

Muconic acid production from glucose using enterobactin precursors in *Escherichia coli*

Jie Wang · Pu Zheng

Received: 3 September 2014 / Accepted: 29 December 2014 / Published online: 8 February 2015
© Society for Industrial Microbiology and Biotechnology 2015

Abstract Muconic acid (MA) is a promising bulk chemical due to its extensive industrial applications in the production of adipic acid and other valuable, biodegradable intermediates. MA is heretofore mainly produced from petrochemicals by organic reactions which are not environmentally friendly or renewable. Biological production processes provide a promising alternative for MA production. We designed an artificial pathway in *Escherichia coli* for the biosynthesis of MA using the catechol group of 2,3-dihydroxybenzoate, an intermediate in the enterobactin biosynthesis pathway. This approach consists of two heterologous microbial enzymes, including 2,3-dihydroxybenzoate decarboxylase and catechol 1,2-dioxygenase. The metabolic flow of carbon into the heterologous pathway was optimized by increasing the flux from chorismate through the enterobactin biosynthesis pathway and by regulating the shikimate pathway. Metabolic optimization enabled a concentration of 605.18 mg/L of MA from glucose in a shaking flask culture, a value nearly 484-fold higher than that of the initial recombinant strain. The results indicated that the production of MA from this pathway has the potential for further improvement.

Keywords Muconic acid · 2,3-Dihydroxybenzoate · Enterobactin · Metabolic engineering · *Escherichia coli*

Electronic supplementary material The online version of this article (doi:10.1007/s10295-014-1581-6) contains supplementary material, which is available to authorized users.

J. Wang · P. Zheng (✉)
The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, People's Republic of China
e-mail: zhengpu@jiangnan.edu.cn

Introduction

Muconic acid (MA), also known as 2,4-hexadienedioic acid, has attracted great attention in recent years because of its potential use as a precursor and platform chemical for the production of several bio-plastics [5]. The products, including the commercially important bulk chemicals adipic acid, terephthalic acid, and trimellitic acid, have a wide variety of uses in the production of nylon-6, 6, polytrimethylene terephthalate, polyethylene terephthalate, dimethyl terephthalate, trimellitic anhydride, industrial plastics, pharmaceuticals, plasticizers, cosmetics, etc. [30]. Worldwide production of adipic acid has already exceeded 2.5 million tons per year [26]. Traditional chemical processes for MA production rely on non-renewable, petroleum-based feedstock and high concentrations of heavy metal catalysts, which results in environmental pollution, petroleum depletion, and high-cost separation processes [30]. Thus, a sustainable, environmentally friendly, and cost-effective biotechnological process for MA production, based on inexpensive carbohydrate raw materials, is very desirable.

MA is a naturally occurring metabolic product of some organisms, such as *Acinetobacter* sp., *Pseudomonas* sp., and *Shingobacterium* sp. [17, 25, 28], which have metabolic pathways for the degradation of aromatic compounds. However, no known organisms can naturally produce MA from renewable carbon sources, such as glucose.

So far, four pathways have been designed for MA synthesis from glucose. Draths and Frost [6] reported the first synthetic route for MA production from glucose in *Escherichia coli*. This pathway was based on three heterologous genes, encoding 3-dehydroshikimate dehydratase (AroZ), protocatechuic acid decarboxylase (AroY) from *Klebsiella pneumoniae*, and catechol 1,2-dioxygenase (CatA)

from *Acinetobacter calcoaceticus*, which convert 3-dehydroshikimate to MA via protocatechuic acid and catechol. Afterwards, the Frost group further optimized the strain by increasing the levels of phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P), as well as by knocking out the 3-dehydroshikimate dehydrogenase gene *aroE* to block aromatic amino acid synthesis [18]. Recently, Apler's and Boles' groups reconstructed the same pathway to produce MA in *Saccharomyces cerevisiae* [5, 27]. Moreover, the Yan group assembled two more artificial pathways for MA production. In one of the pathways, tryptophan biosynthesis was shunted and MA was generated via anthranilate

[23]. In the other, salicylic acid served as the precursor of MA, while salicylic acid was generated from isochorismate by isochorismate pyruvate lyase [13].

Some efforts have been made to study the mechanism of the enterobactin pathway [19, 22]. According to Franke and Bujnicki's work, the fermentation of *E. coli* strains AN193/pDF3 (carrying *entB* and *entC*) and F111/pC20 (carrying *aroF*, *entC*, *entB*, *aroB* and *aroL*) showed that (S,S)-2,3-dihydroxy-2,3-dihydrobenzoic acid could be up to 4.6 and 12 g/L, respectively [4, 8], which indicates that the enterobactin pathway can also be practically applied to MA production. Very recently, the Yan group found a prokaryotic 2,3-dihydroxybenzoic acid decarboxylase and applied it to MA production [24]. In this study, we also successfully constructed a synthetic pathway to produce MA using the precursors of enterobactin, 2,3-dihydrobenzoate (2,3-DHB) (Fig. 1a). Two exogenous enzymes, 2,3-dihydroxybenzoate decarboxylase (EntX) and catechol 1, 2-dioxygenase (CatA) were imported from *Klebsiella pneumonia* strain CICIM B7001 and *Pseudomonas putida* strain KT2440, respectively. Metabolic optimization by increasing the flux enterobactin biosynthesis pathway and by regulating the shikimate pathway, as well as optimization of IPTG induction resulted in a concentration of 605.18 mg/L of MA from glucose in a shaking flask culture.

