Engineering *Escherichia coli* for production of 4-hydroxymandelic acid using glucose–xylose mixture

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**Abstract**

**Background:** 4-Hydroxymandelic acid (4-HMA) is a valuable aromatic fine chemical and widely used for production of pharmaceuticals and food additives. 4-HMA is conventionally synthesized by chemical condensation of glyoxylic acid with excessive phenol, and the process is environmentally unfriendly. Microbial cell factory would be an attractive approach for 4-HMA production from renewable and sustainable resources.

**Results:** In this study, a biosynthetic pathway for 4-HMA production was constructed by heterologously expressing the fully synthetic 4-hydroxymandelic acid synthase (*shmaS*) in our l-tyrosine-overproducing *Escherichia coli* BKT5. The expression level of *shmaS* was optimized to improve 4-HMA production by fine tuning of four promoters of different strength combined with three plasmids of different copy number. Furthermore, two genes *aspC* and *tyrB* in the competitive pathway were deleted to block the formation of byproduct to enhance 4-HMA biosynthesis. The final engineered *E. coli* strain HMA15 utilized glucose and xylose simultaneously and produced 15.8 g/L of 4-HMA by fed-batch fermentation in 60 h.

**Conclusions:** Metabolically engineered *E. coli* strain for 4-HMA production was designed and constructed, and efficiently co-fermented glucose and xylose, the major components in the hydrolysate mixture of agricultural biomass. Our research provided a promising biomanufacturing route to produce 4-HMA from lignocellulosic biomass.

**Keywords:** 4-Hydroxymandelic acid, *Escherichia coli*, Co-utilization of glucose and xylose, Synthetic biology, Metabolic engineering

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**Background**

4-Hydroxymandelic acid (4-HMA) is widely used in production of aromatic drugs and flavors. It is employed for the preparation of 4-hydroxyphenylacetic acid, which is the synthetic precursor of selective β1-receptor antagonist drug atenolol [1]. 4-HMA can conjugate cytotoxic drug and enzyme substrate, and such a 4-HMA based adaptor system showed promising application in the targeting drug delivery system [2]. Moreover, 4-HMA derivatives, polyhydroxylated mandelic acid amides, were reported with higher radical scavenging activities than that of antioxidants α-tocopherol and butylated hydroxytoluene [3]. Recently, the recombinant *Escherichia coli* converted 3-ethoxy-4-HMA to ethyl vanillin, a widely used flavor in foods, beverages and cosmetics [4].

4-HMA is currently synthesized by condensation of glyoxylic acid with excessive phenol via chemical approach [5], which is facing many challenges. First, feedstock like phenol origins from fossil energy as coal, which is unsustainable. Second, condensation reaction with sodium or potassium hydroxide is extremely environmentally unfriendly, and causes pollution in its production and refinery. Third, subsequent acidification of the reaction and extraction of excessive phenol make downstream process more complicated. At last, such a process produces a mixture including byproduct orthoisomer 2-hydroxymandelic acid, which is very difficult to separate from 4-HMA. Biotechnological approach would
be an attractive alternative for 4-HMA production from renewable and sustainable bioresources. 4-Hydroxyphenylglycine, 4-HMA derivative, is a natural building block for several nonribosomal peptide antibiotics like chloroeremomycin [6] and vancomycin [7]. A gene involved in the biosynthesis of 4-hydroxyphenylglycine was identified to encode 4-hydroxymandelate synthase (HmaS), which catalyzes the conversion of 4-hydroxyphenylpyruvate (4-HPP) to 4-HMA via the oxidative decarboxylation reaction and adds hydroxyl to the side chain rather than the aromatic ring. Natural phenolic products are derived from aromatic amino acid biosynthetic pathway [8], and 4-HPP is the direct precursor of L-tyrosine. In the last few years, notable advances have been achieved in microbial production of 4-HPP derivatives, such as caffeic acid, L-dopa, 4-hydroxystyrene, phenol, salvianic acid A and flavonoid [9–14].

We previously constructed an E. coli strain BKT5 capable of overproducing L-tyrosine by deleting five genes (ΔptsG, ΔpykA, ΔpykF, ΔtyrR and ΔpheA), and overexpressing aroG⁻br-tyrA⁻br-aroe and gld-tktA-ppsA [13]. In this study, we used BKT5 as the starting strain, and the gene shmaS of Amycolatopsis orientalis with optimized codons was introduced for 4-HMA production (Fig. 1). By using the strategy of synthetic biology [15] to improve 4-HMA biosynthesis, we finely tuned the expression of synthetic shmaS gene by combining promoters (as gap, lacUV5, trc and T7) of various strengths and vectors of various replication origins (as p15A, CloDF13, RSF1030). To further enhance metabolic flux to 4-HMA, we manipulated the genome of E. coli BAK5 by deletion of tyrB and aspC, which would block the competitive pathway from 4-HPP to formation of L-tyrosine. The final engineered E. coli strain HMA15 was employed to produce 4-HMA using glucose and xylose by fed-batch fermentation. To our knowledge, this is the first report describing engineered E. coli for producing 4-HMA using glucose–xylose mixture.

