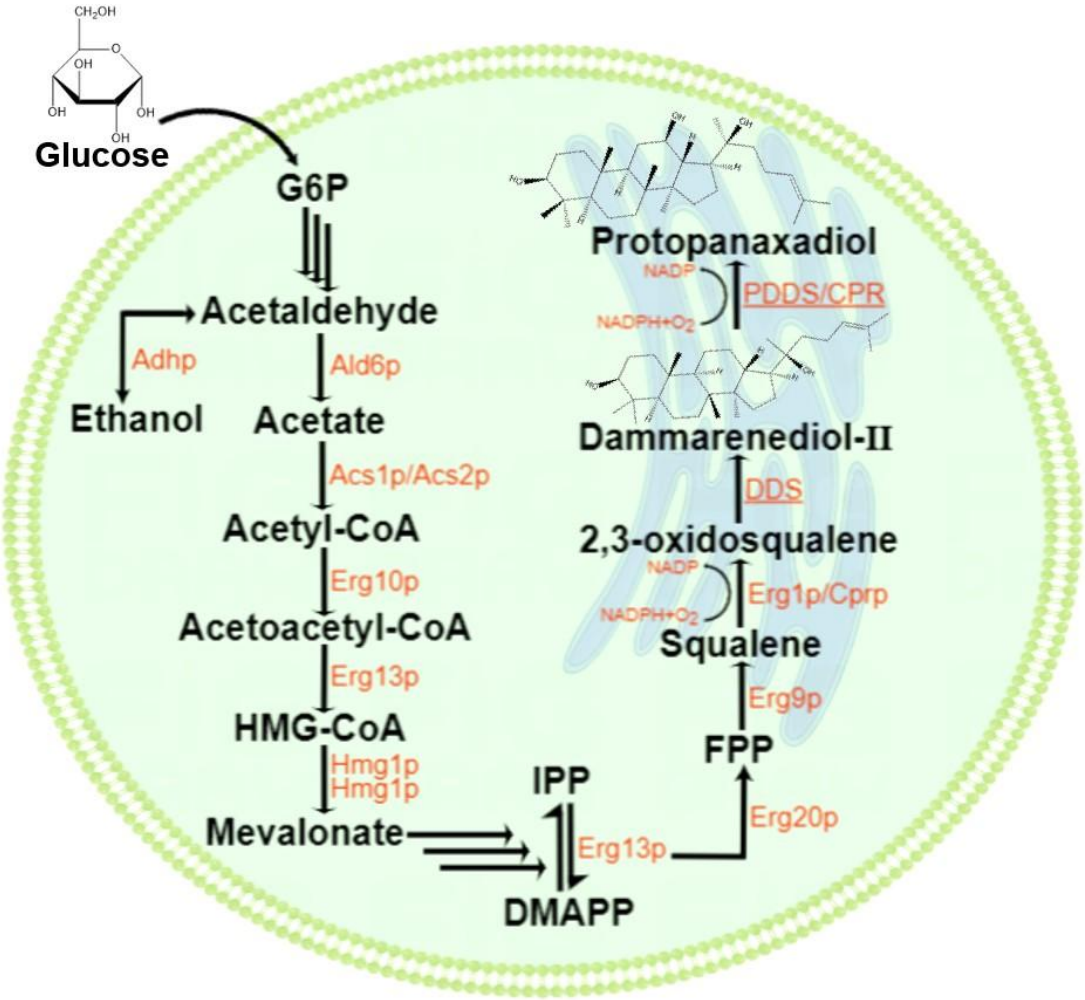


Figure 1. The PPD synthesis pathway in engineered *S. cerevisiae*. The recombinant enzymes are underlined. G6P, glucose 6-phosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; Erg10p, acetyl-CoA acetyltransferase; Erg13p, HMG-CoA synthase; Hmg1p and Hmg2p, HMG-CoA reductase 1 and 2; Idi1p, isopentenyl-diphosphate delta-isomerase; Erg20p, farnesyl diphosphate synthase; Erg9p, squalene synthase; Erg1p, squalene monooxygenase; DDS, dammarenediol-II synthase; PPDS, protopanaxadiol synthase; ER, endoplasmic reticulum. This image was created by Figdraw.

RESULTS AND DISCUSSION

Effect of medium composition and Oxygen availability on DM and PPD Production. The DoE experiment included 34 runs designed using Design Expert 11

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

175 DM production suggests triterpenoid synthesis. However, it is also a potential
176 limitation in the subsequent P450-catalyzed reaction to PPD. Only seven experiments
177 showed DM production (Figure 2B). Among the seven experiments, only runs 21 and
178 27 had DM concentrations above 0.3 g/L. These two runs' glucose, glutamate, and salt
179 concentrations were three-fold that of standard WM8+ medium. The results suggest
180 high glucose and glutamate concentrations support high DM and PPD production. At
181 the same time, low salt concentration, low temperature, and high oxygen
182 concentration might benefit PPD production, indicating that the P450-catalyzed

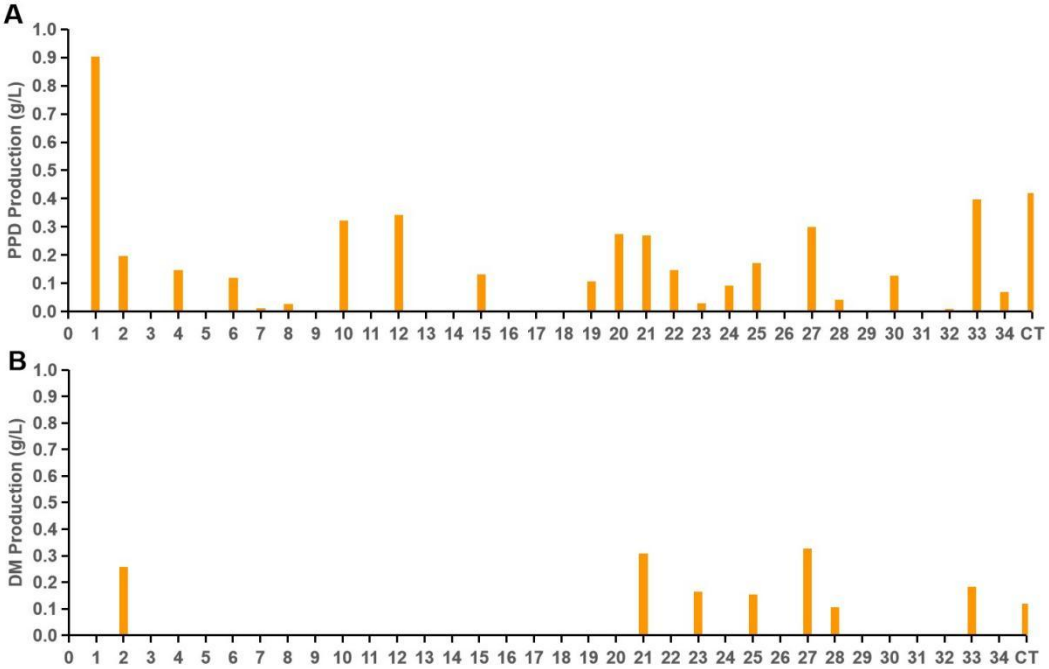
183 hydroxylation is somewhat challenging, with substrate limitation and a slow rate of

184 catalysis.

185 **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

186 Every factor has a low (red), middle (yellow), and high (green) value. For factors A-E, the figures in the table
187 represent fold changes in the actual concentration compared to the standard WM8+ medium. For factor F, the
188 figures in the table represent the actual temperature. For factor G, figures in the table represent the volume of the
189 medium in 24-well plates. CT: Control experiment, standard WM8+ medium.



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

194 **The ordinary least squares (OLS) Estimation of the DoE Data.** DoE data was
195 analyzed with OLS regression to PPD and DM to identify the predictive factors. We
196 tested models containing only the individual factor effects (Ind-OLS) as well as
197 individual factors combined with two-factor interactions (Int-OLS) (Table 2). The
198 p-value of the F-Statistics identified models with sufficient power to explain the
199 variance of the observations (PPD) if the p-value is below 0.05. The Ind-OLS and the
200 more complex Int-OLS explain 41% and 83% of the variance in PPD, respectively

201 (adj. R^2(Ind-OLS)=0.41, adj. R^2(Int-OLS)=0.83). The p-values of the individual
202 factors in each regression model determine whether the factor significantly
203 contributes to the prediction. There are two significant factors for Ind-OLS (A:
204 glucose, C: glutamate) and seven for Int-OLS (B: salt, C: glutamate, AxE (vitamins),
205 AxC, B (salt)xF (temperature), CxD (trace element), BxG (oxygen)) (Figure 3). In the
206 simple Ind-OLS model with individual factors, only glucose and glutamate are the
207 most influential predictors that positively contribute to PPD production with
208 correlation values of 0.4 (Figure 3A). Conversely, salt concentration (B), temperature
209 (F), and medium volume (G) correlate negatively to the final PPD production, albeit
210 with low correlation values at -0.2 (Figure 3A). In the more complex Int-OLS model,
211 glucose is only significant via the two-factor interactions with glutamate (A:C) and
212 vitamin (A:E) but no longer as an individual factor. The most significant single factors
213 in the complex Int-OLS model are the positive correlation with glutamate (C) and the
214 negative correlation with salts (B). While the volume level (G, oxygen) individually is
215 just above the significance value (p=0.05), it negatively correlates with PPD and is
216 also negative with the significant interaction with salts (BxG). Thus, less oxygen via
217 higher volumes decreases PPD (Figure 3A). The yeast increases biomass quickly,
218 while the oxygen concentration decreases fast in the exponential phase.[25] Furthermore,
219 NADPH is oxidized to NADP by a P450 reductase in the presence of oxygen, then
220 transfers electrons to the monooxygenases, such as PPDS.[7] Therefore, controlling the
221 oxygen concentration will also play a critical role in synthesizing the final product.
222 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.