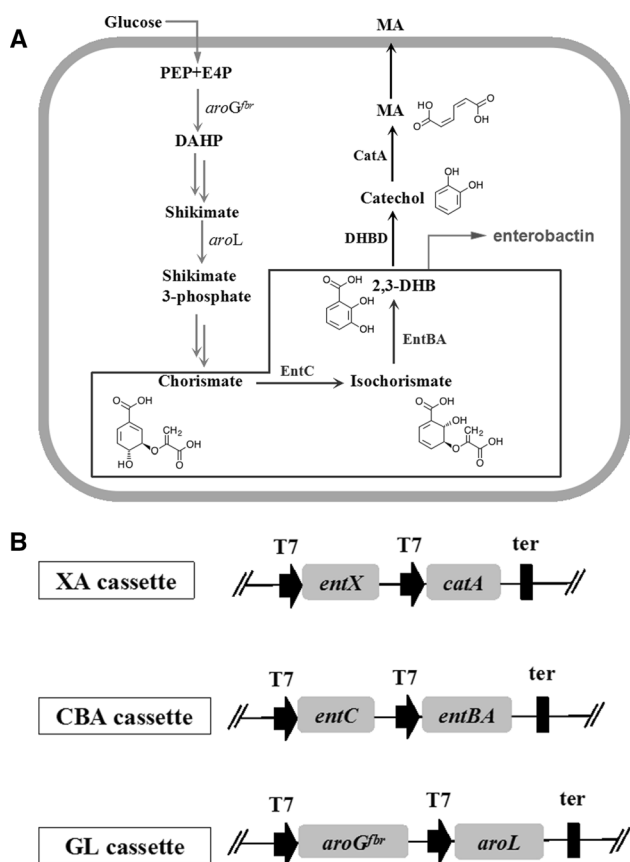


Fig. 1 a. A metabolic pathway for the biosynthesis of MA. Light gray arrows indicate native metabolic pathways of chorismate in *E. coli*, dark gray arrows indicate practical metabolic pathways for enterobactin production in *E. coli*, and black arrows indicate the introduced artificial pathway for MA production. PEP phosphoenolpyruvic acid, E4P D-erythrose 4-phosphate, 2,3-DHB 2,3-dihydroxybenzoate, MA muconic acid, EntC isochorismate synthase, EntB isochorismatase; *aroG^{fbr}*, encoding phenylalanine feedback inhibition-resistant 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase, *aroL* encoding shikimate kinase, EntA 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase, EntX 2,3-dihydroxybenzoate decarboxylase, CatA catechol 1,2-dioxygenase. b. Gene organizations in the three cassettes. Genes *entX*, *catA*, *entC*, *entBA*, *aroG^{fbr}*, and *aroL* encode EntX, CatA, EntC, EntBA, DAHP synthase, and shikimate kinase, respectively

Materials and methods

Microbial strains and plasmids

Escherichia coli strain JM109 was used for plasmid construction, while *E. coli* strain JM109 (DE3) was used for protein expression and MA production. Strains and primers used in this study are summarized in Tables 1 and 2. Standard cloning and bacterial transformations were performed according to Sambrook and Russell [20]. All restriction enzymes, DNA ligase, and the Prime STAR HS DNA Polymerase were purchased from TaKaRa (Dalian, China). The compatible vectors pETDuet-1, pCDFDuet-1, pRSFDuet-1, and pACYCDuet-1 were kindly provided by Prof. Zhou [29]. Plasmids used in this study are listed in Table 1. The gene *entX* (a hypothetical gene, named in this study, encoding 2,3-dihydroxybenzoate decarboxylase) was amplified from *Klebsiella pneumonia* CICIM B7001 genomic DNA by PCR using primers *entX*-F and *entX*-R. Additionally, the *catA* gene, encoding catechol 1,2-dioxygenase, was amplified from *Pseudomonas putida* KT2440 genomic DNA by PCR using primers *catA*-F and *catA*-R. Then, both *entX* and *catA* were sequentially cloned into vector pACYCDuet-1 using *Bgl*III and *Kpn*I, and *Eco*RI and *Hind*III, respectively. The resulting plasmid was named pACYC-XA. pET-XA was constructed by digesting pACYC-XA and pETDuet-1

Table 1 Strains and plasmids used in this study

Plasmids or strains	Characteristics	Source
<i>Plasmids</i>		
pETDuet-1	T7 expression vector, ColE1 origin, Amp ^r	Wu et al. [29]
pCDFDuet-1	T7 expression vector, CloDF13 origin, Sm ^r	Wu et al. [29]
pRSFDuet-1	T7 expression vector, RSF origin, Kan ^r	Wu et al. [29]
pACYCDuet-1	T7 expression vector, P15A origin, Cm ^r	Wu et al. [29]
pKD136	pBR322 origin, Amp ^r , containing <i>aroF</i> , <i>aroB</i> , and <i>tktA</i>	ATCC
pACYC-XA	pACYCDuet-1 vector containing <i>entX</i> and <i>catA</i>	This study
pET-XA	pETDuet-1 vector containing <i>entX</i> and <i>catA</i>	This study
pRSF-CBA	pRSFduet-1 vector containing <i>entC</i> and <i>entBA</i>	This study
pCDF-GL	pCDFDuet-1 vector containing <i>aroG^{fb}</i> and <i>aroL</i>	This study
<i>Strains</i>		
Jm109	<i>recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, Δ (lac-proAB)/F' [traD36, proAB⁺, lacIq, lacZΔM15]</i>	Our lab
Jm109 (DE3)	<i>recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, Δ (lac-proAB)/F' [traD36, proAB⁺, lacIq, lacZΔM15], dcm (DE3)</i>	Our lab
Jm-1a	Jm109 (DE3) harboring pACYC-XA	This study
Jm-1b	Jm109 (DE3) harboring pET-XA	This study
Jm-2	Jm109 (DE3) harboring pACYC-XA and pRSF-CBA	This study
Jm-3	Jm109 (DE3) harboring pET-XA and pRSF-CBA	This study
Jm-4	Jm109 (DE3) harboring pACYC-XA, pRSF-CBA and pCDF-GL	This study
Jm-5	Jm109 (DE3) harboring pET-XA, pRSF-CBA, and pCDF-GL	This study
Jm-6	Jm109 (DE3) harboring pACYC-XA, pRSF-CBA, and pKD136	This study
Jm-7	Jm109 (DE3) harboring pACYC-XA, pRSF-CBA, pCDF-GL, and pKD136	This study

