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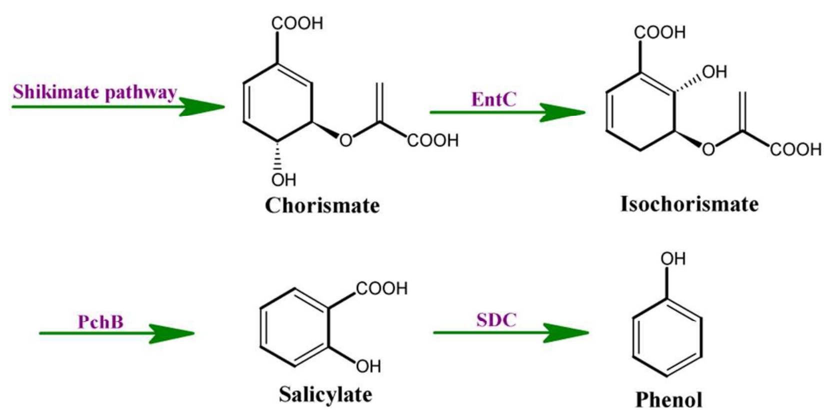

A novel phenol biosynthetic pathway was designed and verified in ***E. coli.***

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1 # Microbial production of phenol via salicylate decarboxylation

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20 **Abstract**

21 Phenol, as an important bulk chemical, is the synthetic precursor to polycarbonates, epoxide
22 resins and phenolic resins. Currently, phenol production utterly relies on chemical processes
23 using benzene as the starting material, which are environmentally incompatible and
24 nonrenewable. Here we designed a novel phenol biosynthetic pathway and achieved phenol
25 production from renewable carbon sources in *Escherichia coli*. A decarboxylase from *Klebsiella*
26 *pneumoniae* was over-expressed and purified. *In vitro* assay was conducted to determine the
27 kinetic parameters toward salicylate. In the bioconversion study, 1154.5 ± 12.0 mg/L of phenol
28 was produced from supplemented salicylate. Connecting salicylate decarboxylation with its
29 biosynthesis led to microbial phenol production. Phenol production was improved by enhancing
30 upstream shikimate pathway and modulating gene expression levels. *E. coli* strain QH4 was a
31 better strain than BW25113 for phenol production. In shake flask experiment the best strain
32 produced 405.6 ± 13.7 mg/L of phenol with only trace amount of salicylate left in the culture.
33 Optimization of cultivation conditions further increase phenol titer by 16.4 % to 472.1 ± 19.8
34 mg/L. This study provides a promising alternative for sustainable production of phenol.

35 Keywords: phenol; salicylate; decarboxylase; metabolic engineering

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38 **1. Introduction**

39 Phenol is an important commodity chemical with many applications. It mainly serves as the
40 precursor to polycarbonates, epoxide resins and phenolic resins. In addition, it can also be used
41 to synthesize herbicides and pharmaceutical drugs. In 2008, over 8 million tons of phenol was
42 produced globally.[1] Currently, the dominant route for phenol production is the cumene process
43 using benzene as the starting material, which is non-renewable and environmentally unfriendly[2].
44 With the concerns on oil crisis and environment pollution, increasing attention has been attracted
45 to develop new sustainable processes for the production of phenol and other fossil-derived
46 chemicals. Metabolic engineering represents a promising alternative and the biological
47 production of a variety of chemicals has been achieved from renewable biomass[3-12].

48 Biological production of phenol has been reported by several research groups. A phenol
49 biosynthetic pathway was constructed in a solvent-tolerant *Pseudomonas putida* S12 strain.[13]
50 Tyrosine was converted to phenol by introducing a foreign tyrosine phenol-lyase (TPL). To
51 increase precursor supply, shikimate pathway was enhanced by over-expressing 3-deoxy-D-
52 arabino-heptolosonate-7-phosphate (DAHP) synthase. The final strain produced 142 mg/L of
53 phenol in shake flask culture. Phenol titer was further improved to 868 mg/L in biphasic fed-
54 batch cultivation using octanol as the extractant. This pathway was also reconstituted in *E. coli.*[1]
55 To improve tyrosine supply, *csrA* and *tyrR* were down-regulated using synthetic regulatory small
56 RNA. The engineered strain produced 419 mg/L of phenol from glucose by flask culture. Phenol
57 titer was increased to 3.8 g/L by biphasic fermentation with tributyrin as an extractant. Recently,
58 a novel phenol production pathway was established in *E. coli* by recruiting 4-hydroxybenzoate
59 decarboxylase[14]. 4-Hydroxybenzoate is a native metabolite in *E.coli* and is synthesized from