Methods
Strains, plasmids, primers and reagents

Strains, plasmids and primers used in this study are listed in Tables 1, 2 and 3, respectively. E. coli BW25113 derivatives were used to construct 4-HMA producing strains. All chemical reagents were purchased from Sigma Aldrich (Beijing, China). Plasmids were isolated using the Tianprep Mini plasmid Kit purchased from Tiangen (Beijing, China). DNA Gel Extraction Kit from Tiangen

![Fig. 1](image_url)

The construction process of recombinant expression vectors and engineered pathway for 4-HMA production. PPP pentose phosphate pathway, 6-P-G 6-phosphate-glucose, 6-P-F 6-phosphate-fructose, PEP phosphoenolpyruvate, E4P erythrose-4-phosphate, DAHP 3-deoxy-D-arabino-heptulosonate-7-phosphate, DHS 3-dehydroshikimic acid, SHK shikimic acid, CHA chorismic acid, 4-HPP 4-hydroxyphenylpyruvic acid, L-Phe l-phenylalanine, L-Tyr l-tyrosine; 4-HMA 4-hydroxymandelic acid, TyrR-tyr TyrR-tyrosine DNA-binding transcriptional repressor
was used to isolate DNA fragments from agarose gels. All PCR fragments were validated via DNA sequencing provided by BGI. Oligonucleotides and synthetic long DNA fragments were ordered from GenScript (Nanjing, China). DNA polymerase Fastpfu and Taq for PCR was purchased from TransGen Biotech (Beijing, China). All restriction enzymes and rapid DNA ligase were purchased from Thermo Scientific (Beijing, China). 

**Codon optimization and assembly of synthetic hmaS gene**

Starting from amino acid sequence of HmaS (GenBank ID CAA11761.1) of *Amycolatopsis orientalis*, we optimized the codon usage for *hmaS* heterologous expression in *E. coli* using a design procedure JCat, and Shine–Dalgarno-like ribosomal pause sequences and selected restriction enzyme recognition sites were also removed. Then, the full DNA sequence of *hmaS* was divided into twenty-six oligonucleotides for each one about 60 bp long with 20 bp region homologous to its adjacent oligonucleotides. The full length of the *hmaS* gene was divided into two fragments and assembled by two-step overlapping PCR. The 5′-terminal and 3′-terminal fragments of the *hmaS* gene optimized were assembled by first-step overlapping PCR with the first fourteen and the latter fourteen oligonucleotides, respectively. PCR reaction system contained fourteen oligonucleotides each with 0.2 μM, 0.2 mM dNTPs, 2.5 units TransFast pfu DNA polymerase, 1 × TransFast pfu buffer in the final volume of 50 μL, and 30 cycles of 95 °C for 5 s, 55 °C for 15 s, 72 °C for 15 s were carried out for PCR amplification program. Then the full-length of the *hmaS* gene was amplified by the second-step overlapping PCR using these two overlapped fragments with the first and last oligonucleotide primers. Synthetic *hmaS* gene determined to be correct by sequencing was designated as *shmaS*, and cloned into vector pEASYBlunt, resulting pEBM. The *shmaS* gene was cloned into expression vector pET28a under control of T7 promoter, resulting pHMA.

**Combinatorial construction of shmaS-regulated expression vectors**

In order to screen superior promoter-copy number combinations for 4-HMA producing, four promoters Pgap, Ptrc, PlacUV5, and P7 with various expression strength and three types of vectors with various copy number (p15A, CloDF13, and RSF1030 as replicon) were selected, and eleven expression vectors were constructed as shown in the table below:

**Table 1 Strains used in this study**

| Strains | Characteristics | Source |
|---------|-----------------|--------|
| BAK5    | *E. coli* BW2513 ΔptsG, ΔtyrR, ΔpykA, pykF, ΔpheA | [13] |
| BAK6    | *BAK5* ΔtyrB | [13] |
| BAK7    | *BAK6* ΔaroC | This study |
| BKT5    | *BKT5* with pYBT5 | [13] |
| BKT6    | *BKT6* with pYBT5 | This study |
| BKT7    | *BKT7* with pYBT5 | This study |
| HMA     | *E. coli* BL21(DE3) with pHMA | This study |
| HMA01   | BKT5 with pAHM1 | This study |
| HMA02   | BKT5 with pAHM2 | This study |
| HMA03   | BKT5 with pAHM3 | This study |
| HMA04   | BKT5 with pAHM4 and pYBH1 | This study |
| HMA05   | BKT7 with pCHM1 | This study |
| HMA06   | BKT7 with pCHM2 | This study |
| HMA07   | BKT7 with pCHM3 | This study |
| HMA08   | BKT7 with pCHM4 and pYBH1 | This study |
| HMA09   | BKT7 with pRHM1 | This study |
| HMA10   | BKT7 with pRHM2 | This study |
| HMA11   | BKT7 with pRHM3 | This study |
| HMA12   | BKT6 with pCHM3 | This study |
| HMA13   | BKT6 with pRHM1 | This study |
| HMA14   | BKT6 with pRHM3 | This study |
| HMA15   | BKT7 with pCHM3 | This study |
| HMA16   | BKT7 with pRHM1 | This study |
| HMA17   | BKT7 with pRHM3 | This study |