Table 2 Primers used in this study

Primers cloning genes	Sequence (5'–3')	Digestion site	Primers RT-qPCR	Sequence (5'–3')
<i>entX</i> -F	GAAGATCTCATGCGCGGAAAAATTGCTT	<i>Bgl</i> III	<i>entX</i> -qF	GCCACCCTGGCATTGCAGAA
<i>entX</i> -R	GGGGTACCTTATTTAATGTTAAATACGCGG	<i>Kpn</i> I	<i>entX</i> -qR	GCCATGCTCGTATCTTTAACG
<i>catA</i> -F	GGAATTCGATGACCGTGAAAATTTCCAC	<i>Eco</i> RI	<i>catA</i> -qF	GCACCATCGAAGGCCGTTGTA
<i>catA</i> -R	CCCAAGCTTTTAGCCCTCCTGCAACGCCCG	<i>Hind</i> III	<i>catA</i> -qR	CATCGAACACCTGGCCCTGA
<i>entC</i> -F	GAAGATCTCATGGCTATTCCAAAATTAC	<i>Bgl</i> III	<i>entC</i> -qF	CTGAGGAAGTACAGCAGACCAT
<i>entC</i> -R	GGGGTACCTTATGCCCCAGCGTTGAG	<i>Kpn</i> I	<i>entC</i> -qR	TTTGCTGGAAGGGACTGTCTG
<i>entBA</i> -F	GGAATTCGATGGCTATTCCAAAATTAC	<i>Eco</i> RI	<i>entB</i> -qF	CTTACGCACTGCCGAGTCT
<i>entBA</i> -R	CCCAAGCTTTTATGCCCCAGCGTTGAG	<i>Hind</i> III	<i>entB</i> -qR	TCGCCCCAGAAGCTGACAAA
<i>aroG</i> -F	GAAGATCTCATGAATTATCAGAACGACG	<i>Bgl</i> III	<i>entA</i> -qF	TAGCTGAAACGGAGCGACTG
<i>aroG</i> _m -down	ATTCAGGAATTCACCCGCCGCTG	None	<i>entA</i> -qR	AAAGTCTGCTGCCAGTCTCTC
<i>aroG</i> _m -up	CAGCGGCGGGTGAATTCCTGAATATGATCA	None	<i>aroG</i> -qF	TACTTCTCCTGTCTGCATTG
<i>aroG</i> -R	GGGGTACCTTACCCGCGACGCGCTTT	<i>Kpn</i> I	<i>aroG</i> -qR	CAACCAACAGGCGATCATCA
<i>aroL</i> -F	CGAGCTCGATGACACAACCTCTTTTTTC	<i>Sac</i> I	<i>aroL</i> -qF	CAACCTCTTTTTCTGATCGGGC
<i>aroL</i> -R	CCCAAGCTTTTAAACAATTGATCGTCTG	<i>Hind</i> III	<i>aroL</i> -qR	CAACCACTGATCGGTATCGAC
			16S-qF	CCGCGTGTATGAAGAAGGCC
			16S-qR	TTCTTCTGCGGGTAACGTCA

with *EcoRI* and *KpnI*, followed by ligation of the appropriate fragments.

The *E. coli* K-12 *entC* gene, encoding isochorismate synthase, was isolated by PCR using primers *entC*-F and *entC*-R. *E. coli* K-12 *entBA*, encoding isochorismatase and 2,3-dihydro-2, 3-dihydroxybenzoate dehydrogenase, was isolated by PCR using primers *entBA*-F and *entBA*-R. Then, both *entC* and *entBA* were sequentially cloned into vector pRSFDuet-1 using *BglIII* and *KpnI*, and *SacI* and *HindIII*, respectively. The resulting plasmid was named pRSF-CBA.

E. coli K-12 *aroG^{fbr}*, encoding a feedback inhibition-resistant 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase, was isolated by fusion PCR using primers *aroG*-F, *aroGm*-down, *aroGm*-up, and *aroG*-R. *E. coli* K12 *aroL*, encoding shikimate kinase, was isolated by PCR using primers *aroL*-F and *aroL*-R. Then, both *aroG^{fbr}* and *aroL* were sequentially cloned into vector pCDFDuet-1 using *BglIII* and *KpnI*, and *SacI* and *HindIII*, respectively. The resulting plasmid was named pCDF-GL.

The plasmid pKD136, which expresses *aroF* (encoding 3-deoxy-7-phosphoheptulonate synthase), *aroB* (encoding 3-dehydroquinate synthase), and *tktA* (encoding transketolase), was isolated from strain *E. coli* ATCC 69875, which produces MA [6].

Strains Jm-1a and Jm-1b were constructed by transforming strain *E. coli* JM109 (DE3) with the plasmids pACYC-XA and pET-XA, respectively, to allow expression of *entX* and *catA* upon induction with isopropyl β -D-1-thiogalactopyranoside (IPTG). Similarly, strains Jm-2 and Jm-3 were constructed by transforming Jm-1a and Jm-1b with the plasmid pRSF-CBA, which expresses *entC* and *entBA* upon induction with IPTG. Strains Jm-4 and Jm-5 were constructed by transforming strain Jm-2 and Jm-3 with the plasmid pCDF-GL, which expresses *aroG^{fbr}* and *aroL* upon induction with IPTG. Finally, strains Jm-6 and Jm-7 were created by transforming Jm-2 and Jm-4 with the plasmid pKD136, which expresses *aroF*, *aroB*, and *aroL* upon induction with IPTG.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Overnight culture of *E. coli* Jm-1a were inoculated into 30 mL LB medium and grew at 37 °C till OD₆₆₀ reached 0.6, then induced with 0.20 mM IPTG. After 4 h induction, cells were harvested by centrifugation for 2 min at 12,000×g and suspended to 5 mL with 50 mM sodium phosphate buffer (pH 7.0), and treated by the ultrasonicator. SDS-PAGE was carried out on 12 % (w/v) polyacrylamide slab gels, which were stained with Coomassie brilliant blue R-250.

Reverse transcription-quantitative PCR (RT-qPCR)

Strains were grown in minimal medium. The cells were harvested by centrifugation for 2 min at 12,000×g and 4 °C and washed once with distilled water. The RNA was isolated using an RNAisoPlus kit (TaKaRa, Dalian, China). 1,000 ng of total RNA was treated with a iScript™ cDNA synthesis kit (Bio-Rad, Shanghai, China). The obtained cDNA was subjected to quantitative PCR (qPCR) using the LightCycler 2.0 system (Roche, Basel, Switzerland) and SYBR Premix Ex Taq (TaKaRa, Dalian, China). The 16S rRNA gene was set as reference for normalization. Primers used in RT-qPCR were listed in Table 2.

Culture conditions

LB medium (composed of 5 g/L yeast extract, 10 g/L peptone, and 10 g/L NaCl) and TB medium (composed of 24 g/L yeast extract, 12 g/L peptone, 40 g/L glycerol, 17 mM KH₂PO₄, and 72 mM K₂HPO₄) were used for inoculations and cell propagation. The synthetic minimal medium as described in [16] was used for MA production. Various combinations of ampicillin (100 mg/L), kanamycin (40 mg/L), chloramphenicol (20 mg/L), and streptomycin (40 mg/L) were added to cultures of plasmid-bearing *E. coli* strains. All strains were cultivated with 220 rpm orbital shaking. Strains were stored at –70 °C in 15 % glycerol.