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60 chorismate by the catalysis of chorismate pyruvate lyase (UbiC). DAHP synthase and UbiC were
61 shown to be rate-limiting, and over-expressing of their encoding genes resulted in 7- and 69-fold
62 increase of phenol titer, respectively. The pathway genes were integrated into the chromosome to
63 obtain genetically stable strains. By modulating gene expression levels, phenol titer reached 250
64 mg/L in shake flask culture. Five different solvents were tested for biphasic extractive
65 fermentation and dibutyl phthalate and tributyrin were the best two solvents for improving
66 phenol production. By two-phase fed-batch fermentation, the best strain produced 9.5 g/L phenol
67 in a 7 L fermentor.[14]

68 In this study, we designed a novel phenol biosynthetic pathway by connecting salicylate
69 synthesis and its decarboxylation. After pathway and strain optimization, the final titer reached
70 472.1 mg/L in shake flask experiment.

71 **2. Materials and methods**

72 **2.1 Media, strains and plasmids**

73 Luria-Bertani (LB) medium was used for cell propagation and protein expression. Modified M9
74 medium was used for microbial production of phenol. LB medium contains 10 g tryptone, 5 g
75 yeast extract and 10 g NaCl per liter. The modified M9 (M9Y) medium contains 10 g/L glycerol,
76 2.5 g/L glucose, 6 g/L Na_2HPO_4, 0.5 g/L NaCl, 3 g/L KH_2PO_4, 1 g/L NH_4Cl, 1 mM $MgSO_4$, 0.1
77 mM $CaCl_2$, 2 g/L yeast extract and 2 g/L MOPS. When needed, ampicillin and kanamycin were
78 added to the medium to the final concentration of 100 mg/L and 50 mg/L, respectively. *E. coli*
79 strain XLBlue was used for plasmid construction and propagation. *E. coli* BL21 (DE3) Star was
80 used for protein expression and purification. *E. coli* BW25113 and QH4 were used for phenol
81 production. Plasmid pETDuet-1 was employed for protein expression and purification. Plasmids

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82 pZE12-luc (high-copy) and pCS27 (medium-copy) were used for pathway assembly. The details
83 of the strains and plasmids used in this study are depicted in Table 1.

84 **2.2 DNA manipulation**

85 Plasmids pET-kpBDC, pZE-EP and pCS-APTA were constructed in our previous studies[15, 16].
86 Plasmid pZE-kpBDC was constructed by inserting BDC encoding gene into *Kpn* I/*Xba* I sites of
87 plasmid pZE12-luc. Plasmids pZE-kpBDC-EP and pCS-APTA-EP were constructed by inserting
88 the expressing cassette $P_L$lacO1-EP into *Spe* I/*Sac* I sites of pZE-kpBDC and pCS-APTA,
89 respectively. Plasmid pCS-APTA-kpBDC was constructed by inserting the expressing cassette
90 $P_L$lacO1-kpBDC into *Spe* I/*Sac* I sites of pCS-APTA.

91 **2.3 Salicylate decarboxylase assay**

92 *E. coli* strain BL21 (DE3) Star was transformed with plasmid pET-kpBDC. Overnight cultures
93 were inoculated into 50 mL of LB medium and incubated at 37 ℃ with shaking. When OD_{600}
94 reached 0.4, cultures were induced with 0.25 mM IPTG and grown for 5 h at 30 ℃. Cell pellets
95 were harvested by centrifugation. After re-suspended in 800 μL of His-binding buffer, the cells
96 were disrupted with 0.1 mm glass beads. The protein was purified using His-Spin protein
97 miniprep kit (ZYMO Research). Protein concentration was estimated using BCA kit (Pierce
98 Chemicals). For measurement of decarboxylase activity, the following reaction conditions were
99 used: the mixture contained 20 μL of purified protein, salicylate as substrate in 66.7 mM
100 Na_2HPO_4-KH_2PO_4 buffer (pH 5.0) with a final volume of 1 mL in a 1.5-mL micro-centrifuge
101 tube and incubated at 30 ℃ for 4 h. The substrate concentrations varied from 0 to 2.5 mM. The
102 enzyme activity was monitored by measuring the formation of phenol by HPLC. The kinetic

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103 parameters of decarboxylase were calculated through non-linear regression of the Michaelis-
104 Menten equation.