**Table 2 Plasmids used in this study**

| Plasmids | Characteristics | Source |
|----------|-----------------|--------|
| pBldgbrick1 | pMB1 ori with Pgap and Prec ApR | [13] |
| pYBT5 | pBldgbrick1 with PlacUV5 and Pgap ApRaroE, Prec ppsA tktA glik | [13] |
| pYBH1 | pYSC1 with Pgap T7 RNA polymerase | [13] |
| pEBM | pEASY-Blunt with shmaS, pUC ori, KanR | This study |
| pHMA | pET28a with shmaS | This study |
| pACYCDuet-1 | p15A ori, CmR | Novagen |
| pCDFDuet-1 | CloDF13 ori, StrR | Novagen |
| pRSFDuet-1 | RSF1030 ori, KanR | Novagen |
| pAHM | p15A ori, CmR | This study |
| pCHM | CloDF13 ori, StrR | This study |
| pRHM | RSF1030 ori, KanR | This study |
| pAHM1 | pAHM with Pgap-shmaS | This study |
| pAHM2 | pAHM with PlacUV5-shmaS | This study |
| pAHM3 | pAHM with Ptrc-shmaS | This study |
| pAHM4 | pACYCDuet-1 with Ptrc-shmaS | This study |
| pCHM1 | pCHM with PGAP-shmaS | This study |
| pCHM2 | pCHM with PlacUV5-shmaS | This study |
| pCHM3 | pCHM with Ptrc-shmaS | This study |
| pCHM4 | pCDFDuet-1 with P7-shmaS | This study |
| pRHM1 | pRHM with PGAP-shmaS | This study |
| pRHM2 | pRHM with PlacUV5-shmaS | This study |
| pRHM3 | pRHM with Ptrc-shmaS | This study |

**Table 3 Strains used in this study**

| Strains | Characteristics | Source |
|---------|-----------------|--------|
| BA05    | *E. coli* BW2513 | ΔptsG, ΔtyrR, ΔpykA, pykF, ΔpheA | [13] |
| BA06    | *BAK5* ΔtyrB | [13] |
| BA07    | *BAK6* ΔtyrB | [13] |
| BA08    | *BAK7* ΔaroC | This study |
| BA09    | *BAK8* ΔaroC | This study |
| BA10    | *BAK9* ΔaroC | This study |
| BA11    | *BAK10* ΔaroC | This study |
| BA12    | *BAK11* ΔaroC | This study |
| BA13    | *BAK12* ΔaroC | This study |
| BA14    | *BAK13* ΔaroC | This study |
| BA15    | *BAK14* ΔaroC | This study |
| BA16    | *BAK15* ΔaroC | This study |
| BA17    | *BAK16* ΔaroC | This study |

**Table 4 Plasmids used in this study**

| Plasmids | Characteristics | Source |
|----------|-----------------|--------|
| pBldgbrick1 | pMB1 ori with Pgap and Pprec ApR | [13] |
| pYBT5 | pBldgbrick1 with PlacUV5 and Pgap ApRaroE, Pprec ppsA tktA glik | [13] |
| pYBH1 | pYSC1 with Pgap T7 RNA polymerase | [13] |
| pEBM | pEASY-Blunt with shmaS, pUC ori, KanR | This study |
| pHMA | pET28a with shmaS | This study |
| pACYCDuet-1 | p15A ori, CmR | Novagen |
| pCDFDuet-1 | CloDF13 ori, StrR | Novagen |
| pRSFDuet-1 | RSF1030 ori, KanR | Novagen |
| pAHM | p15A ori, CmR | This study |
| pCHM | CloDF13 ori, StrR | This study |
| pRHM | RSF1030 ori, KanR | This study |
| pAHM1 | pAHM with Pgap-shmaS | This study |
| pAHM2 | pAHM with PlacUV5-shmaS | This study |
| pAHM3 | pAHM with Ptrc-shmaS | This study |
| pAHM4 | pACYCDuet-1 with P7-shmaS | This study |
| pCHM1 | pCHM with PGAP-shmaS | This study |
| pCHM2 | pCHM with PlacUV5-shmaS | This study |
| pCHM3 | pCHM with P7-shmaS | This study |
| pCHM4 | pCDFDuet-1 with P7-shmaS | This study |
| pRHM1 | pRHM with PGAP-shmaS | This study |
| pRHM2 | pRHM with PlacUV5-shmaS | This study |
| pRHM3 | pRHM with P7-shmaS | This study |
Table 3 Primers used in this study