Feeding experiments

Feeding experiments were conducted to examine the enzyme efficiency of EntX and CatA, which converted 2,3-DHB into MA. A single colony of strain Jm-1a, harboring pACYC-XA, was inoculated into 5 mL LB medium containing 20 μ g/mL chloramphenicol and grown overnight at 37 °C. Two percent (v/v) of the overnight cultures were inoculated into 50 mL of TB medium containing 20 μ g/mL chloramphenicol. The cultures were grown at 37 °C until the OD₆₆₀ reached 0.6 and then they were induced at 30 °C with 0.20 mM IPTG. After 4 h, cells were harvested and re-suspended in 50 mL minimal medium. The cultures were supplemented with 2 mM 2,3-DHB and incubated at 30 °C with shaking, a 50 μ L of 2 M 2,3-DHB solution was added into the cultures per hour. Samples were taken at 2, 4, 6, and 8 h. MA concentrations were analyzed by high-performance liquid chromatography (HPLC).

Microbial production of MA from glucose

Single colonies of the MA-producing strains were inoculated into 5 mL of LB medium containing appropriate antibiotics and grown overnight at 37 °C. Four percent (v/v) of

the overnight cultures were inoculated into 50 mL minimal medium containing appropriate antibiotics and cultivated at 37 °C with shaking at 220 rpm in baffled shaker flasks. When the OD₆₆₀ values of the cultures reached 0.6, IPTG was added to a final concentration of 0.20 mM to induce MA production. Samples were taken every 12 h. OD₆₆₀ values were measured, and the products were analyzed by HPLC. All liquid cultivations were conducted in triplicate, at least.

HPLC analysis

Muconic acid (MA) (Sigma Aldrich, Shanghai, China) was used as the standard. Both the standard and samples were analyzed using a HPLC system (Waters, Milford, MA, USA) with a reverse-phase Agilent C18 column and a refractive index detector (Waters). A mobile phase of 0.33 mM H₂SO₄ solution at a flow rate 0.5 mL/min was applied to the column. The column temperature was set to 35 °C. Quantification of MA was based on the peak areas at 260 nm absorbance.

Results

Assembly of the MA biosynthetic pathway

In this study, a MA biosynthesis pathway using precursors of enterobactin was presented, which included the upstream pathway from glucose to shikimate to chorismate, the midstream pathway from chorismate to 2,3-DHB, and the downstream pathway from 2,3-DHB to MA (Fig. 1a). In this pathway, 2,3-DHB was regarded as the precursor of catechol that was subsequently transformed to MA, even though 2,3-DHB was the intermediate of enterobactin in *E. coli*, and chorismate was converted into 2,3-DHB by the enzymes EntC and EntBA. Considering there is no 2,3-DHB decarboxylase in *E. coli*, which converts 2,3-DHB into catechol, we assembled the downstream pathway of MA by co-expressing the heterologous genes *entX* and *catA* in *E. coli*. To accumulate 2,3-DHB from the chorismate pool, the partial enterobactin pathway (the midstream pathway) was reinforced by overexpressing *entC* and *entBA*. Moreover, to increase the carbon flow in the upstream pathway, a phenylalanine feedback inhibition-resistant gene, *aroG^{fbr}* (which has a D146N mutation), encoding 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase, was cloned and co-expressed with shikimate kinase gene (*aroL*). The above genes, organized into three cassettes, are shown in Fig. 1b. Therefore, the MA biosynthetic pathway from glucose was constructed by relying upon the degradation of 2,3-DHB via EntX and CatA.

Bioconversion of 2,3-DHB to MA in *E. coli*

The downstream pathway from 2,3-DHB to MA was critical in the aforementioned MA biosynthetic pathway because *E. coli* lacks EntX activity, which converts 2,3-DHB into catechol. Earlier reports have shown that some fungi, such as *Aspergillus niger* and *Trichosporon cutaneum*, have EntX activity [2, 21]. Nevertheless, eukaryotic protein production in *E. coli* is limited because of problems associated with transcription, translation, and proper folding of proteins [7]. According to the KEGG database, the *entX* gene product of *Klebsiella pneumoniae sub sp. pneumoniae* NTUH-K2044 was defined as putative amidohydrolase, which was homologue 2,3-dihydroxybenzoate decarboxylase. A new literature of Yan group amplified *entX* from *K. pneumoniae*, and verified the protein, which 35.9 % similarity with that from *A. niger*, showed decarboxylase activity [24]. In addition, catechol 1,2-dioxygenase from *Pseudomonas putida* strain KT2440 has a high CatA activity described by Sun et al. [13]. Therefore, to assemble the downstream pathway for MA production, we co-expressed the heterologous genes *entX* and *catA* in *E. coli*, the genes *entX* (999 bp) from *K. pneumoniae* CICIM B7001 and *catA* (936 bp) from *P. putida* KT2440 were cloned into the double T7 expression vector pACYCDuet-1, resulting in the expression vector pACYC-XA (Fig. 2a), and thereby generating strain Jm-1a.

SDS-PAGE analysis of supernatant protein lysates from strain Jm-1a showed that the two recombinant proteins (EntX and CatA) were expressed with molecular weight of 35.7 and 36.1 kDa, respectively (Fig. 2b), which were of the expected molecular weight similar with that reported by Sun et al. (EntX, 38 kDa band) [24] and Guzik et al. (CatA, 33.1 kDa band) [10]. Because 2,3-DHB was found toxic to the growth of *E. coli* cells in our previous work (see Supporting Information), a substrate feeding strategy was used to evaluate its potential in the bioconversion of 2,3-DHB to MA. Strain Jm-1a was pre-cultivated and transferred to the minimal medium containing 2,3-DHB. As seen in Fig. 3, by the end of 2, 4, 6, and 8 h incubation, the concentrations of MA reached 596.55, 1,173.92, 1,617.79, and 1,919.52 mg/L, respectively. The conversion rate of 2,3-DHB to MA reached about 100 % after 2 h and was 83.3 % after 8 h, while there is no MA detected by *E. coli* JM109 (DE3) harboring pACYCDuet-1, demonstrating the enzymatic efficiency of EntX and CatA with this plasmid. Accumulation of MA by reinforcing the enterobactin pathway and copy numbers of the XA cassette. Six genes, *entA* to *-F*, encode enzymes for the biosynthesis of enterobactin from the precursor chorismate. In the initial stage of enterobactin biosynthesis, the enzymes EntC, EntB, and EntA converted chorismate to 2,3-DHB. The remaining enzymes,