105 **2.4 Feeding experiments**

106 Feeding experiments were carried out to examine phenol production from salicylate. *E. coli*
107 strain BW25113 was transformed with plasmid pZE-kpBDC. Single colonies were inoculated
108 into 3 mL LB medium containing 100 μg/mL of ampicillin and grown overnight at 37 ℃.
109 Overnight cultures were inoculated into 50 mL of M9Y medium containing ampicillin. The
110 cultures were left to grow at 37 ℃ till OD_{600} reached 0.6 and then induced with 0.25 mM IPTG.
111 Salicylate (final concentration 400 mg/L) was fed into the culture at 8 h, 16 h, 24 h and 36 h after
112 induction. Samples were taken at four different time points and the product concentrations were
113 measured by HPLC.

114 **2.5 *De novo* production of phenol**

115 Overnight cultures of phenol-producing strains were inoculated into the M9Y medium
116 containing appropriated antibiotics with a ratio of 1% or 2 % and cultivated at 37 ℃ with shaking
117 for 3 h. Then, the cultures were induced with 0.25 mM IPTG and continued to be cultivated at 30℃
118 or 37℃ for 48 h. Samples were taken every 12 hours. OD_{600} values were measured and the
119 concentrations of phenol and salicylate were analyzed by HPLC.

120 **2.6 HPLC analysis**

121 Salicylate (from Sigma Aldrich) and phenol (from Alfa Aesar) were used as the standards. Both
122 the standards and samples were analyzed and quantified by HPLC (Hitachi Chromaster)
123 equipped with a reverse-phase Diamonsil C18 column and a Hitachi 5420 UV-VIS detector.

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124 Solvent A was water with 0.1% formic acid and solvent B was methanol. The column
125 temperature was set to 30 ℃. For salicylate and phenol detection, the following gradient was
126 used at a flow rate of 1 ml/min: 50 to 80 % solvent B for 22 min, 80 to 50 % solvent B for 3 min,
127 and 50 % solvent B for an additional 5 min. Quantification of salicylate and phenol was based on
128 the peak areas at specific wavelengths (300 nm for salicylate, 274 nm for phenol).

129 **3. Results and disussion**

130 **3.1 Design of a novel phenol biosynthetic pathway**

131 Salicylate is an important naturally-occurring compound that has various physiological functions
132 and wide commercial applications. In our previous study, a salicylate biosynthetic pathway was
133 designed and verified in *E. coli*， in which chorismate was converted to salicylate by the action
134 of two enzymes isochorismate synthase (encoded by *entC*) and isochorismate pyruvate
135 lyase(endoded by *pchB*)[16]. After systematic pathway and strain optimization, 1.2 g/L of
136 salicylate was produced in shake flasks[17]. This pathway was further extended for 4-
137 hydroxycoumarin and muconic acid production[16, 17]. In nature, salicylate decarboxylase(SDC)
138 catalyzes the decarboxylation of salicylate to form phenol and CO_2. Although the reaction is
139 reversible, the escape of CO_2 from the reaction system makes it prefer phenol production. Based
140 on this, we designed a new phenol biosynthetic pathway by connecting salicylate synthesis and
141 its decarboxylation (Figure 1).

142 **Insert Figure 1**

143 **3.2 SDC characterization and bioconversion of salicylate to phenol**

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144 So far, only one SDC, which is from *Trichosporon moniliiforme*, is recorded in BRENDA
145 enzyme database. Formerly, our group characterized a decarboxylase, which showed activity
146 toward several hydroxybezoic acids[15]. To further investigate the kinetic parameters, we purified
147 the enzyme and did *in vitro* assay using salycylate as the substrate. The K_m, V_{max}, and k_{cat} under
148 optimal conditions were determined to be 1.03 mM, 1.85 μm min^{-1}, and 3.93 min^{-1}, respectively.