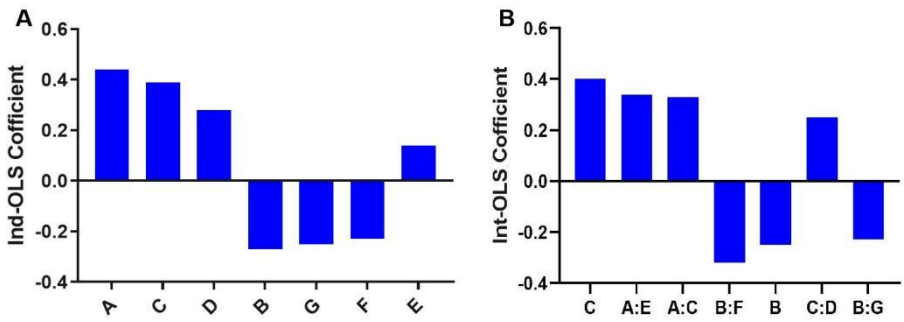


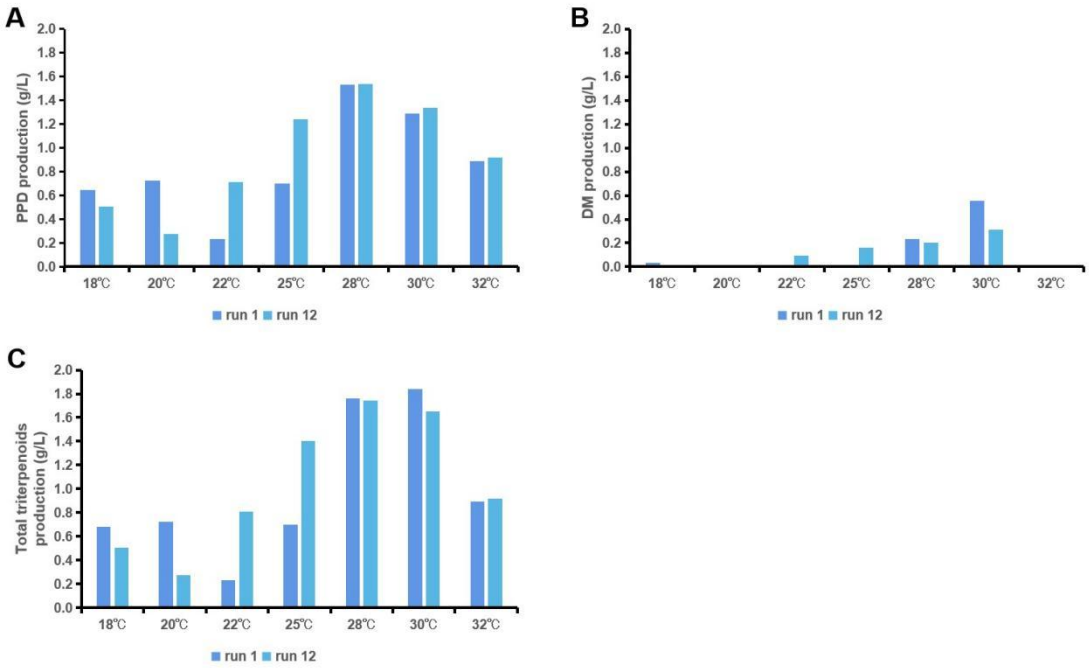
Figure 3. The OLS regression result of the DoE data with prediction of PPD production. (A) The OLS regression result of the Ind-OLS. (B) The OLS regression result of the Int-OLS. The coefficients with decreasing absolute values are shown for factors (Ind-OLS) and factor interactions (Int-OLS), significantly explaining the variance (p-values below 0.05). The larger the coefficient, 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.

Effect of Temperature on DM and PPD Production. Sesquiterpenoid production

238 by engineered *S. cerevisiae* was reported to be better at 25 °C than at 30 °C.[32] We also
239 investigated temperature as an optimization parameter for PPD production. Here, the
240 highest PPD production was observed at 20 ℃ in the 34 runs DoE experiment (Figure
241 2A). Temperatures above 30 °C were reported to cause stress to baker´s yeast and
242 decrease cell viability.[25] Therefore, the 15 runs of the DoE experiment cultured at
243 20 ℃ were selected to investigate the effect of different temperatures on DM and PPD
244 production in 24-well plates. To explore the optimum temperature, 18 ℃, 20 ℃,
245 22 ℃, 25 ℃, 28 ℃, 30 ℃, and 32 ℃ were selected. The other fermentation and
246 medium conditions remained constant. In general, the production of PPD increased
247 with increasing temperature and reached the highest value at 28°C, then decreased
248 with increasing temperature (Figure 4A, Figure S1). Especially the PPD productions
249 of runs 1 and 12 are the highest among the 15 runs, reached 1.5 g/L at 28 ℃ (Figure
250 4A). But for run 13, run 14, run 18, run 20, run 24, run 30, and run 32, the PPD
251 production reached the highest value at 32 ℃. However, the PPD production for these
252 seven runs is lower than runs 1 and 12 (Figure S1). So, we can speculate that the
253 optimum temperature for PPD production is 28 ℃.

254 The DM production of run 1, run 12, and run 24 reached the highest when cultured
255 at 30 ℃. However, the optimum temperature of the run 6 and run 32 is 25 ℃, run 10,
256 run 20, run 22, and run 28 is 28 ℃, and run 30 is 32℃ (Figure 4B, Figure S1). The
257 first reason why the optimum temperatures of PPD and DM synthesis in all the runs
258 are different may be that the different concentrations of compounds in the medium
259 can affect the DM conversion to PPD at different temperatures. Another reason may

260 be that the different medium compositions affect the enzyme activities involved in
261 PPD and DM synthesis at different temperatures. The DM production of run 28 is the
262 highest when cultured at 28 °C, which reached 1.1 g/L (Figure S1). The DM
263 production of run 22, run 28, and run 30 are all over 0.8 g/L. The total triterpenoid
264 production of run 1 is the highest among all the runs, cultured at 30 °C and reached
265 2.3 g/L (Figure 4C). The total triterpenoid production of run 1, run 12, and run 30 is
266 over 2 g/L. However, the total triterpenoid production of run 1 and run 12 reached 2
267 g/L when cultured at 28 °C or 30 °C, while run 30 reached 2 g/L at 32 °C (Figure 4C,
268 Figure S1). Hence, optimum temperature for DM and total triterpenoid production is
269 in the range of 28-32 °C.



270

271 **Figure 4.** The effect of different temperatures on DM and PPD production. (A) The comparison of PPD production
272 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
273 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
274 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

275 runs' data is shown in Figure S1.

276 **Effect of Glucose, Glutamate, and Salt Concentration on DM and PPD**
277 **Production.** According to the results above, runs 1 and 12 have the highest PPD and
278 total triterpenoid production. The conditions of runs 1 and 12 were chosen for further
279 investigation (Figure 4A). Glucose, glutamate, and salt were selected to be varied in
280 concentration, while all other parameters were kept constant. The optimum salt
281 concentration for PPD production seems to be 0.33, 0.44 or 2 times the WM8+
282 medium, with the highest PPD production is 2 g/L (Figure 5A). Here, it is indicated
283 that the optimum concentration of glucose and glutamate for PPD synthesis is two
284 times that of the standard WM8+ medium. Higher concentrations might cause osmotic
285 stress and deflect resources from PPD production (Figure 5A).