| Name  | Sequence (5’–3’)          |
|-------|---------------------------|
| gap F1| CCATGGTTTTAGGAGGATTACAAATGCAGAATCTCGAATATC- GACTACG |
| gap R1| CCGGATCCCTCAAGACGGCCACCCCAACGGCA          |
| gap F2| CCGGATCCCTCAAGACGGCCACCCCAACGGCA          |
| gap R2| ACTGCTTTTCTTTGTTTCATATATTTCCACCAGCTATT |
| lacUV5F1| GGAATCTAATGCAAGAATTCTGAATGACTACG          |
| lacUV5R1| CCGGATCCCTCAAGACGGCCACCCCAACGGCA          |
| lacUV5F2| CCGGATCCCTCAAGACGGCCACCCCAACGGCA          |
| lacUV5R2| CCGGATCCCTCAAGACGGCCACCCCAACGGCA          |
| trcF1 | GGGGTACCTTAAGGCTGACTACG                  |
| trc F2| GGGGTACCTTAAGGCTGACTACG                  |
| T7 F  | GGAATCTAATGCAAGAATTCTGAATGACTACG          |
| T7 R  | GGGGTACCTTAAGGCTGACTACG                  |
| aspC F| CCGGATCCCTTTTCTTGTACCAATATATCCGACTATG    |
| aspC R| GTGACTCTATGCAAGAATTCTGAATGACTACG          |
| aspC F1| CGGGATCCCTTTTCTTGTACCAATATATCCGACTATG    |
| aspC R1| CGGGATCCCTTTTCTTGTACCAATATATCCGACTATG    |
| aspCR | GTGACTCTATGCAAGAATTCTGAATGACTACG          |
| aspCVF| AGCCCGCTTTTCTGTTACCAATATATCCGACTATG      |
| aspCVR | GGAAATTTGCTAAGGCTGACTACG                  |
| aspCZF| CCGGATCCCTTTTCTGTTACCAATATATCCGACTATG    |
| aspCZR | CCGGATCCCTTTTCTGTTACCAATATATCCGACTATG    |

Table 2. The skeleton vectors pAHM, pCHM and pRHM without lacI were obtained by PCR with pACYCDuet-1, pCDFDuet-1 and pRSFDuet-1 as templates, respectively.

For the construction of pAHM1, pCHM1 and pRHM1, the shmaS gene was amplified from pEBM by PCR with primers gapF1 and gapR1. Promoter gap was cloned from genome of E. coli BW25113 with primers gapF2 and gapR2. The shmaS gene and promoter gap were spliced by overlapping extension PCR, constructing P-gap-shmaS, which was then ligated at the restriction sites HindIII and BamHI of skeleton vectors pAHM, pCHM and pRHM, respectively. For the construction of pAHM2, pCHM2 and pRHM2, the shmaS gene was amplified from pEBM with primers lacUV5F1 and lacUV5R1, which was then cloned into pBludgBrick1 obtaining recombinant, and with it as template, lacUV5F2 and lacUV5R2 as primers yielding P-lacUV5-shmaS, which was then cloned into the skeleton vectors pAHM, pCHM and pRHM, respectively. The same method was used for construction of pAHM3, pCHM3 and pRHM3. For the construction of pAHM4 and pCHM4, the shmaS gene was amplified from pEBM by PCR with primers T7F and T7R, which was then cloned into the restriction sites Ndel and KpnI of pACYCDuet-1 and pCDFDuet-1, respectively, under the regulation of T7 promoter directly. Due to the same antibiotic resistance of pRHM and pYBH (for expression of T7 RNA polymerase), the expression vector pRHM4 with combination of P-T7-shmaS and the skeleton vector pRHM was not constructed.