149 To evaluate its application potential for phenol production, feeding experiment was carried out.
150 The result showed that 1154.5 ± 12.0 mg/L of phenol was produced from 1580 mg/L of
151 salicylate (Figure 2). The conversion continued even when cell growth entered stationary phase,
152 which indicates that this enzyme can keep active for a long time and requires no cofactors from
153 cell metabolism.

154 **Insert Figure 2**

155 **3.3 Microbial production of phenol in *E.coli* strain BW25113**

156 After achieving phenol production from salicylate, we then moved forward to *de novo* phenol
157 production. First, *E. coli* BW25113 was transformed with plasmid pZE-EP-kpBDC, yielding
158 strain BP1. In 48 h, this strain only produced 67.7 ± 1.7 mg/L of phenol with 209.8 ± 1.4 mg/L
159 of salicylate remained unconverted in the cell culture (Figure 3A). Sufficient supply of
160 precursors is vital to achieve high production of target compounds. To this end, strain BP1 was
161 transformed with plasmid pCS-APTA, yielding strain BP2. Plasmid pCS-APTA was constructed
162 previously, containing four key genes (*aroL*, *ppsA*, *tktA* and *aroG^{fbr}*) in the shikimate pathway.
163 Expression of these genes had been shown to be effective to increase cabon flux through
164 shikimate pathway, resulting in increased titer and yield of final products[18, 19]. As expected,
165 phenol titer was doubled to 132.8 ± 1.3 mg/L (Figure 3A). However, we observed that higher

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166 amount of salicylate was accumulated. To solve this problem, we tried to modulate the
167 expression level of pathway genes. In the first strategy, to decrease salicylate supply, EP module
168 was moved to medium-copy number plasmid (pCS-APTA-EP) while BDC remained on high
169 copy number plasmid (pZE-kpBDC). Compared to strain BP2, *E. coli* strain BP3 containing
170 these two new plasmids produced even less amount of phenol (8.6 ± 0.4 mg/L) although
171 salicylate accumulation was alleviated (359.6 ± 2.4 mg/L). In the second strategy, an extra copy
172 of BDC encoding gene was cloned into plamid pCS-APTA, yielding plasmid pCS-APTA-BDC.
173 *E. coli* strain BP4 co-transformed with pZE-EP-kpBDC and pCS-APTA-kpBDC produced 220.6
174 ± 9.0 mg/L of phenol with only 40.1 ± 0.1 mg/L of salicylate left in the culture, which indicated
175 that increasing BDC expressing level did promote salicylate conversion (Figure 3A).

176 **Insert Figure 3**

177 **3.4 Microbial production of phenol in *E. coli* strain QH4**

178 Using *E.coli* BW25113 as the host, we achieved *de novo* phenol production. However, the titer is
179 not satisfactory. We then tested the production capacity of another *E. coli* strain QH4, which is a
180 derivative of a phenylalanine overproducing strain ATCC 31884. Strain QH4 has been
181 successfully used for the production of several valuable compounds derived from shikimate
182 pathway, such as caffeic acid and muconic acid[17, 20]. Strain QH4 was transformed with the same
183 sets of plasmids, generating strains QP1 to QP4. Compared with BP1, QP1 performed much
184 better and phenol titer reached 296.3 ± 32.2 mg/L with only trace amount of salicylate
185 accmulated. QP2 with enhanced upstream pathway produced 405.6 ± 13.7 mg/L of phenol,
186 which is the best among all the constructed strains (Figure 3B). Compared with their

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187 counterparts (BP3 and BP4), strains QP3 and QP4 performed slightly better or similar in phenol
188 production (Figure 3).

189 **3.5 Optimization of cultivating conditions to futher improve phenol production**

190 As mentioned above, the best strain QP2 produced 405.6 ± 13.7 mg/L of phenol in shake flasks.
191 Originally, 8 h-old inoculum was used with a inoculation ratio of 1 % and the induction
192 temperature was 30 ℃. Changing inoculum cultivation time from 8 h to 12 h improved phenol
193 titer to 424.5 ± 6.6 mg/L. Increasing inoculation ratio from 1 % to 2 % led to further
194 improvement of phenol titer to 472.1 ± 19.8 mg/L in 48 h. Shifting the induction temperature
195 from 30℃ to 37℃ resulted in decreased phenol production although cell growth was improved
196 (Figure 4).