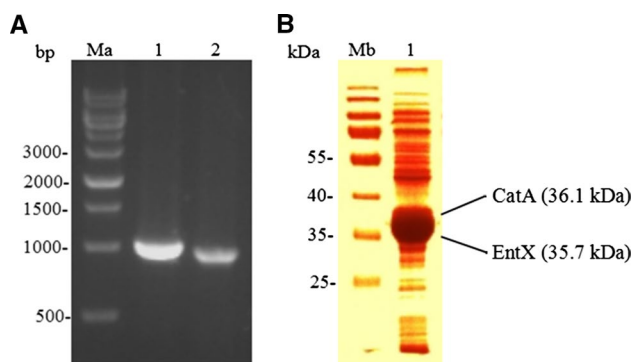


Fig. 2 Identification of cloning and co-expressing EntX and CatA in *E. coli*. **a** Agarose gel electrophoresis of *entX* and *catA* amplified from pACYC-XA. Ma, 1 kb Ladder; Lane 1 *entX* gene of 999 bp, Lane 2 *catA* gene of 936 bp. **b** SDS-PAGE analysis of supernatant protein lysates. Mb, PageRuler Prestained Protein Ladder. Lane 1 recombinant EntX and CatA proteins, 35.7 and 36.1 kDa, respectively

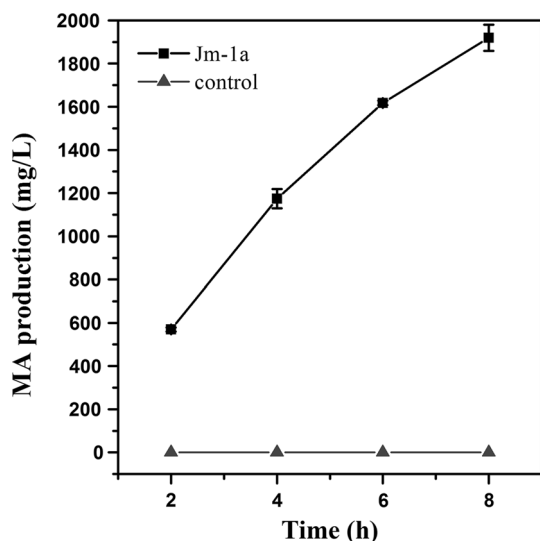


Fig. 3 Fed-batch experiment of the conversion of 2,3-DHB to MA by strain Jm-1a (filled square) and control *E. coli* JM109 (DE3) harboring pACYCDuet-1 (filled triangle). The data were generated from three independent experiments

Ent-D to -F, together with the bifunctional enzyme EntB, convert 2,3-DHB to enterobactin [15]. In *E. coli*, *entC*, *entE*, *entB*, and *entA* are adjacent on chromosome, according to the KEGG database. Besides, EntC is more active than its isozyme MenF [14]. Thus, we cloned *entC*, but not *menF*, and *entBA* into pRSFDuet-1, for the purpose of accumulating more 2,3-DHB.

As seen in Fig. 4, the titer of MA produced by strain Jm-1a, which only harbors pACYC-XA, was 1.25 mg/L. To increase flux through the enterobactin pathway from the chorismate pool, both EntC and EntBA were

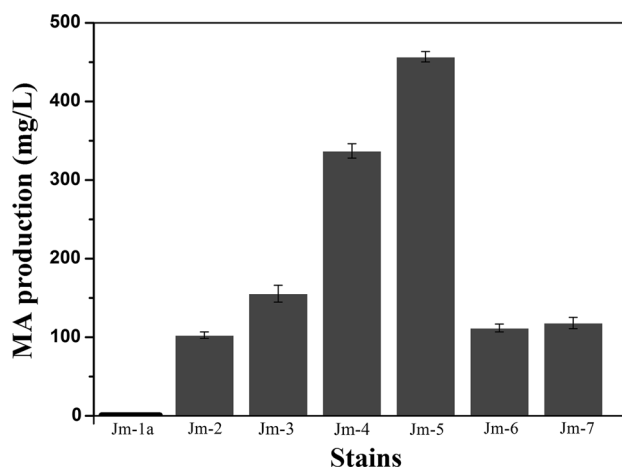


Fig. 4 MA production from glucose by all recombinant strains used in this study. Strains are described in more detail in Table 1. All values were determined by HPLC after at least 72 h of batch flask cultures. Standard deviations are based on triplicate experiments

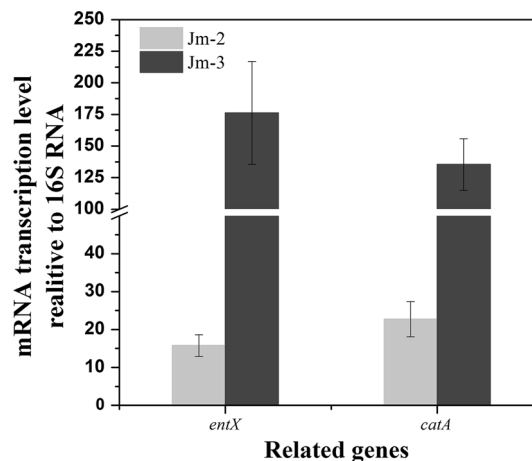


Fig. 5 The transcription levels of *entX* and *catA* genes relative to 16S RNA in strains Jm-2 (gray) and Jm-3 (black). The 16S rRNA was set as 1.0. Standard deviations are based on triplicate experiments

overexpressed by strain Jm-2, which resulted in a MA titer of 102.71 mg/L. Considering the aromatic compound 2,3-DHB, which is produced by EntCBA, is potentially toxic to *E. coli* [3], we further altered the copy number of the XA cassette by subcloning *entX* and *catA* from a low copy plasmid, pACYCDuet-1, to a relatively high copy number plasmid, pETDuet-1, thereby yielding strain Jm-3. As a result, MA production increased to 155.47 mg/L. The transcript levels of *entX* and *catA* in strain Jm-3 was much higher than those in Jm-2 (Fig. 5), which demonstrated that increase of copy numbers of XA cassette could improve the mRNA transcript levels of *entX* and *catA*, thereby, promote production of MA.