Fermentation media and cultivation conditions

LB medium was used for cultivating the general strains for cloning or preparation of fermentation seeds. The fermentation of engineered E. coli strain was operated at 37 °C and 220 rpm with 50 ml medium in 250 ml shake flasks with synthetic minimal salt medium. The salt medium contains 17.1 g/L Na₂HPO₄·12H₂O, 3.0 g/L KH₂PO₄, 0.5 g/L NaCl, 3.0 g/L NH₄Cl, 0.2 g/L L-phenylalanine, 3.0 g/L L-aspartic acid, 1.0 g/L yeast extract, 1.0 g/L MgSO₄·7H₂O, 0.03 g/L CaCl₂·2H₂O, 0.02 g/L FeSO₄·7H₂O. The initial medium pH was adjusted to 7.0 by the addition of 10 M NaOH. Sole glucose (4.0 g/L), xylose (4.0 g/L), or mixture of glucose (2.0 g/L) and xylose (2.0 g/L) were added as carbon source supply. For shmaS expression controlled by promoter T7, the preliminary experiments showed that IPTG induction inhibited cell growth, and shmaS was expressed without additional inducer IPTG in the fermentation process. For shmaS expressions controlled by the promoters gap, trc and lacUV5, they were constitutively expressed throughout the fermentation.

For bioreactor fermentation, the 5 L fermenter (Bailun, Shanghai) was employed, and contained 2 L the same medium as described in shake flask except for initial 5.0 g/L glucose, 5.0 g/L xylose and 5.0 g/L yeast extract. 300 ml seed culture was inoculated into the bioreactor, yielding an initial OD₆₀₀ of ~0.3. The dissolved oxygen (DO) level was maintained at 30 % (v/v) by flowing 2 L/min of air with automatically changing the agitation.
speed from 300 to 700 rpm. The feeding solution contained 250 g/L glucose, 250 g/L xylose, 60 g/L yeast extract, 3.0 g/L l-aspartate, 0.2 g/L l-phenylalanine. The speed of feeding was regulated to keep the concentration of residual sugars at appropriate level. Fed-batch fermentation was performed twice and average data were shown.

Broth samples were withdrawn periodically to determine the cell biomass, concentrations of residual sugars and metabolites produced. Each fermentation was conducted in triplicates. Antibiotics were supplemented to the media when needed at the following concentrations: 50 mg/L ampicillin, 30 mg/L kanamycin, 30 mg/L streptomycin, 20 mg/L chloramphenicol.

**Analytical methods**

Cell growth was monitored by measuring the absorbance at 600 nm (OD$_{600}$) using an UV–VIS spectrophotometer. Glucose consumption was quantified by a biosensor SBA-90 (Biology Institute of Shandong Academy of Sciences, China). Residual concentration of xylose was measured using Waters 1515 HPLC system, equipped with a Bio-Rad HPX-87H column and a refractive index detector (Waters 2414, Milford, USA), and the column was eluted at 65 °C with 5 mM sulfuric acid at 0.6 mL/min.

The broth samples were centrifuged, and supernatants were filtered through 0.22 μm syringe filter, and injected to the HPLC system. 4-HMA and l-tyrosine were measured using Agilent 1200 HPLC system equipped with a C18 column (250 × 4.6 mm, Agilent) and a PDA detector (Agilent) at 196 nm with a mobile phase (10% methanol-90% H$_2$O, addition of 0.1% formic acid) at 1.0 mL/min. The structure of 4-HMA was identified using LC–MS (Agilent 1200 HPLC system and 6310 Ion Trap mass spectrum system, Agilent) under negative ion mode. All of the HPLC analysis were quantified using a six point standard curve and the $R^2$ coefficient for the standard curve was higher than 0.99.

**Fig. 2**  HPLC and LC–MS analysis of 4-HMA produced by shmsS-expressing strain HMA.  

- **a** HPLC analysis of standard 4-HMA.  
- **b** LC–MS analysis of standard 4-HMA.  
- **c** Identity of 4-HMA detected in the fermentation supernatant by HPLC analysis, compared the retention time to standard 4-HMA.  
- **d** The identity of 4-HMA detected in the fermentation supernatant by LC–MS analysis, compared m/z to standard 4-HMA.
Results and discussion

Expression of synthetic shmaS gene with optimized codons led to 4-HMA production

The hmaS gene encodes an α-keto acid-dependent dioxygenase in the biosynthesis of polycyclic nonribosomal glycopeptide antibiotic chloroeremomycin [6, 7]. It acts on phenyl α-keto acid to decarboxylate, and one oxygen from molecular oxygen is incorporated into the carbonyl group and one into the benzylic hydroxyl group to form α-hydroxy acid [17]. HmaS can catalyze the conversion of 4-HPP and phenylpyruvate to 4-HMA and mandelate, respectively, and the biosynthetic pathways for production of D-/L-phenylglycine, and S-/R-mandelic acid from phenylpyruvate were constructed in E. coli [18–20]. Enzymatic activity of A. orientalis HmaS towards 4-hydroxyphenylpyruvate (4-HPP) was higher than that of S. coelicolor HmaS [18]. Thus, we chose hmaS from actinomycetes A. orientalis to construct a heterologous 4-HMA biosynthetic pathway in E. coli.