197 **Insert Figure 4**

198 Time course of phenol production showed that salicylate production was cell growth dependent.
199 Salicylate titer experienced an increasing phase and began to decline due to the continued
200 conversion to phenol. Phenol production is independent on cell growth and its titer kept
201 increasing untill trace amount of salicylate was left in the culture (Figure 5).

202 **Insert Figure 5**

203 **4. Conclusion**

204 In this study, a novel phenol biosynthetic pathway was established. Modulating gene expression
205 levels is an effective way to balance metabolic pathway, reduce intermediate accumulation and
206 realize significant improvement of product titer. Strain QH4 is superior to strain BW25113 for
207 phenol production. Through systematic optimization of pathway, host strain and cultivation

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208 conditions, 472.1 ± 19.8 mg/L was produced, which is, to the best of our knowledge, the highest
209 reported titer obtained in flask culture. To meet the standards for industrial application, furture
210 work will include improving genetic stability by chromosome integration, increasing strain
211 tolerance to toxic products by direct evolution, and maximizing titer and productivity by process
212 engineering.

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242 **Table 1** Plasmids and strains used in this study

Plasmids and strains	Description	Source
Plasmids		
pZE12-luc	$P_L$lacO1, *colE* ori, *luc*, Amp^r	Ref. 16
pCS27	$P_L$lacO1,P15A ori, Kan^r	Ref. 16
pETDuet-1	pT7, PBR322 ori, Amp^r	Ref. 16
pET-kpBDC	pETDuet-1, salicylate decarboxylase gene from *K. pneumoniae*	Ref. 15
pZE-EP	pZE12-luc, *entC* from *E. coli* and *pchB* from *Pseudomonas fluorescens*	Ref. 16
pCS-APTA	pCS27, *aroL*, *ppsA*, *tktA*, $aroG^{fbr}$ from *E. coli*	Ref. 16
pZE-kpBDC	pZE12-luc, salicylate decarboxylase gene from *K. pneumoniae*	This study
pZE-kpBDC-EP	pZE12-luc, salicylate decarboxylase gene, *entC*, *pchB*, two operons	This study
pCS-APTA-EP	pCS27, *aroL*, *ppsA*, *tktA*, $aroG^{fbr}$, *entC*, *pchB*, two operons	This study
pCS-APTA-kpBDC	pCS27, *aroL*, *ppsA*, *tktA*, $aroG^{fbr}$, *entC*, *pchB*, salicylate decarboxylase gene, two operons	This study
Strains		
XL1-Blue	*recA*1 *endA*1 *gyrA*96 thi-1 *hsdR*17 *supE*44 *relA*1 *lac* [F' proAB lacIqZΔM15Tn10 (Tet^r)]	Stratagene
BW25113	rrnBT14 ΔlacZWJ16 hsdR514 ΔaraBADAH33 ΔrhaBADLD78	Coli genome stock center
QH4	*E. coli* ATCC 31884 with *pheA* and *tyrA* disrupted	Ref. 20
BP1	BW25113 with pZE-kpBDC-EP	This study
BP2	BW25113 with pZE-kpBDC-EP and pCS-APTA	This study
BP3	BW25113 with pZE-kpBDC and pCS-APTA-EP	This study
BP4	BW25113 with pZE-kpBDC-EP and pCS-APTA-kpBDC	This study
QP1	QH4 with pZE-kpBDC-EP	This study
QP2	QH4 with pZE-kpBDC-EP and pCS-APTA	This study
QP3	QH4 with pZE-kpBDC and pCS-APTA-EP	This study
QP4	QH4 with pZE-kpBDC-EP and pCS-APTA-kpBDC	This study

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249 **Figure legends**

250 **Figure 1** A novel biosynthetic pathway for phenol production. EntC, isochorismate synthase;

251 PchB, isochorismate pyruvate lyase; BDC, salicylate decarboxylase.

252 **Figure 2** Bioconversion of salicylate to phenol using recombinant *E. coli* strain BW25113
253 harboring pZE-kpBDC. Salicylate (400 mg/L) was supplemented to the cell culture at 8 h, 16 h,
254 24 h and 36 h after induction. Experiments were carried out in triplicate.