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

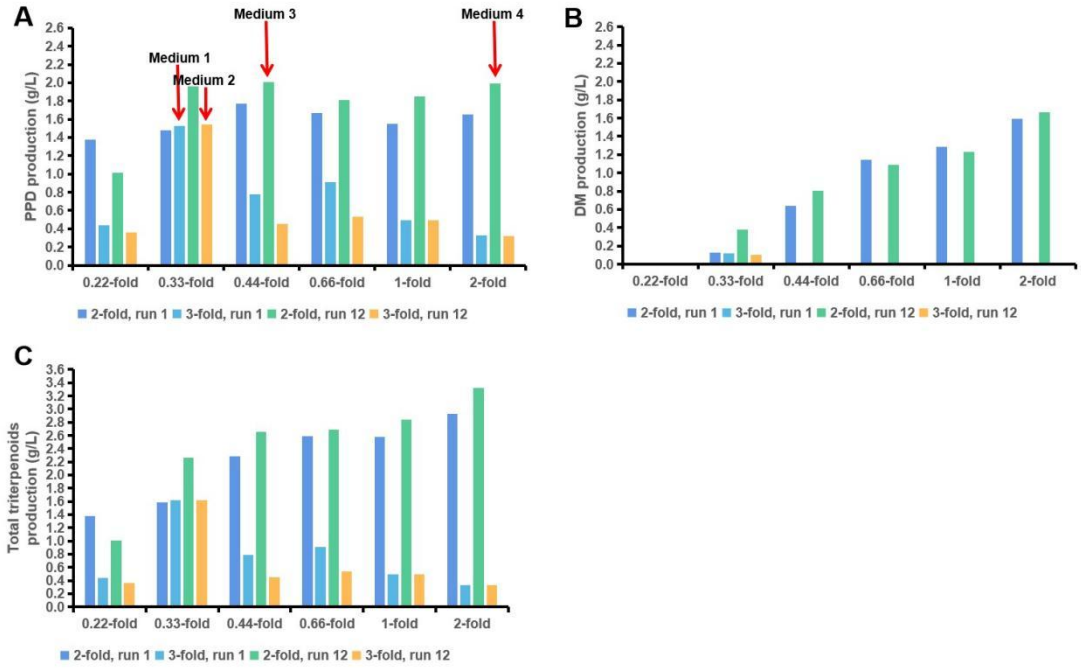
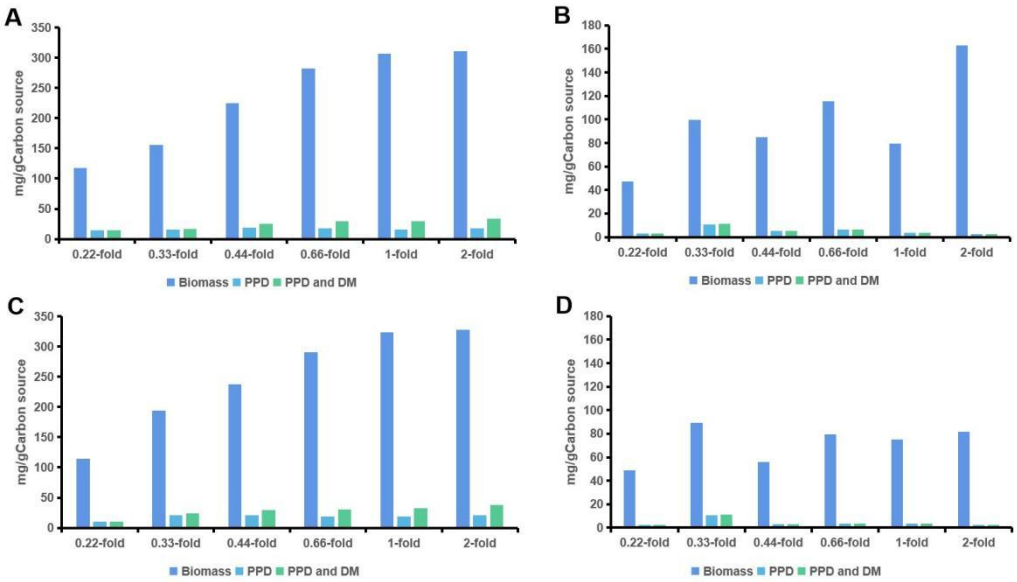


Figure 5. The effect of glucose, glutamate, and salt concentration on PPD and DM production (A) PPD production depends on glucose, glutamate, and salts. (B) DM production is dependent on glucose, glutamate, and salts. (C) The total triterpenoid production. The glucose and glutamate concentration is two 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 6). The highest biomass yield per gram carbon source is 328 $mg_{CDW}/g_{glucose\ and\ glutamate}$ in two-fold salt concentration (Figure 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 6A and C). The highest total triterpenoid yield is 38 $mg/g_{glucose\ and\ glutamate}$ (Figure 6C). At the same time, the highest

307 PPD yield is 21 mg/$g_{glucose\ and\ glutamate}$ (Figure 6C).

308 The total triterpenoid content of ripe ginseng root is 0.1-3 %.[3] We here estimated
309 the CDW content of PPD, DM, and total triterpenoids. The CDW content of the total
310 triterpenoid of three runs is above 120 mg/g_{CDW} (Figure 7A and C). The carbon source
311 concentration of these three runs is two times that of the standard WM8+ medium.
312 The salt concentration is 0.22, 0.33, or 0.44 times the reference. All the data indicates
313 that low salt concentrations favor high CDW content of PPD. In contrast, high salt
314 concentrations can contribute to increased CDW content of DM (Figure 7A and C).
315 The optimum glucose and glutamate concentration for the CDW content of PPD and
316 total triterpenoids is two times of the standard WM8+ medium.

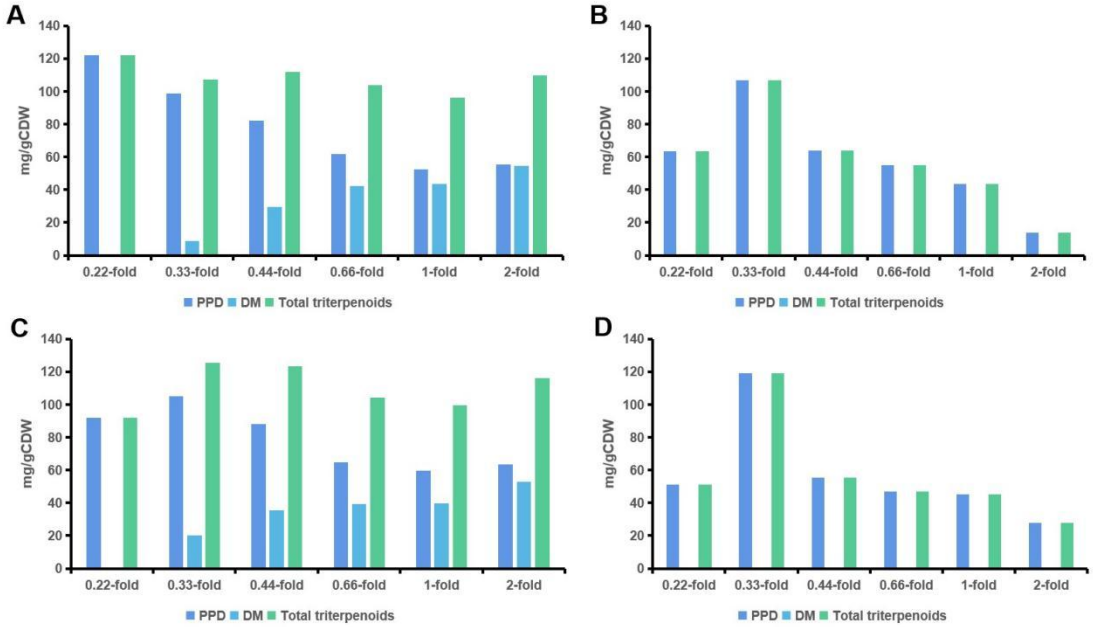


317

318 **Figure 6.** The effect of glucose, glutamate, and salt concentration on triterpenoid yield. (A) The glucose and
319 glutamate concentration is two times the standard WM8+ medium. This medium is modified from run 1. (B) The
320 glucose and glutamate concentration is three times the standard WM8+ medium. This medium is modified from
321 run 1. (C) The glucose and glutamate concentration is twice the standard WM8+ medium. This medium is
322 modified from run 12. (D) The glucose and glutamate concentration is three times the standard WM8+ medium.

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

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



325

326 **Figure 7.** The effect of glucose, glutamate, and salt concentration on CDW content of triterpenoids. (A) The

327 glucose and glutamate concentration is two times the standard WM8+ medium. This medium is modified from run

328 1. (B) The glucose and glutamate concentration is three times the standard WM8+ medium. This medium is

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

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

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

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

333 **Triterpenoid Production in 500 mL Shake Flasks.** While microtiter plate

334 cultivation allows strain comparison, shake flask cultivations can be designed to test

335 product purification ideas and synthesize the first products for use. For this scale increase, we

336 selected four media from the above experiments, which are medium 1 (3-fold, run 1),

337 medium 2 (3-fold, run 12), medium 3 (2-fold, run 12), and medium 4 (2-fold, run 12)

338 (Figure 5A). Yeasts were cultivated in two types of 500 mL shake flasks. The shake
339 flask was closed by either an aluminum or a membrane lid (Figure 8A). The
340 membrane lid facilitates better aeration when compared to the aluminum lid. Two
341 enzymes in PPD synthesis require oxygen: the conversion of squalene to
342 2,3-oxidosqualene, catalyzed by Erg1p, and the conversion of DM to PPD, catalyzed
343 by PPDS (Figure 1).[33,34] First, yeasts were cultivated in the shake flask with an
344 aluminum lid. The smaller the filling volume of the shake flask, the better the aeration.
345 Two different filling volumes were used, i.e., 25 mL and 50 mL. The yeast was
346 cultured in the four improved media. The results showed the highest PPD production
347 of 0.8 g/L occurred in medium 3 with 25 mL filling volume. The PPD production
348 decreased with the increase in the medium. The PPD production in all four media
349 tested is higher than that in WM8+ medium, which is 0.16 g/L. It can be speculated
350 that the higher oxygen concentration benefits the synthesis of PPD, which can
351 increase the final PPD production in the shake flask (Figure 8B).