Regulation of the shikimate pathway

In *E. coli*, the genes *aroF*, *aroG*, and *aroH* encode three 3-Deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase isozymes that are sensitive to tyrosine, phenylalanine, and tryptophan, respectively. Carbon flow through the shikimate pathway is regulated initially through repression and feedback inhibition by these three amino acids. In wild-type cells grown in minimal medium, Phe-, Tyr-, and Trp-sensitive DAHP synthases constitute about 80, 20, and 1 % of the total DAHP synthase activity, respectively [12]. Therefore, we overexpressed *aroG^{fbr}* (which has a D146N mutation) in pCDFDuet-1 to relieve phenylalanine feedback repression. Additionally, shikimate kinase (*aroL*), which was reported to stimulate the shikimate pathway [4], was co-expressed in pCDFDuet-1 along with *aroG^{fbr}*. Strains Jm-4 and Jm-5 were obtained, respectively. Furthermore, as the plasmid pKD136 from the strain ATCC 69875, which was reported to improve MA production [6], carries the genes encoding transketolase (*tktA*), DAHP synthase (*aroF*), and DHQ synthase (*aroB*), we also chose pKD136 as a candidate to regulate the shikimate pathway. We co-expressed pKD136 with the plasmids pRSF-CBA and pACYC-XA, thereby resulting in strain Jm-6.

Although pKD136 intensified the shikimate pathway, it failed to show a notable effect on MA accumulation in the presence of pACYC-XA and pRSF-CBA, which resulted in a MA yield by Jm-6 of only 111.75 mg/L. A possible reason for this low yield was that *aroF* in pKD136 does not relieve tyrosine feedback inhibition and relies upon weak, autologous promoters, and *aroF* comprised only about 20 % of the total DAHP synthase activity, while *aroG* comprised 80 % [12], which may have a much more poor effect on regulating shikimate pathway than pCDF-GL. Additionally, even when pCDF-GL was transformed into Jm-6, the titer of MA by Jm-7 was only 118.13 mg/L, which was not a significant improvement. The reason may be that pKD136 is a high copy number plasmid with a pBR322 origin, resulting in a metabolic burden with other 3 plasmids in Jm-7 from the expression of plasmid-borne genes and the replication of the plasmids. Strains Jm-4 and Jm-5, which overexpressed *aroG^{fbr}* and *aroL*, showed a remarkable increase in MA production. Strain Jm-4 yielded up to 337.00 mg/L of MA, a 328 % increase compared to strain Jm-2, while the titer of MA in Jm-5 reached 456.77 mg/L, which was a 249 % increase by compared to Jm-3, and a 365-fold increase over Jm-1a (Fig. 4).

Relieving the metabolic burden of three plasmids in *E. coli* Jm-5

Because of expression of plasmid-borne resistance and replication of the plasmids, the three plasmids in *E. coli*

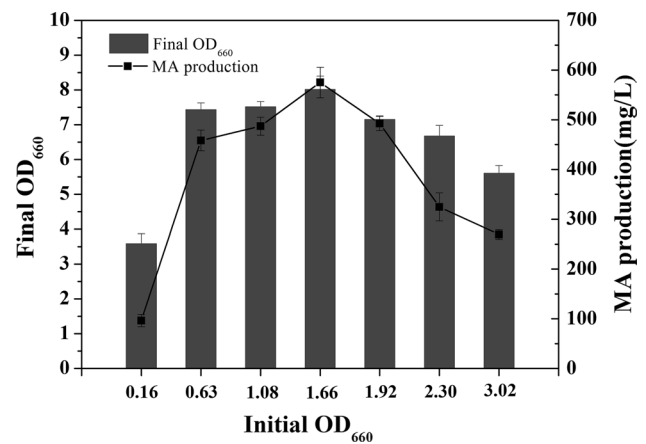


Fig. 6 Effect of IPTG induction at different cells growth stages (OD₆₆₀ values) on MA production titers and final OD₆₆₀ of strain Jm-5. MA (filled square), OD₆₆₀ (filled column)

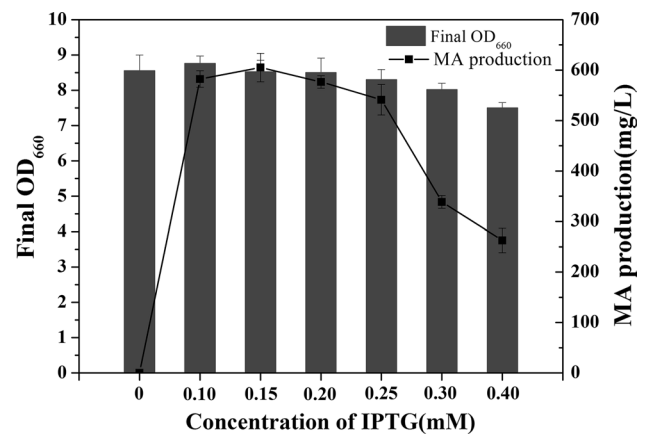


Fig. 7 Effect of IPTG induction at different IPTG concentration on MA production titers and final OD₆₆₀ of strain Jm-5. MA (filled square), OD₆₆₀ (filled column)

Jm-5 could impose a metabolic burden on the host strain. One solution to alleviate the metabolic burden was to delay IPTG induction and change the concentration of IPTG [1, 11, 29]. Strain Jm-5 were cultured and induced by 0.20 mM IPTG at an OD₆₆₀ value of 0.16, 0.63, 1.08, 1.66, 1.92, 2.30, or 3.02, respectively. As shown in Fig. 6, when IPTG induction was delayed, the final OD₆₆₀ was increasing, but the production titer increased until an OD₆₆₀ of 1.66. The addition of IPTG at an OD₆₆₀ of 1.66 yielded the highest production titer (575.02 mg/L), and therefore, inductions in subsequent experiments were performed at an OD₆₆₀ of 1.6.