255 **Figure 3** Pathway optimization for phenol production in *E. coli* strains BW25113(A) and
256 QH4(B). Detailed information for strains used was shown in Table 1. Data were generated from
257 samples collected 48 h after induction. Experiments were carried out in triplicate.

258 **Figure 4** Optimization of cultivation conditions to further improve phenol titer. Inoculum age,
259 inoculation ratio and induction temperature were three variables investigated. Condition A, 8 h,
260 1 % and 30 ℃; Condition B, 12 h, 1 % and 30 ℃; Condition C, 12 h, 2 % and 30 ℃; Condition
261 D, 12 h, 2% and 37 ℃. Data were generated from samples collected 48 h after induction.
262 Experiments were carried out in triplicate.

263 **Figure 5** Time courses of phenol production using strain QP2 under optimized conditions.

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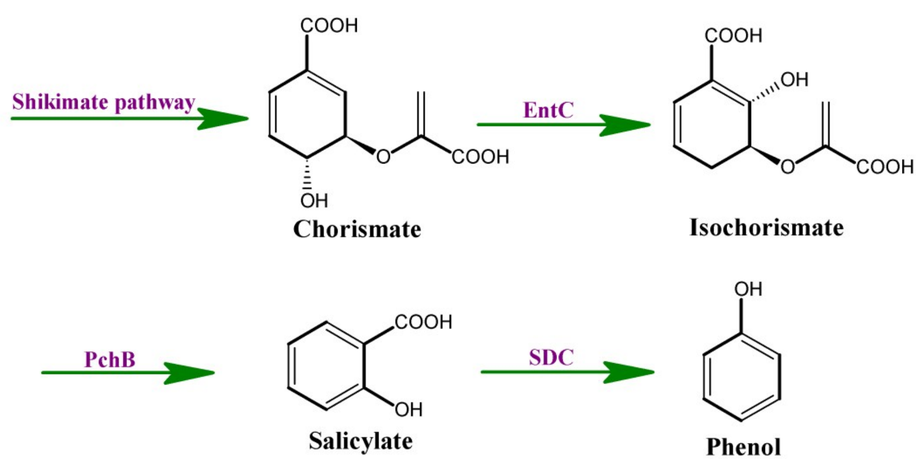
265 **Fig. 1**



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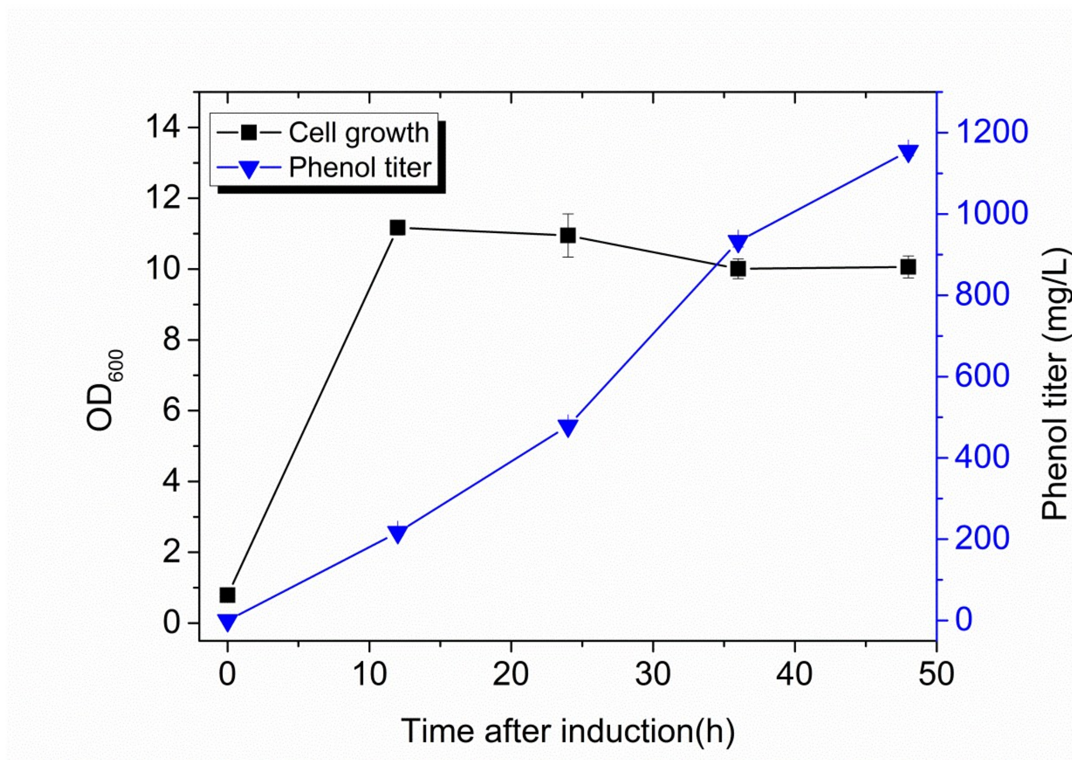
268 **Fig. 2**