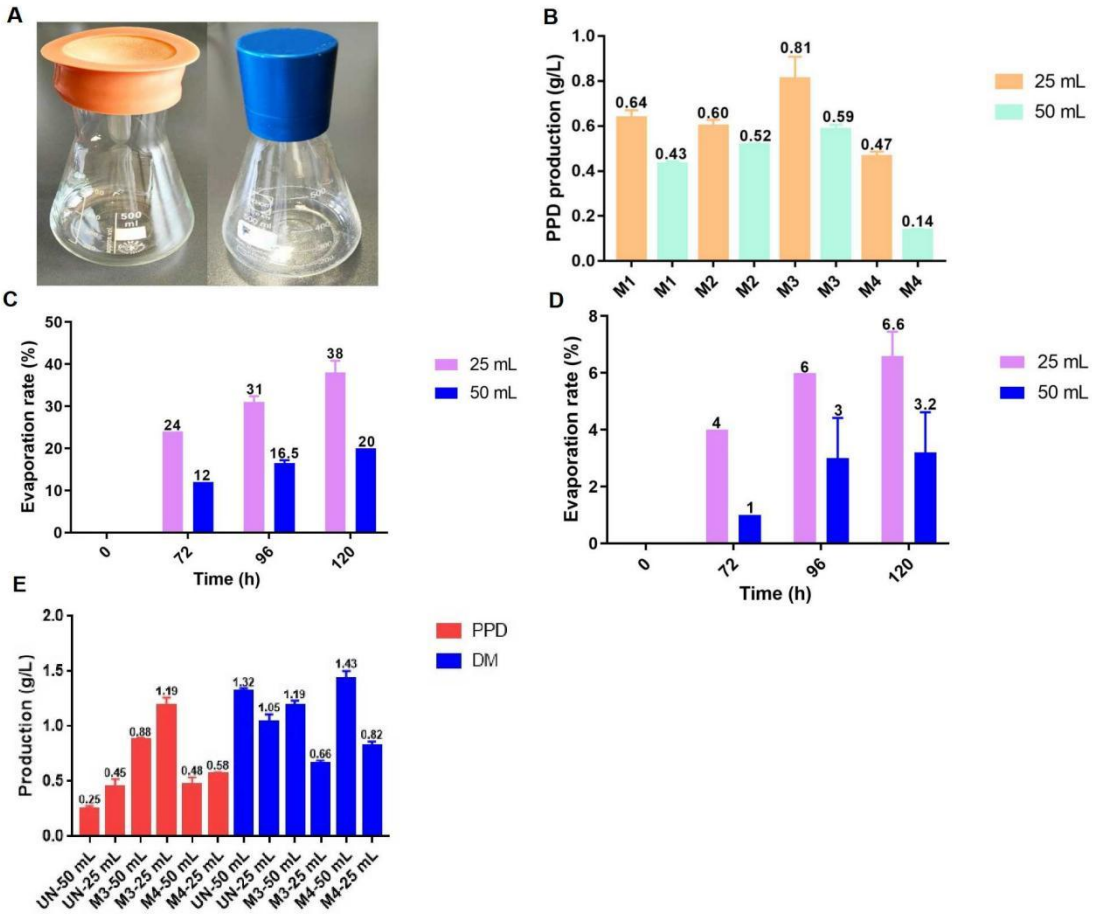


Figure 8. Shake flask configuration influences PPD production. (A) Shake flasks with membrane and aluminum lid. (B) PPD production depends on the filling volume using a shake flask with an aluminum lid. (C) Water evaporation rate in the 500 mL shake flask with a membrane lid. (D) Water evaporation rate 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

364 8C and D). We measured the volume after 120 h and calculated the product
365 concentration without evaporation. The corrections were as follows:

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

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

369 The results indicated that the PPD production also decreased with the increase of
370 medium volume. The PPD productions of optimum media 3 and 4 in this membrane
371 lid shake flask are higher than the PPD production of the same media with an
372 aluminum lid, reaching 1.2 g/L and 0.6 g/L when the volume is 25 mL. At the same
373 time, the DM production increased with medium volume (Figure 8E). This indicated
374 that the membrane lid and smaller volume allow more oxygen to enter the shake flask,
375 which supplies more oxygen for improving the conversion of squalene to
376 2,3-oxidosqualene and the conversion of DM to PPD (Figure 1). The highest PPD
377 productions of unoptimized standard WM8+ medium in the shake flask with an
378 aluminum lid or membrane lid are 0.16 g/L or 0.45 g/L, respectively (Figure 8E). This
379 indicated that the highest PPD production of the optimum medium 3 is 2.6-fold
380 compared to the unoptimized standard WM8+ medium in the shake flask with a
381 membrane lid. However, the PPD productions in media 1 and 2 with this membrane
382 lid shake flask are much lower than the PPD production in medium 3 (data not
383 shown). This may be due to the higher glucose and glutamate concentration (three
384 times the standard WM8+ medium) in media 1 and 2, limiting the PPD and DM

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

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

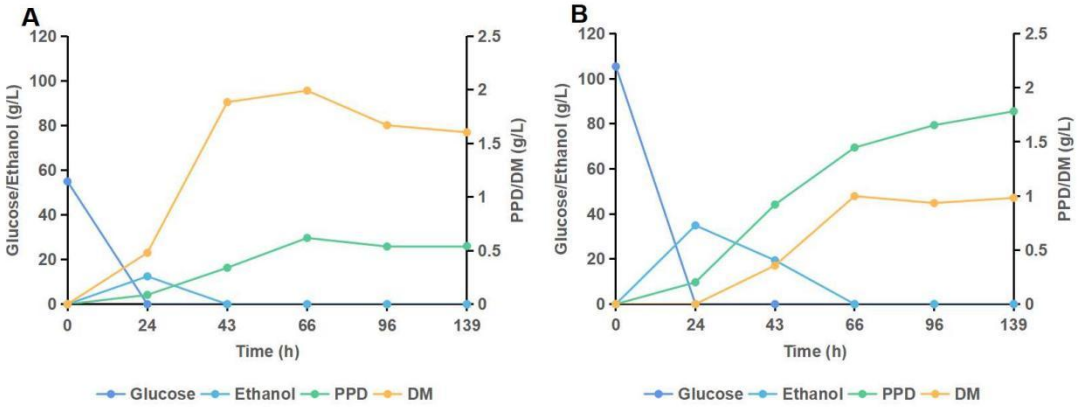
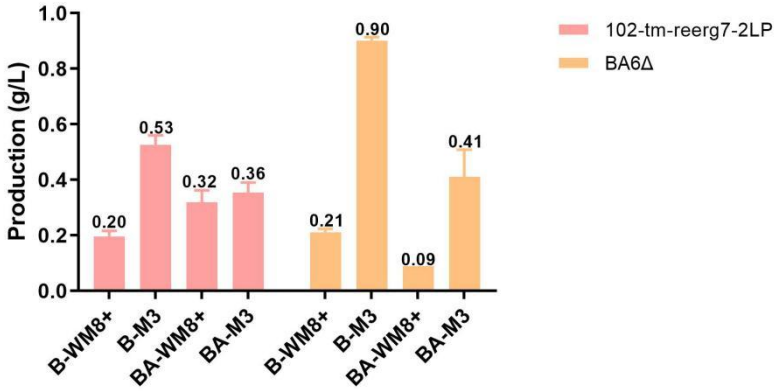


Figure 9. Fermentation kinetics of PPD and DM in engineered yeast. (A) The concentration of glucose, ethanol, PPD, and DM during the fermentation in engineered yeast cultured in the standard WM8+ medium. (B) The concentration of glucose, ethanol, PPD, and DM during the fermentation in engineered yeast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

424 growth conditions.



425

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

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

428 In conclusion, we identified the optimum temperature for PPD production at 28 °C.
429 The M3 medium presented here for producing PPD by yeast was built by stepwise
430 evaluation and optimization of the WM8+ medium. Furthermore, we used a
431 membrane lid to improve the final PPD production, which facilitated increased
432 aeration. Eventually, a remarkable titer of 1.2 g/L PPD was achieved in the 500 mL
433 shake flask, a 7.5-fold improvement compared to the unoptimized standard WM8+
434 medium with original fermentation conditions (0.16 g/L). As the medium and growth
435 conditions are easily established and transferable to other triterpenoid-producing
436 baker´s yeast, the study might facilitate the simple evaluation of engineered strains in
437 microtiter plates and shake flask cultivations.

438 **MATERIALS AND METHODS**

439 **Strains, Medium, and Reagents.** The engineered yeast 102-tm-reerg7-2PP for PPD
440 synthesis was modified from *S. cerevisiae* CEN.PK 102-5B and originated from a
441 previous study.[16,36] *S. cerevisiae* BA6Δ (unpublished) is a pah1 deletion mutant of

442 BA6, generated through replacement of PAH1 with a hygromycin resistance cassette
443 (YMR165CΔ:hphMX).[37,38] The engineered yeast BA6Δ for betulin synthesis was
444 modified from *S. cerevisiae* Simo1575.[16] Standard WM8+ medium included 50 g/L
445 glucose, salt solution, 10 g/L glutamate, trace elements solution, vitamins solution,
446 verduyn-trace elements solution, and verduyun-vitamins solution. In addition, the salt
447 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
448 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
449 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
450 $CuSO_4 \cdot 5H_2O$, 0.1 mg/L $MnCl_2 \cdot 4H_2O$, 0.1 mg/L $Na_2MoO_4 \cdot 2H_2O$, and EDTA (0.04
451 mM). The vitamins solution includes 10 mg/L $C_6H_5NO_2$ (nicotinic acid), 25 mg/L
452 $C_8H_{11}NO_3$ (pyridoxine), 10 mg/L $C_{12}H_{17}N_4OS$ (thiamine), 2.5 mg/L $C_{10}H_{16}N_2O_3S$
453 (biotin), and 50 mg/L $C_9H_{17}NO_5$ (calcium-d-pantothenate). Verduyn-Trace elements
454 solution includes 15 mg/L Na_2EDTA, 4.5 mg/L $CaCl_2 \cdot 2H_2O$, 4.5 mg/L $ZnSO_4 \cdot 7H_2O$,
455 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
456 $Na_2MoO_4 \cdot 2H_2O$, 0.3 mg/L $CoCl_2 \cdot 6H_2O$, 1 mg/L H_3BO_3, and 0.1 mg/L KI.
457 Verduyn-vitamins solution includes 25 mg/L $C_6H_{12}O_6$ (myo-inositol), 1 mg/L
458 $C_6H_5NO_2$ (nicotinic acid), 1 mg/L $C_8H_{11}NO_3$ (pyridoxine), 1 mg/L $C_{12}H_{18}Cl_2N_4OS$
459 (thiamin-HCl), 0.05 mg/L $C_{10}H_{16}N_2O_3S$ (biotin), 1 mg/L $C_9H_{17}NO_5$
460 (calcium-pantothenate), and 0.2 mg/L $C_7H_7NO_2$ (p-aminobenzoic acid).[10] The PPD
461 standard was purchased from PhytoLab. The DM standard was purchased from
462 AOBIOUS.