Native hmaS sequence of A. orientalis is characterized with high GC content (over 72%) and not suitable to express in E. coli. Optimization of the coding sequence of hmaS is necessary for the efficient heterologous expression. According to codon usage bias of E. coli, and elimination of the secondary structure of its mRNA, we designed the full length DNA sequence of hmaS and designated as shmaS. Full sequence of shmaS was conveniently assembled by overlapping PCR (detailed description in “Methods” section). The expression vector pHMA was constructed and introduced to strain BL21 (DE3), resulting the recombinant strain HMA.

Strain HMA was cultivated in mineral salt medium with addition of 0.4% glucose and 0.5 g/L 1-tyrosine. After 24 h of cultivation, the supernatant of fermentation broth was analyzed by HPLC, and 4-HMA was identified by LC–MS analysis. Strain HMA carrying shmaS gene in the biosynthesis of 4-HMA via its expression in E. coli.

Among three types of plasmids, copy numbers with replication origin p15A, CloDF13 and RSF1030 are estimated about 10, 30 and 100, respectively [25]. When weak promoter lacUV5 drove expression of shmaS on the low or high copy number vectors, the lowest titer of 4-HMA was produced, compared with promoters T7, trc and gap. Hybrid promoter trc is popularly used in metabolic engineering [26] and its strength is considered to be stronger than that of lacUV5. When the shmaS was expressed under the control of promoter trc, the titers of 4-HMA were approximately three-fold of increase, compared to promoter lacUV5 on middle and high copy-number vectors. Promoter T7 is well known as a very strong promoter, and widely used in production of heterologous proteins and metabolic engineering. Herein, the expression of shmaS on the middle copy-number vector under control of promoter T7 could be beneficial for production of 4-HMA, but not optimal. Promoter gap is a strong constitutive promoter regulating expression of glyceraldehyde 3-phosphate dehydrogenase in glycolysis pathway, and ever been used for high production of resveratrol [27]. The expression of shmaS driven by the
gap promoter on high copy-number vector resulted in the production of 4-HMA up to 146.68 mg/L in strain HMA09, however promoter gap was less efficient to express shmaS for 4-HMA production, compared with promoter trc on the same vector.

Although fine tuning of shmaS expression through combination of promoters and gene copy-numbers improved the production of 4-HMA in l-tyrosine over-producing background strains by using glucose, as shown in Fig. 3b (Additional file 1: Table S1), byproduct l-tyrosine was unexpectedly accumulated in fermentation broth. HmaS can also convert phenylpyruvate to mandelic acid [18–20], however, no phenylpyruvate and its derivative byproducts were detectable in our pheA deficient strains. Incomplete conversion of metabolic intermediate 4-HPP to 4-HMA would lead to accumulation of l-tyrosine (Fig. 1). It indicated that metabolic flux to l-tyrosine could compete the formation of 4-HMA. We speculated that blocking the flux from 4-HPP to l-tyrosine might further improve the production of 4-HMA.

Genome modification enhanced 4-HMA production by blocking byproduct formation

In order to enhance the production of 4-HMA, a logical possibility can be considered by blocking the conversion of 4-HPP to l-tyrosine through modifying the genome of strain BAK5, which was constructed by deleting ptsG, tyrR, pheA, pykA, and pykF in our previous study [13]. In E. coli, two aminotransferases are involved in the last step of the biosynthesis of l-tyrosine [28]. Under normal conditions, the aromatic amino acid aminotransferase encoded by tyrB is the main enzyme for l-tyrosine biosynthesis from 4-HPP. In case of higher 4-HPP pool, the aspartate aminotransferase encoded by aspC could contribute to the biosynthesis of l-tyrosine as well. In order to verify our hypothesis, sequentially inactivating the tyrB and aspC genes from the l-tyrosine biosynthetic pathway was implemented. We deleted tyrB and aspC in strain BAK5 background in sequence by λ-red recombinant method and constructed two new strains BAK6 and BAK7 for expression of shmaS. After introduction of pYBT5 (Table 2) and three shmaS expression vectors (pCHM3, pRHM1 and pRHM3), resulting engineered strains were cultivated for fermentation 4-HMA. 3.0 g/L of L-aspartate was added in mineral salt medium to support growth of strains with aspC deletion.