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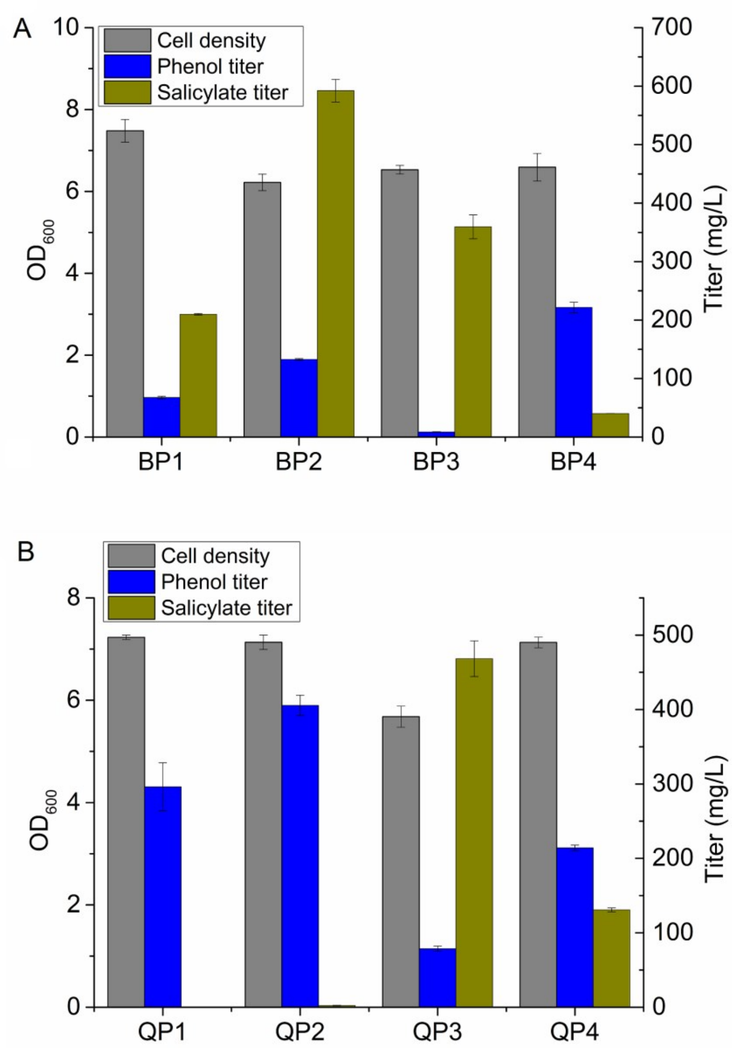
271 **Fig. 3**