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

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

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

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

486 200 rpm, and 50 mm shaking diameter). The pre-culture medium with an OD_{600} of
487 6-10 was used as the inoculum of the main culture. The start OD_{600} of the main
488 culture was set to 0.4, measured with a single-use cuvette (polystyrene, semi-micro,
489 1.6 mL, Carl Roth, Germany) in a photometer (Biochrom Ltd., United Kingdom). All
490 experiments were carried out in the 500 mL shake flasks with aluminum or membrane
491 lids. The filling volume differed between 25 and 50 mL (see text for details). The
492 cultivation temperature differed between 28 °C and 30 °C (200 rpm and 50 mm
493 shaking diameter). At least duplicates were performed.

494 **Determination of PPD, DM, and Biomass concentrations.** 800 µL of cell broth
495 were sampled, transferred into 2 mL reaction tubes (Eppendorf, Germany), and frozen
496 at -20 °C until further use. For cell disruption, 250 µL of glass beads (0.5 mm
497 diameter, Carl Roth, Germany) were added, followed by 800 µL chloroform:
498 methanol (80:20 ratio) and 80 µL HCl (1 M). The samples were then placed into the
499 MinibeadBeater (Biospec Products, USA) for 2 minutes twice. For phase separation,
500 samples were centrifuged for 10 minutes at 4 °C, 13,000 rpm (Eppendorf Centrifuge
501 5418 R, Eppendorf, Germany). 500 µL of the lower organic phase containing the PPD
502 and DM were filtered (4 mm syringe filters RC membrane, pore size 0.2 µm,
503 Phenomenex, Germany) and transferred into HPLC vials (Flat N9 vials, screw neck,
504 1.5 mL, clear, Macherey-Nagel, Germany) and stored at 4 °C until HPLC analysis.
505 PPD and DM concentration were determined with an HPLC system (UltiMate 3000
506 HPLC system, Thermo Fisher Scientific, USA) coupled to a Dionex Corona Veo
507 Charged Aerosol Detector. The column was an EC150/4.6 NUCLEODUR C18

508 gravity column (Macherey-Nagel GmbH and Co. KG, Germany), the mobile phase
509 was Acetonitrile:0.2 % (v/v) formic acid, the column temperature was set to 40 °C, the
510 flow rate was programmed to 1.2 mL/min, the injection volume was set to 5 μL, the
511 runtime was 23 min. To measure the CDW, 2 mL culture was harvested by
512 centrifugation for 5 minutes at 4 °C, 13,000 rpm (Eppendorf Centrifuge 5418 R,
513 Eppendorf, Germany). The collected yeast cells were washed with deionized water
514 and oven-dried to a constant weight. An OD_{600} to CDW correlation of CDW (g/L)=0.2
515 $\times OD_{600}$+1.3 was estimated. All values are reported as the average of three replicates.
516 **Water evaporation.** Water evaporation was monitored using 500 mL shake flasks
517 with either the aluminum or membrane lid. Different water volumes (25 mL and 50
518 mL) were incubated in a shaker for 120 h (28 °C, 200 rpm, and 50 mm shaking
519 diameter).

520 **Author Contributions**

521 Shangkun Qiu completed all the experiments, including the DoE design, OLS
522 estimation analysis, triterpenoids measurement, and data analysis. Shangkun Qiu also
523 wrote the first draft of the manuscript. Mariam Dianat Sabet Gilani helped with the
524 yeast fermentation and triterpenoid measurements. Conrad Müller advised on the DoE
525 experiment design and DoE data analysis. Ulf Liebal advised the OLS estimation
526 analysis. Lars M. Blank supervised the study, discussed the results, and revised the
527 manuscript.

528 **Declaration of Competing Interest**

529 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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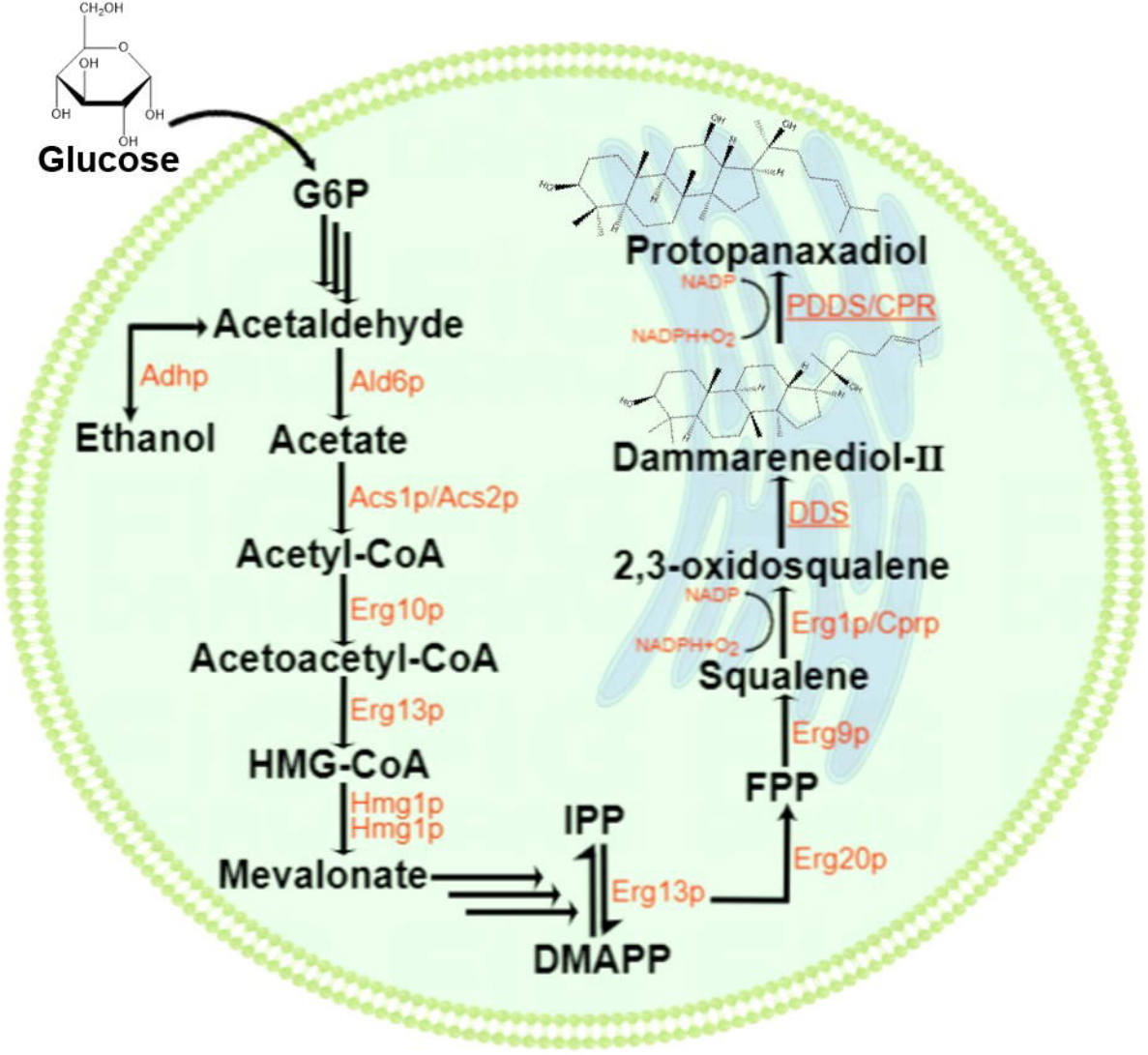
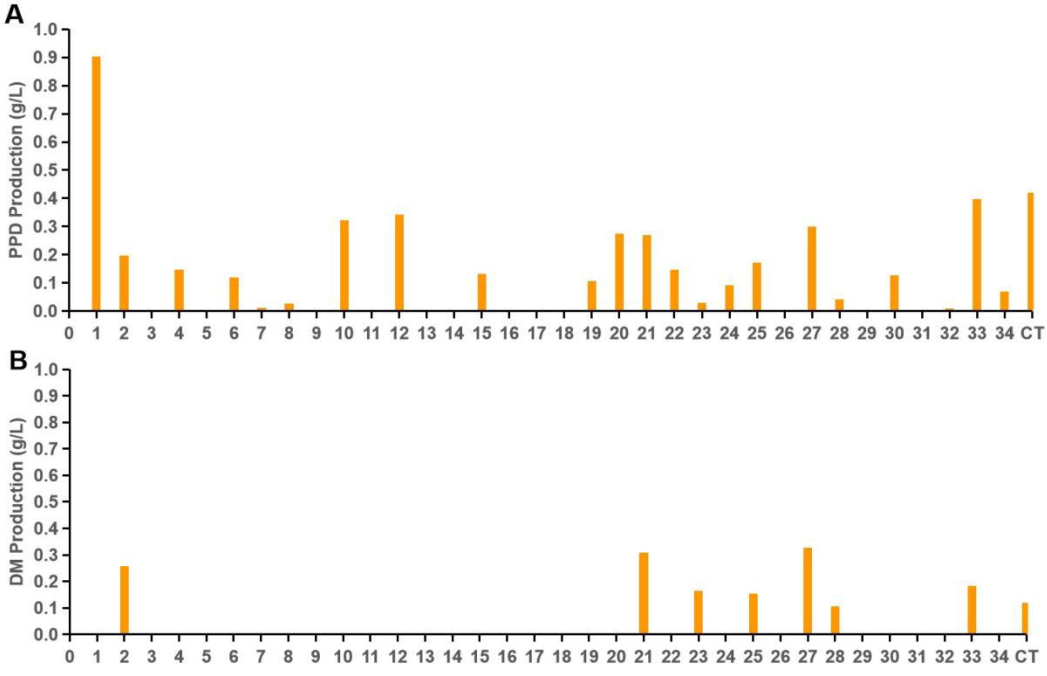
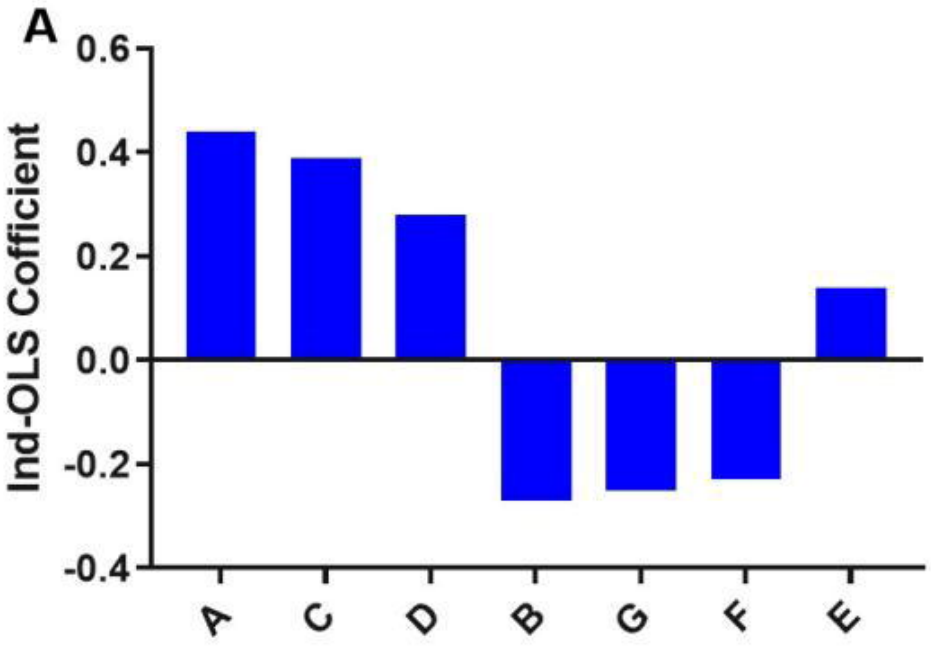
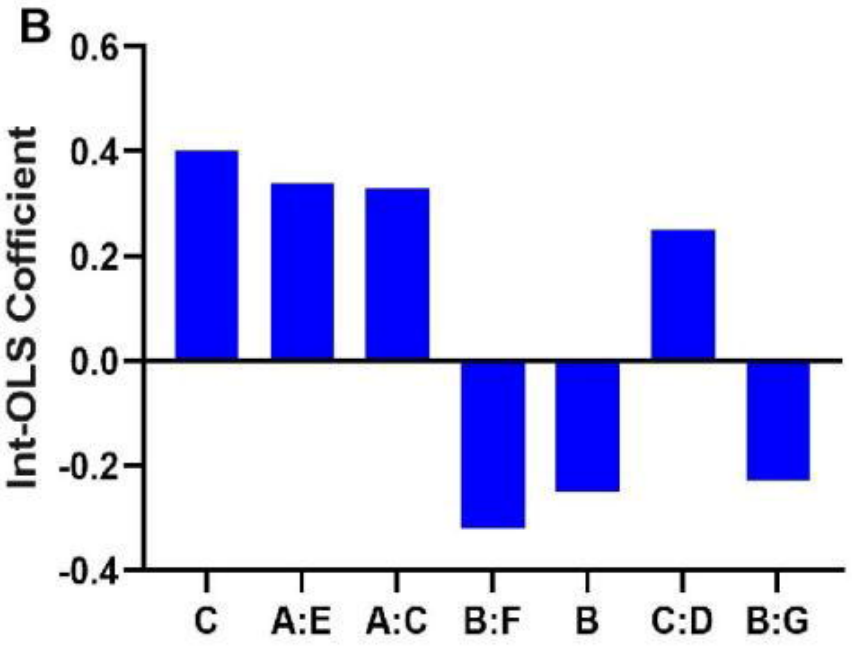
631 microbial triterpenoid production using nonlinear microscopy techniques. *Front Bioeng Biotechnol.*
632 **2023**, *11*, 1106566.
633 (38) Kerstin, W. *Compartment engineering to increase metabolic flux through the mevalonate pathway*
634 *in Saccharomyces cerevisiae*; RWTH Aachen University, 2018.