As expected, for tyrB or/and aspC deletion strains, byproduct l-tyrosine was totally undetectable, meanwhile an obvious increase in 4-HMA production was observed (Fig. 4), in comparison with the unmodified strains. Deletion of tyrB led to 1.45-fold increase of 4-HMA titer of 376.26 mg/L in strain HMA12 (pCHM3) at 24 h fermentation. Further deletion of aspC contributed to improvement of 4-HMA production, but the efficacy depended on the expression vectors of shmaS. Compared to expression vectors pRHM1 and pRHM3, vector pCHM3 gave the highest titer of 4-HMA production in strain BKT7 background with double deletions of tyrB and aspC. Strain HMA15 (pCHM3) produced 594.07 mg/L of 4-HMA, 30.8 and 59.2 % higher than strains HMA16 (pRHM1) and HMA17 (pRHM3), respectively.
The ptsG gene is responsible for transportation of glucose into cytoplasm and deleted in strain HMA15 (Table 1). Thus fermentation of 4-HMA in ptsG deficient strain HMA15 was comparatively carried out in shake flasks by three fed-batch modes, glucose or/and xylose used as sole or mixed carbon source. As shown in Fig. 5b, when glucose was used as sole carbon source, strain HMA15 produced 601.56 mg/L of 4-HMA at 24 h fermentation. With additional amount of glucose fed, the titer of 4-HMA was continuously increased to 747.13 mg/L at 48 h. When sole xylose was fed as carbon source, strain HMA15 produced 820.18 mg/L of 4-HMA, slightly higher than fed with glucose at 48 h (Fig. 5c). Furthermore, Fermentation was carried out by feeding glucose–xylose mixture. Different ratios of glucose and xylose as carbon source showed that sugar mixture of glucose (2.0 g/L) and xylose (2.0 g/L) at equal amount resulted in the highest titer of 4-HMA at 48 h fermentation (Additional file 1: Figure S1). When equal mass amounts of both glucose and xylose were fed as mixed carbon source, strain HMA15 produced 1.11 g/L of 4-HMA (Fig. 5d), displaying 48.6 and 35.3 % higher than that fed with glucose and xylose, respectively.

Feeding fermentation of strain HMA15 was further performed for production of 4-HMA in 5 L bioreactor. Feeding solution was added into the bioreactor based on concentration of residual sugars to maintain it lower than 2.0 g/L. In all, 42.5 g/L glucose and 42.5 g/L xylose were consumed in bioreactor fermentation. The production of 4-HMA showed a cell growth-dependent manner (Fig. 6). The maximum cell density (OD600) was ~34.0 at 60 h, and 4-HMA titer was 15.8 g/L with a productivity of 0.26 g/L h−1. During the fermentation, byproduct acetate (3.4 g/L) was detected. It might be caused by the overflux of sugar metabolism. Reduction of acetate accumulation [36] would further improve the yield and titer of 4-HMA.

E. coli utilizes xylose as a secondary sugar by the general control of CCR. PTS is the most efficient system for transporting sugars, and plays a crucial role in CCR. PEP-dependent glucose-specific PTS is composed of four components including the soluble non-sugar-specific enzymes EI and HPr (encoded by ptsI and ptsH, respectively), and glucose-specific soluble enzyme EIIA (encoded by crr) and membrane-integral permease EIICB (encoded by ptsG). Inactivating one of the PTS components could abolish CCR in E. coli; and the effects of different CCR-insensitive mutants on cell growth and sugar consumption were varied, which showed different application for production of various products [37]. Here we showed that ptsG negative strain HMA15 simultaneously utilized glucose and xylose (Fig. 5d). It was consistent to previous studies when equal mass of glucose–xylose mixture was employed [38, 39]. In E. coli without ptsG, GalP

**Fed-batch fermentation for production of 4-HMA by co-utilization of xylose and glucose**

Lignocellulosic biomass is the most abundant biorenewable resource in nature, utilization of lignocellulosic hydrolysate in fermentation industry for the production of fine chemicals and biofuels is very promising [33]. However, simultaneous utilization of glucose and xylose in lignocellulosic hydrolysate is still a challenge, because E. coli metabolizes glucose in preference to other sugars [34]. As shown in Fig. 5a, carbon catabolite repression (CCR) by glucose was obvious in the wild type E. coli BW25113, and sequential consumption of carbon sources was evident. When cultivated with glucose or xylose as the sole carbon source, the wild type BW25113 consumed glucose faster than xylose. However, when glucose and xylose mixture was provided, the wild type BW25113 consumed glucose rapidly while xylose was used after glucose was significantly depleted.