Based on the result of delaying IPTG induction, strains were induced at an OD₆₆₀ value of 1.6 by different concentration of IPTG of 0, 0.10, 0.15, 0.20, 0.25, 0.30 or 0.40 mM. Figure 7 showed that low concentration of IPTG

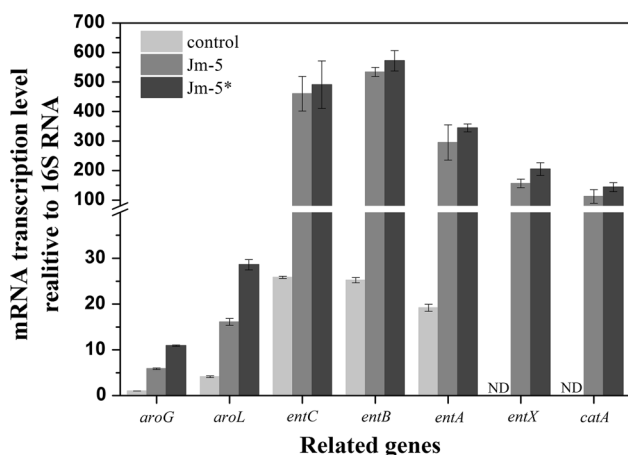


Fig. 8 The transcription levels of *aroG*, *aroL*, *entC*, *entB*, *entA*, *entX* and *catA* relative to 16S RNA in strains control *E. coli* JM109 (DE3) harboring pETDuet-1, pRSFDuet-1, and pCDFDuet-1 (Light gray) and Jm-5. Jm-5 cells were induced at an OD₆₆₀ value of 0.6 by 0.20 mM IPTG (dark gray), that of Jm-5* were at an OD₆₆₀ value of 1.6 by 0.15 mM IPTG. The 16S rRNA was set as 1.0 (black). Standard deviations are based on triplicate experiments

was beneficial to MA production. The highest production titer of MA reached 605.18 mg/L by 0.15 mM IPTG.

Furthermore, the transcript levels of all expressed genes in the recombination strain Jm-5 were investigated. It can be seen from Fig. 8, comparing to control strain (*E. coli* JM109 (DE3) harboring pETDuet-1, pRSFDuet-1, and pCDFDuet-1) the transcript levels of overexpressed *entC*, *entB*, and *entA* in enterobactin biosynthesis pathway were increased more than 15 times, while that of *aroG*^{fbt} and *aroL* in the shikimate pathway raised about five times. After optimization of IPTG induction, the transcript levels of *aroG*^{fbt} and *aroL*, which related the regulating shikimate pathway improved about 50 %, and the genes encoding chorismate to MA were expressed more than 5–15 %, indicating the metabolic burden was lightened.

Discussion

Muconic acid (MA) is a promising bulk chemical due to its extensive industrial applications [30]. In this study, we assembled a synthetic, composite pathway to produce MA by comprising two distinct enzymes, EntX and CatA. We also used several strategies to improve MA production. First, we strengthened the enterobactin pathway by overexpressing EntC and EntBA. Second, the copy numbers of the XA cassette were regulated. Then, the upstream pathway was intensified by overexpressing *aroG*^{fbt} and *aroL*.

As can be observed, this pathway was based on the shikimate pathway, which leads to the biosynthesis of aromatic compounds that are essential in *E. coli*. Prior to

this study, three artificial pathways derived from the shikimate pathway were reported for the microbial production of MA. Draths and Frost first reported the creation of metabolism of glucose to MA by introducing three heterologous enzymes, namely AroZ, AroY, and CatA, respectively, as well as disrupting shikimate dehydrogenase gene to accumulate more 3-dehydroshikimate. 16.8 mM of MA was produced in shake flask by *E. coli* AB2834/pKD136/pKD8.243A/pKD8.292 [6]. They further used this pathway to construct *E. coli* WN1/pWN2.248, which synthesized 36.8 g/L of MA after 48 h by culturing under fed-batch fermentations [18]. However, this blocked the shikimate pathway, which had the effect of turning the strain into an auxotroph for aromatic amino acids (phenylalanine, tyrosine, and tryptophan) and aromatic vitamins or vitamin-like intermediates (4-hydroxybenzoate, 4-aminobenzoate, and 2,3-dihydroxybenzoate) [6, 18]. Aromatic amino acids are relatively expensive, and their requirement would result in a large increase in the cost of MA production. Thus, there is also a need for a process that does not require these expensive nutrients to be added to the growth medium. Two studies used *Saccharomyces cerevisiae* to produce MA from glucose by the same pathway. However, the yields were 140 and 1.5 mg/L, respectively [5, 27]. In another MA producing pathway that shunted tryptophan biosynthesis from anthranilate, reported by the Yan group, there is a rate-limiting transamination step that converts chorismate into anthranilate. This reaction requires glutamine, which limited the efficiency of the whole pathway [23]. In the third pathway, MA was synthesized via salicylic acid. The yield of MA reached 1.5 g/L [13]. Although the formation of catechol from salicylic acid requires the coenzyme NADH.

Similarly to our work of MA production approach, the Yan group is the first to achieve the de novo production of 2,3-DHB and the conversion of 2,3-DHB to MA with plasmids pCS-entCBA-APTA and pZE-kpBDC-CDO, they assembled the two partial pathways in *E. coli* strain BW25113 Δ *entE* for microbial production of MA from glucose, and MA titer reached up to 480 mg/L at fermentation temperatures of 37 °C [24]. Here, we imported three cassette (pET-XA, pRSF-CBA, and pCDF-GL) to assemble strain Jm-5. Although the transcript levels of the expressed genes were not balanced (Fig. 5), the highest titer of MA reached 605.18 mg/L after optimization of IPTG induction. The transcriptional levels of the midstream pathway from chorismate to 2,3-DHB, and the downstream pathway from 2,3-DHB to MA was higher than the upstream pathway from glucose to shikimate to chorismate (Fig. 8), suggesting the upstream pathway may be continued to improve for production of MA.

Further work can also be performed on this pathway to enhance MA production. For example, AhpC, an alkyl hydroperoxide reductase, may participate in 2,3-DHB

synthesis by increasing the availability of chorismate or increasing the efficiency with which EntC uses chorismate [15]. Thus, AhpC could be a potential key enzyme to improve MA production from this pathway. Gerstle et al. [9] reported that, RybA (one of the orphan sRNAs), downregulates aromatic amino acid biosynthesis under peroxide stress, and might increase the availability for other downstream products, such as products in the enterobactin pathway.

In summary, we constructed a MA biosynthetic approach by making use of the precursors of enterobactin. The final production of MA reached 605.18 mg/L, indicating that this biosynthetic pathway is promising for further modification and use in industrial MA production.

Acknowledgments This work was supported by a Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions, the 111 Project (No. 111-2-06), and the Jiangsu province “Collaborative Innovation Center for Advanced Industrial Fermentation” industry development program.