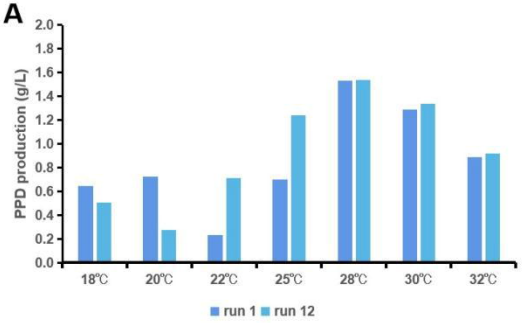
635

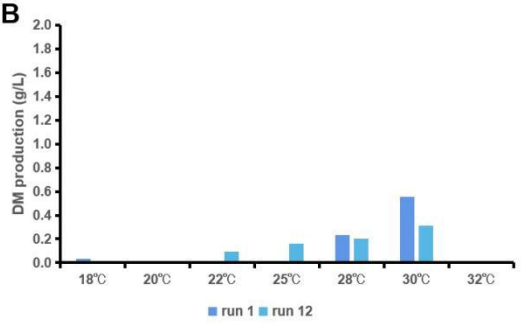

Glucose
G6P
Acetaldehyde
Adhp
Ethanol
Ald6p
Acetate
Acs1p/Acs2p
Acetyl-CoA
Erg10p
Acetoacetyl-CoA
Erg13p
HMG-CoA
Hmg1p
Hmg1p
Mevalonate
IPP
Erg13p
DMAPP
Erg20p
FPP
Erg9p
Squalene
NADP
NADPH+O2
Erg1p/Cprp
2,3-oxidosqualene
DDS
Dammarenediol-II
NADP
NADPH+O2
PDDS/CPR
Protopanaxadiol

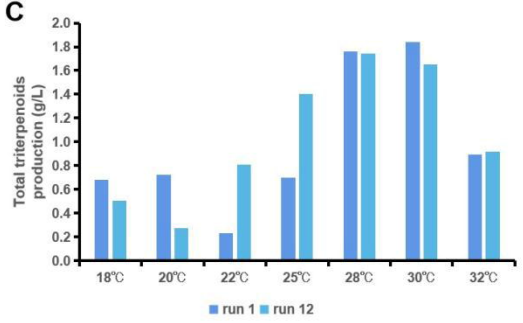

A
PPD Production (g/L)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 CT
B
DM Production (g/L)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 CT

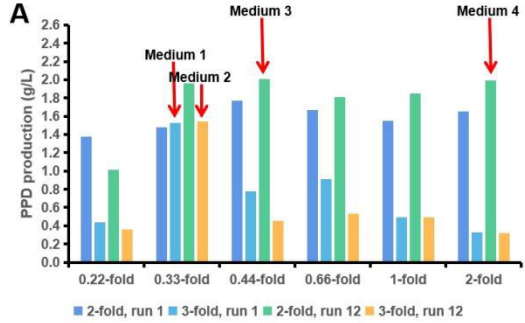

A
Ind-OLS Cofficient
0.6
0.4
0.2
0.0
-0.2
-0.4
A
C
D
B
G
F
E

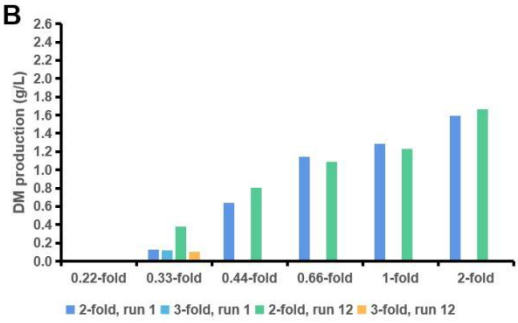

B
Int-OLS Cofficient
0.6
0.4
0.2
0.0
-0.2
-0.4
C
A:E
A:C
B:F
B
C:D
B:G

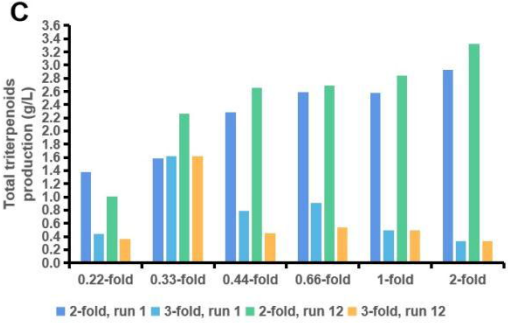

A
PPD production (g/L)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
18℃
20℃
22℃
25℃
28℃
30℃
32℃
run 1
run 12