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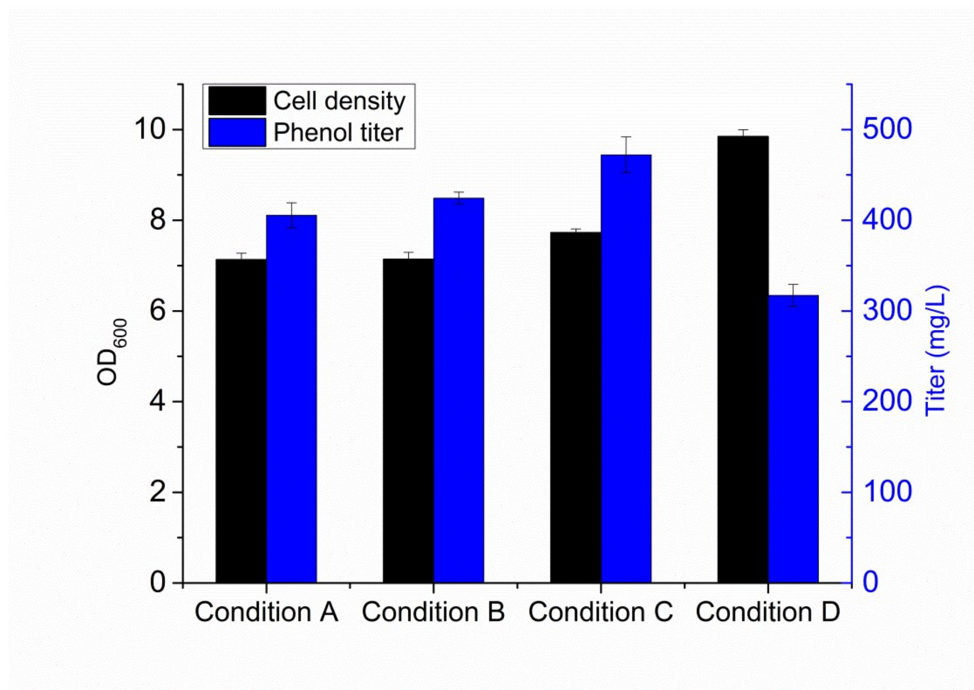
274 **Fig. 4**



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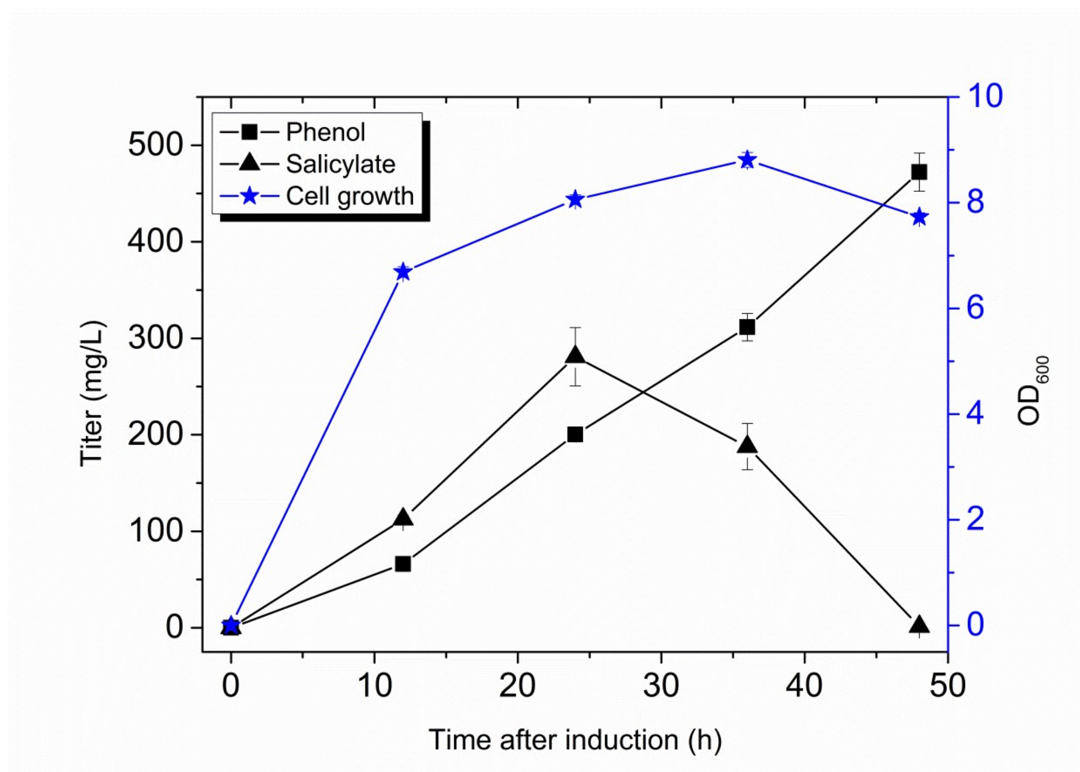
277 **Fig. 5**



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