References

- Alonso-Gutierrez J, Chan R, Bath TS, Adams PD, Keasling JD, Petzold CJ, Lee TS (2013) Metabolic engineering of *Escherichia coli* for limonene and perillyl alcohol production. *Metab Eng* 19:33–41
- Anderson JJ, Dagley S (1981) Catabolism of tryptophan, anthranilate, and 2, 3-dihydroxybenzoate in *Trichosporon cutaneum*. *J Bacteriol* 146:291–297
- Bartsch M, Bednarek P, Vivancos PD, Schneider B, von Roepenack-Lahaye E, Foyer CH, Kombrink E, Scheel D, Parker JE (2010) Accumulation of isochorismate-derived 2,3-dihydroxybenzoic 3-O-beta-D-xyloside in arabidopsis resistance to pathogens and ageing of leaves. *J Biol Chem* 285:25654–25665
- Bujnicki RP, Bongaerts JJ, Raeven LJ, Sprenger G, Kuhm A, Takors R Improved biosynthetic production of 2, 3-trans-CHD. EP 1734130 A1
- Curran KA, Leavitt JM, Karim AS, Alper HS (2013) Metabolic engineering of muconic acid production in *Saccharomyces cerevisiae*. *Metab Eng* 15:55–66
- Draths KM, Frost JW (1994) Environmentally compatible synthesis of adipic acid from D-glucose. *J Am Chem Soc* 116:399–400
- Finkelstein J, Antony E, Hingorani MM, O'Donnell M (2003) Overproduction and analysis of eukaryotic multiprotein complexes in *Escherichia coli* using a dual-vector strategy. *Anal Biochem* 319:78–87
- Franke D, Lorbach V, Esser S, Dose C, Sprenger GA, Halfar M, Thömmes J, Müller R, Takors R, Müller M (2003) (S, S)-2,3-dihydroxy-2,3-dihydrobenzoic acid: microbial access with engineered cells of *Escherichia coli* and application as starting material in natural-product synthesis. *Chem Eur J* 9:4188–4196
- Gerstle K, Klätschke K, Hahn U, Piganeau N (2012) The small RNA RybA regulates key-genes in the biosynthesis of aromatic amino acids under peroxide stress in *E. coli*. *RNA Biol* 9:458–468
- Guzik U, Greń I, Hupert-Kocurek K, Wojcieszynska D (2011) Catechol 1, 2-dioxygenase from the new aromatic compounds-degrading *Pseudomonas putida* strain N6. *Int Biodeter Biodegr* 65(3):504–512
- Jones KL, Kim SW, Keasling JD (2000) Low-copy plasmids can perform as well as or better than high-copy plasmids for metabolic engineering of bacteria. *Metab Eng* 2(4):328–338
- Kikuchi Y, Tsujimoto K, Kurahashi O (1997) Mutational analysis of the feedback sites of phenylalanine-sensitive 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase of *Escherichia coli*. *Appl Environ Microbiol* 63:761–762
- Lin Y, Sun X, Yuan Q, Yan Y (2014) Extending shikimate pathway for the production of muconic acid and its precursor salicylic acid in *Escherichia coli*. *Metab Eng* 23:62–69
- Lin Y, Shen X, Yuan Q, Yan Y (2013) Microbial biosynthesis of the anticoagulant precursor 4-hydroxycoumarin. *Nat Commun* 4:2603. doi:10.1038/ncomms3603
- Ma L, Payne SM (2012) AhpC is required for optimal production of enterobactin by *Escherichia coli*. *J Bacteriol* 194:6748–6757
- Neidhardt FC, Bloch PL, Smith DF (1974) Culture medium for *Enterobacteria*. *J Bacteriol* 119:736–747
- Neidle EL, Hartnett C, Bonitz S, Ornston LN (1988) DNA sequence of the *Acinetobacter calcoaceticus* catechol 1, 2-dioxygenase I structural gene catA: evidence for evolutionary divergence of intradiol dioxygenases by acquisition of DNA sequence repetitions. *J Bacteriol* 170:4874–4880
- Niu W, Draths KM, Frost JW (2002) Benzene-free synthesis of adipic acid. *Biotechnol Prog* 18:201–211
- Raymond KN, Dertz EA, Kim SS (2003) *Enterobactin*: an archetype for microbial iron transport. *Proc Natl Acad Sci USA* 100:3584–3588
- Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York
- Santha R, Savithri HS, Rao NA, Vaidyanathan CS (1995) 2,3-Dihydroxybenzoic acid decarboxylase from *Aspergillus niger*: a novel decarboxylase. *Eur J Biochem* 230:104–110
- Sikora AL, Wilson DJ, Aldrich CC, Blanchard JS (2011) Kinetic and inhibition studies of dihydroxybenzoate-AMP ligase (EntE) from *Escherichia coli*. *Biochemistry* 49:3648–3657
- Sun X, Lin Y, Huang Q, Yuan Q, Yan Y (2013) A novel muconic acid biosynthesis approach by shunting tryptophan biosynthesis via anthranilate. *Appl Environ Microbiol* 79:4024–4030
- Sun X, Lin Y, Yuan Q, Yan Y (2014) Biological production of muconic acid via a prokaryotic 2,3-dihydroxybenzoic acid decarboxylase. *Chem Sus Chem*. doi:10.1002/cssc.201402092
- van Duuren JB, Wijte D, LePrince A, Karge B, Puchałka J, Wery J, Dos Santos VA, Eggink G, Mars AE (2011) Generation of a catR deficient mutant of *P. putida* KT2440 that produces cis, cis-muconate from benzoate at high rate and yield. *J Biotechnol* 156:163–172
- van Duuren JB, Wittmann C (2014) Bioprocessing of renewable resources to commodity bioproducts. In: Bisaria VS, Kondo A (eds) First and second generation production of bio-adipic acid, 1st edn. Wiley, New York, pp 519–540
- Weber C, Brückner C, Weinreb S, Lehr C, Essl C, Boles E (2012) Biosynthesis of cis, cis-muconic acid and its aromatic precursors, catechol and protocatechuic acid, from renewable feedstocks by *Saccharomyces cerevisiae*. *Appl Environ Microbiol* 78:8421–8430
- Wu CM, Lee TH, Lee SN, Lee YA, Wu JY (2004) Microbial synthesis of cis, cis-muconic acid by *Shingobacterium sp.* GCG generated from effluent of a styrene monomer (SM) production plant. *Enzyme Microb Technol* 35:598–604
- Wu J, Du G, Zhou J, Chen J (2013) Metabolic engineering of *Escherichia coli* for (2S)-pinocebrin production from glucose by a modular metabolic strategy. *Metab Eng* 16:48–55
- Xie NZ, Liang H, Huang RB, Xu P (2014) Biotechnological production of muconic acid: current status and future prospects. *Biotechnol Adv* 32:615–622