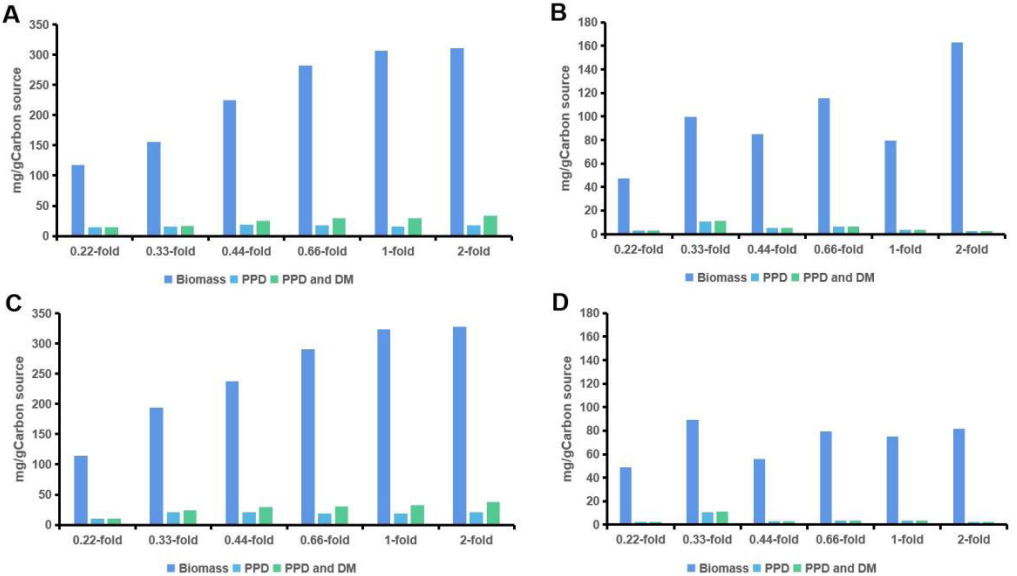

B
DM production (g/L)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
18℃
20℃
22℃
25℃
28℃
30℃
32℃
run 1
run 12

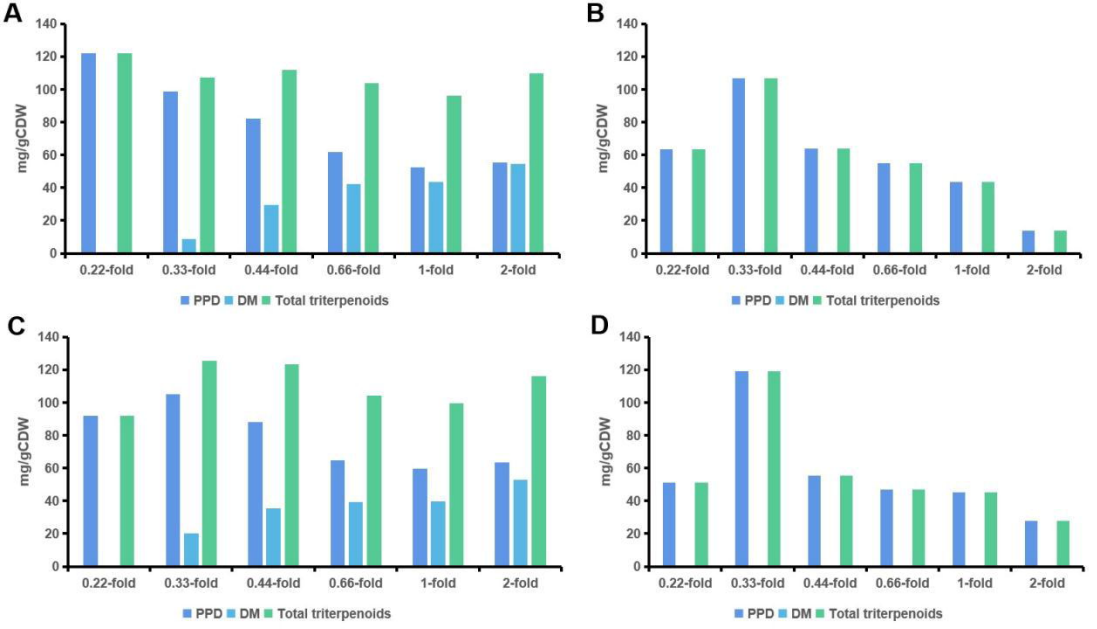

C
Total triterpenoids production (g/L)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
18℃
20℃
22℃
25℃
28℃
30℃
32℃
run 1
run 12

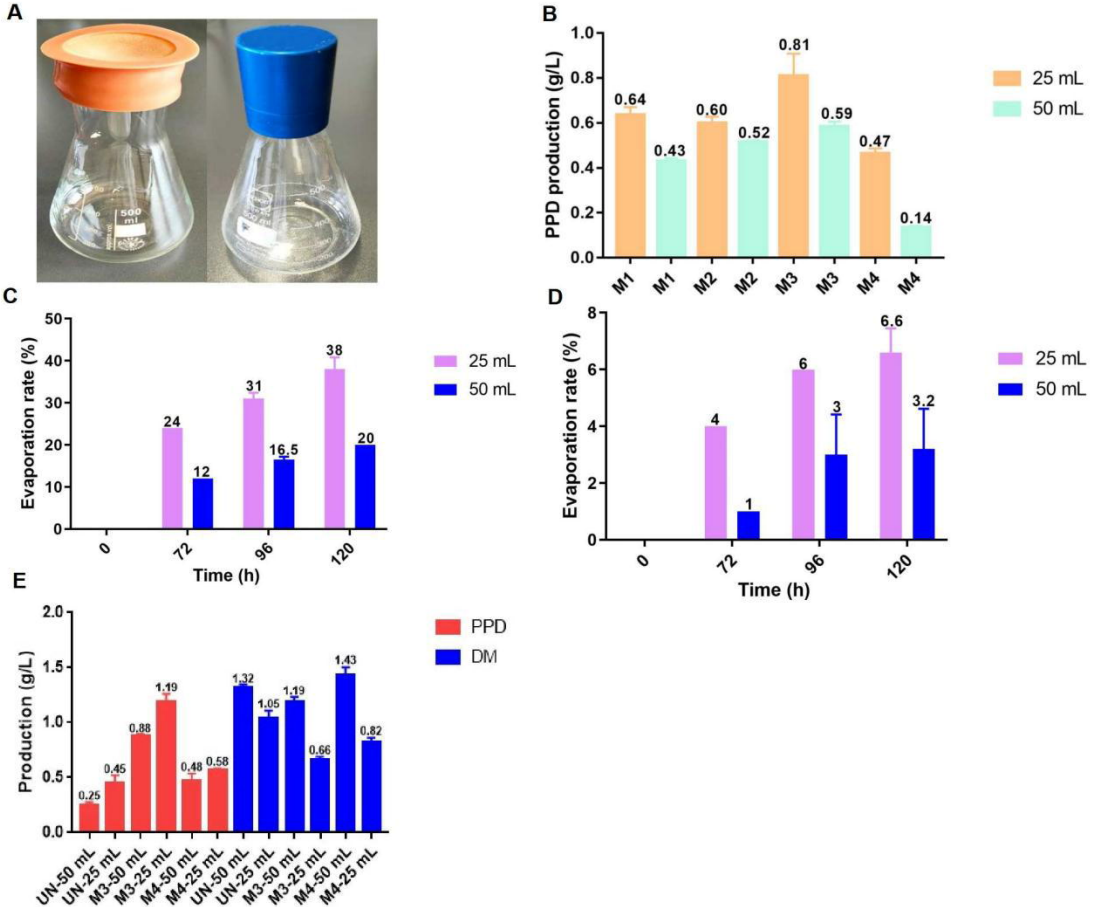

A
Medium 1
Medium 2
Medium 3
Medium 4
PPD production (g/L)
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.22-fold
0.33-fold
0.44-fold
0.66-fold
1-fold
2-fold
2-fold, run 1
3-fold, run 1
2-fold, run 12
3-fold, run 12

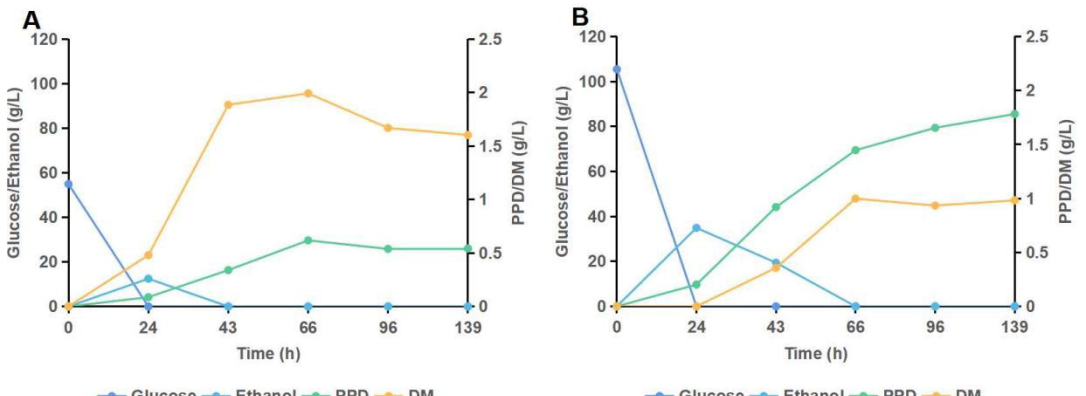

B
DM production (g/L)
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.22-fold
0.33-fold
0.44-fold
0.66-fold
1-fold
2-fold
2-fold, run 1
3-fold, run 1
2-fold, run 12
3-fold, run 12