To release glucose repression, phosphoenolpyruvate-carbohydrate phosphotransferase system (PTS) was often focused as an important target of genetic modification [35]. TyrB and AspC are identified as isoenzymes [29] and also involved in biosynthesis of L-phenylalanine [30]. In previous reports, tyrB and aspC were deleted to block the biosynthesis of L-phenylalanine, and titers of phenylpyruvate [31] and its derivatives R-/S-mandelic acid [19], D-/L-phenylglycine [18, 20] and benzyl alcohol [32] were greatly improved. In this study, deletion of tyrB and aspC completely blocked the formation of byproduct L-tyrosine from precursor 4-HPP and resulted in significant increase of 4-HMA production. Therefore, double deletions of tyrB and aspC could benefit for the fermentative production of aromatic 4-HMA from 4-HPP and its analogs, mandelic acid, phenylglycine and benzyl alcohol from phenylpyruvate.
and MglABC are involved in glucose transport, but they are less effective than PtsG [40]. In addition, pykF- and pykA-deficiency in strain HMA15 might lead to slow glucose metabolism as corresponding pyruvate kinase catalyze formation of pyruvate and release energy ATP for glucose phosphorylation and cell growth. When the CCR was abolished, xylose could be transported via non-PTS XylE and XyFGH [34] and catabolized via pentose phosphate pathway (PPP). Furthermore, overexpression of tktA coding transketolase A in strain HMA15 would enhanced xylose metabolism and benefit to the formation of precursor E4P for aromatic compound production [41]. Meanwhile, inactivation of ptsG, pykF and pykA in strain HMA15 would increase carbon flux from glucose to PEP via glycolysis, which is precursor for aromatic compound biosynthesis (Fig. 1). Taking together, co-utilization of glucose and xylose would further promote the production of aromatic 4-HMA in strain HMA15.

Recently, several alternative strategies have been proposed to achieve co-utilization of glucose and xylose in E. coli. Constitutive expression of xylose metabolism was required from the araC mutant strain by adaptive evolution in xylose minimal medium, which showed the simultaneous co-utilization of glucose and xylose and possessed the same growth pattern as the wild type [39]. Similarly, growth adaptation of ptsG deficient E. coli strain on arabinose resulted in the simultaneous

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**Fig. 5** 4-HMA production, cell growth and sugar consumption in shake flasks. **a** Consumption of sole sugar and sugars mixture of the wild type E. coli BW25113. **b** Fed-batch fermentation of HMA15 with sole glucose as carbon source. **c** Fed-batch fermentation of HMA15 with sole xylose as carbon source. **d** Fed-batch fermentation of HMA15 with mixture of glucose and xylose as carbon sources.

**Fig. 6** Fed-batch fermentation of HMA15 with glucose–xylose mixture in 5 L bioreactor.
utilization of glucose, xylose and arabinose [42] and was used to produce 3-hydroxybutyric acid [43]. By using SIMUP algorithm, growth phenotype of E. coli was predicted; co-utilization of glucose and xylose was constructed by deleting pgi, rpe and eda [44]. It is the first report to achieve co-utilization of two sugars without targeting the regulatory pathways of CCR. Protein engineering of the hexose- or pentose-specific transporters might be orthogonal strategy to confer the co-utilization of glucose and xylose [45, 46].

Conclusions

Biosynthesis of 4-HMA was achieved through heterologous expression of fully synthetic shmaS gene in E. coli. Expression of shmaS was optimized to improve production of 4-HMA by combinatorial regulation with various promoters and copy numbers. Production of 4-HMA was further increased by deletion of tyrB and aspC on E. coli genome to block the formation of byproduct L-tyrosine. Furthermore, fed-batch fermentation mode with both sugars showed that engineered strain can utilize xylose and glucose simultaneously, and glucose-xylose mixture gave high production of 4-HMA with the titer of 15.8 g/L at 60 h fermentation in 5 L bioreactor. It is expected that 4-HMA production would be further improved through control of acetate biosynthesis and optimization of the fermentation process. Co-utilization of glucose and xylose will be promising for the production of other aromatic products of interest.

Additional file

**Additional file 1: Table S1.** Fine tuning of shmaS expression by combinatorial regulation of promoters and copy numbers. Figure S1. Production of 4-HMA with different ratio of glucose and xylose.

**Abbreviations**

CCR: carbon catabolite repression; E4P: erythrose-4-phosphate; 4-HMA: 4-hydroxymandelic acid, HmaS: 4-hydroxymandelate synthase; 4-HPP: 4-hydroxyphenylpyruvic acid; PEP: phosphoenolpyruvate; PPP: pentose phosphate pathway; PTS: phosphotransferase system.

**Authors’ contributions**

FFL, JJQ and GRZ designed research and experiments. FFL and YZ carried out the metabolite analysis and data processing. FFL, BZL, JJQ and GRZ wrote the paper. All authors read and approved the final manuscript.

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**Acknowledgements**

This work was supported by the National Basic Research Program of China (2011CBA00800), the National High-Tech R&D Program of China (2012AA02A701), the National Natural Science foundation of China (31570807), and the Natural Science Foundation of Tianjin (13JCZDJC27600). We would like to thank Professor Wen-Hai Xiao for his technical assistance for bioreactor fermentation and suggestions to the manuscript.

**Availability of data and material**

The data supporting their findings can be found in the main paper.

**Competing interests**

The authors declare that they have no competing interests.

**Ethics approval and consent to participate**

Not applicable.

Received: 22 February 2016 Accepted: 13 May 2016

**Published online:** 27 May 2016

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