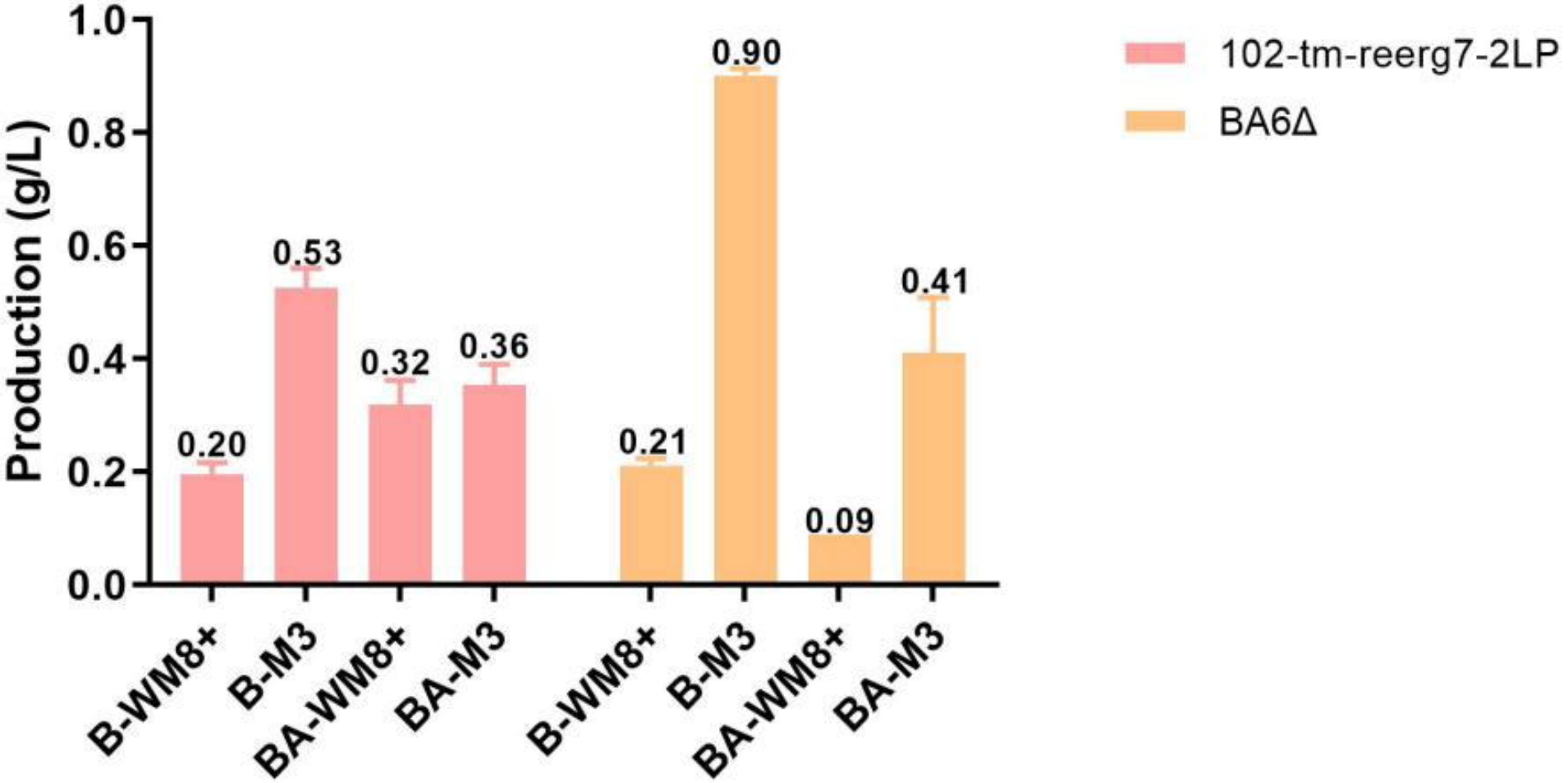

C
Total triterpenoids production (g/L)
3.6
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.22-fold
0.33-fold
0.44-fold
0.66-fold
1-fold
2-fold
2-fold, run 1
3-fold, run 1
2-fold, run 12
3-fold, run 12

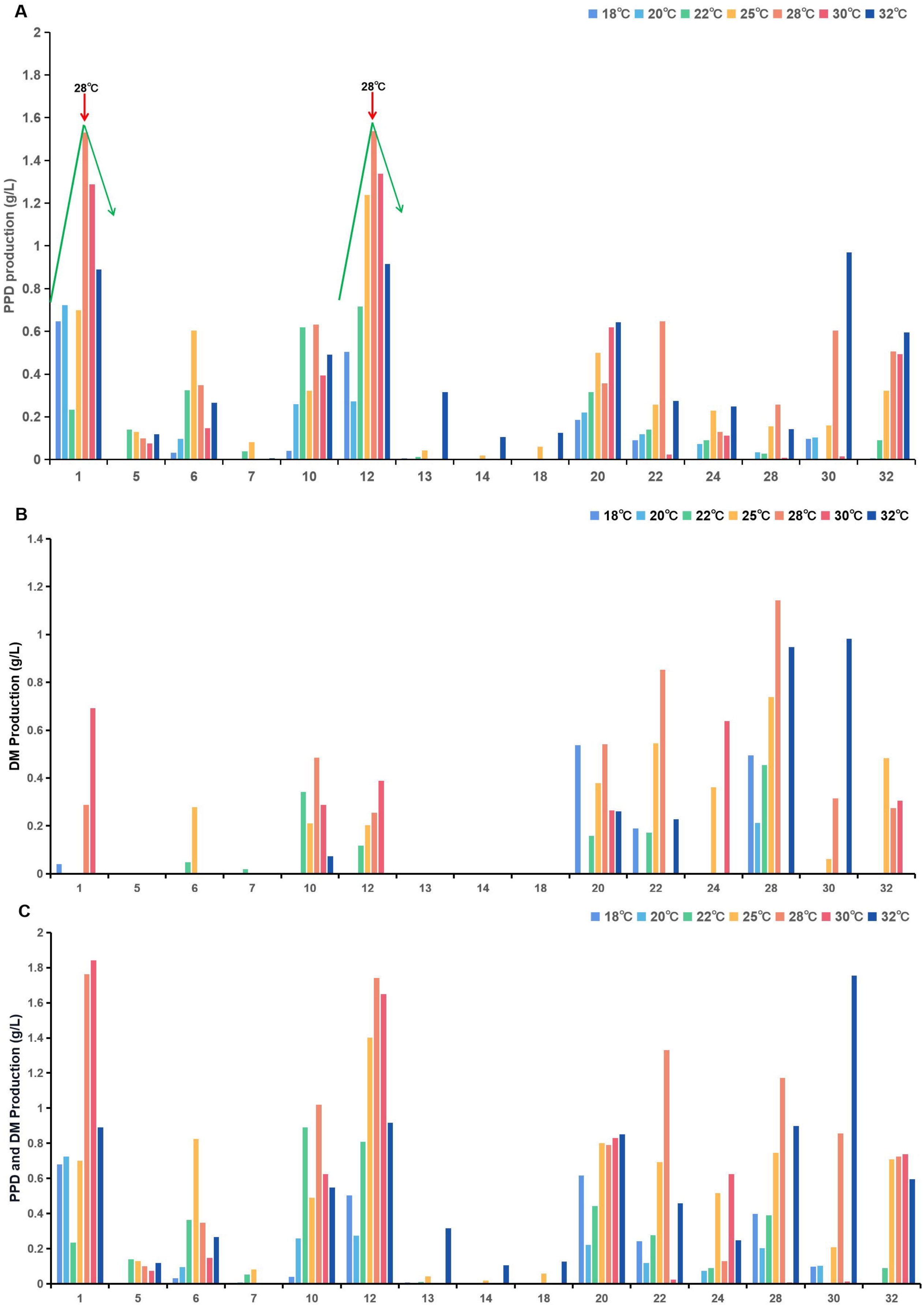

A
B
C
D
mg/gCarbon source
0.22-fold
0.33-fold
0.44-fold
0.66-fold
1-fold
2-fold
Biomass
PPD
PPD and DM


A
B
C
D
mg/gCDW
0
20
40
60
80
100
120
140
0.22-fold
0.33-fold
0.44-fold
0.66-fold
1-fold
2-fold
PPD
DM
Total triterpenoids


A
500 ml
B
PPD production (g/L)
1.0
0.8
0.6
0.4
0.2
0.0
0.64
0.43
0.60
0.52
0.81
0.59
0.47
0.14
M1
M1
M2
M2
M3
M3
M4
M4
25 mL
50 mL
C
Evaporation rate (%)
50
40
30
20
10
0
24
12
31
16.5
38
20
0
72
96
120
Time (h)
25 mL
50 mL
D
Evaporation rate (%)
8
6
4
2
0
4
1
6
3
6.6
3.2
0
72
96
120
Time (h)
25 mL
50 mL
E
Production (g/L)
2.0
1.5
1.0
0.5
0.0
0.25
0.45
0.88
1.19
0.48
0.58
1.32
1.05
1.19
0.66
1.43
0.82
UN-50 mL
UN-25 mL
M3-50 mL
M3-25 mL
M4-50 mL
M4-25 mL
UN-50 mL
UN-25 mL
M3-50 mL
M3-25 mL
M4-50 mL
M4-25 mL
PPD
DM


A
B
Glucose/Ethanol (g/L)
PPD/DM (g/L)
Time (h)
0
24
43
66
96
139
Glucose
Ethanol
PPD
DM


Production (g/L)
1.0
0.8
0.6
0.4
0.2
0.0
0.20
0.53
0.32
0.36
0.21
0.90
0.09
0.41
B-WM8+
B-M3
BA-WM8+
BA-M3
B-WM8+
B-M3
BA-WM8+
BA-M3
102-tm-reerg7-2LP
BA6Δ


A
18℃ 20℃ 22℃ 25℃ 28℃ 30℃ 32℃
28℃
28℃
PPD production (g/L)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
1 5 6 7 10 12 13 14 18 20 22 24 28 30 32
B
18℃ 20℃ 22℃ 25℃ 28℃ 30℃ 32℃
DM Production (g/L)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1 5 6 7 10 12 13 14 18 20 22 24 28 30 32
C
18℃ 20℃ 22℃ 25℃ 28℃ 30℃ 32℃
PPD and DM Production (g/L)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
1 5 6 7 10 12 13 14 18 20 22 24 28 30 32