
**Modular enzyme assembly for enhanced cascade biocatalysis and
metabolic flux**

Kang and Ma et al.

Contents

| Items | Page No. |
|--|------------|
| Supplementary Table 1. Plasmids used in this study | S3 |
| Supplementary Table 2. Strains used in this study | S5 |
| Supplementary Table 3. Strains and plasmids construction | S6 |
| Supplementary Table 4. Primers used in this study | S11 |
| Supplementary Figure 1. Purified MenH _{RD} (lane 1), MenH _{RA} (lane 2), MenD _{RA} (lane3), MenD _{RA2} (lane 4) and MenD _{RD} (lane 5) analyzed by SDS-PAGE. | S14 |
| Supplementary Figure 2. Purified MenD elutes as a tetramer, and MenH as a monomer on Superdex 200 10/300 GL in SEC. | S15 |
| Supplementary Figure 3. Peptide tagging did not change the activity of MenD and MenH. | S16 |
| Supplementary Figure 4. TEM images of the Assembly B. | S17 |
| Supplementary Figure 5. Strains Car3 and Car4 have identical carotenoids production rates and growth rates as the untagged control Car1 in shake-flask fermentation. | S18 |
| Supplementary Figure 6. Independent fed-batch fermentations showing the same trend of carotenoid production. | S19 |
| Supplementary Figure 7. Comparison of enzyme activity of the MenD-MenH fusion enzyme and free MenD and MenH in 1:1 ratio, showing the enzyme fusion has significantly reduced activity. | S20 |
| Supplementary Figure 8. Comparison of the assembly strains and fusion strains, showing that strains with enzyme assembly have higher product titer during shake-flask fermentation. | S21 |
| Supplementary References | S22 |

Supplementary Table 1. Plasmids used in this study

| Plasmids | Backbone | Description | Source |
|--------------------------|-----------------|---|------------------------|
| pETM-MenD _{RA} | pET32a(+) | N-terminal His-tagged MenD, (GGGGS) ₃ linker and RIAD, Amp ⁺ | this study |
| pETM-MenD _{RD} | pET32a(+) | N-terminal His-tagged MenD, (GGGGS) ₃ linker and RIDD, Amp ⁺ | this study |
| pETM-MenH _{RA} | pET32a(+) | N-terminal His-tagged MenH, (GGGGS) ₃ linker and RIAD, Amp ⁺ | this study |
| pETM-MenH _{RD} | pET32a(+) | N-terminal His-tagged MenH, (GGGGS) ₃ linker and RIDD, Amp ⁺ | this study |
| pETM-MenD _{RA2} | pET32a(+) | N-terminal His-tagged MenD, (GGGGS) ₃ linker, RIAD, (GGGGS) ₃ linker and RIAD, Amp ⁺ | this study |
| pETM-MenD-MenH | pET21a(+) | N-terminal His-tagged MenD, (GGGGS) ₃ linker, MenH, Amp ⁺ | this study |
| pMH1 | pBBR1MCS-1 | AtoB, ERG13 and N-terminal His-tagged tHMG1, Cam ⁺ | reference ¹ |
| pFZ81 | pBBR1MCS-2 | ERG12, ERG8, MVD1 and N-terminal His-tagged Idi, Kan ⁺ | reference ¹ |
| pFZ153 | pETDuet-1 | CrtE, CrtI, CrtB, Idi, CrtY, CrtZ, CrtW, Amp ⁺ | reference ² |
| pFZ82 | pBBR1MCS-2 | ERG12, ERG8, MVD1 and N-terminal His-tagged Idi _{RA} , Kan ⁺ | this study |
| pFZ1532 | pETDuet-1 | CrtE _{RD} , CrtI, CrtB, Idi, CrtY, CrtZ, CrtW, Amp ⁺ | this study |
| pFZ81f | pBBR1MCS-2 | ERG12, ERG8, MVD1 and N-terminal His-tagged Idi-CFP, Kan ⁺ | this study |

| | | | |
|----------|------------|---|------------|
| pFZ82f | pBBR1MCS-2 | ERG12, ERG8, MVD1 and N-terminal His-tagged Idi _{RA} -CFP, Kan ⁺ | this study |
| pFZ1532f | pETDuet-1 | CrtE _{RD} -YFP, CrtI, CrtB, Idi, CrtY, CrtZ, CrtW, Amp ⁺ | this study |
| pFZ1530 | pETDuet-1 | CrtE _{RD} , CrtI, CrtB, CrtY, CrtZ, CrtW, Amp ⁺ | this study |
| pFZ15301 | pETDuet-1 | CrtE-Idi, CrtI, CrtB, CrtY, CrtZ, CrtW, Amp ⁺ | this study |
| pFZ15302 | pETDuet-1 | Idi-CrtE, CrtI, CrtB, CrtY, CrtZ, CrtW, Amp ⁺ | this study |
| pFZ15322 | pETDuet-1 | CrtE _{RD} , CrtI, CrtB, Idi _{RA} , CrtY, CrtZ, CrtW, Amp ⁺ | this study |

Supplementary Table 2. Strains used in this study

| Strains | Description | Source |
|----------------|---|------------------------|
| Car1 | <i>E. coli</i> MG1655 (DE3) with pMH1, pFZ81 and pFZ153 | this study |
| Car2 | <i>E. coli</i> MG1655 (DE3) with pMH1, pFZ82 and pFZ1532 | this study |
| Car3 | <i>E. coli</i> MG1655 (DE3) with pMH1, pFZ82 and pFZ153 | this study |
| Car4 | <i>E. coli</i> MG1655 (DE3) with pMH1, pFZ81 and pFZ1532 | this study |
| Car1f | <i>E. coli</i> MG1655 (DE3) with pMH1, pFZ81f and pFZ1532f | this study |
| Car2f | <i>E. coli</i> MG1655 (DE3) with pMH1, pFZ82f and pFZ1532f | this study |
| Car5 | <i>E. coli</i> BL21 (DE3) with pMH1, pFZ81 and pFZ15322 | this study |
| Car6 | <i>E. coli</i> BL21 (DE3) with pMH1, pFZ81 and pFZ15301 | this study |
| Car7 | <i>E. coli</i> BL21 (DE3) with pMH1, pFZ81 and pFZ15302 | this study |
| TM606 | CEN.PK2-1D, <i>gal1Δ</i> , <i>gal7Δ</i> , <i>gal10Δ:: TRP1_P_{GAL10}-tHMG1</i> <i>leu2Δ:: LEU2_P_{GALI}-TmCrtE</i> <i>ura3Δ:: HIS3_P_{GALI}-PaCrtB</i> , <i>P_{GAL10}-BtCrtI</i> <i>YPRCdelta15Δ:: KanMX_P_{GAL7}-BtCrtI</i> , <i>P_{GAL10}-TmCrtE</i> , <i>XI-3Δ:: P_{GALI}-POS5</i> , <i>X-3Δ:: P_{GALI}-ADH2</i> , <i>P_{GAL10}-SeACS</i> , <i>P_{GAL7}-ALD6</i> , <i>ypl062wΔ:: HphMX</i> , <i>exg1Δ:: KanMX</i> | reference ³ |
| TM624 | TM606, <i>Idi::Idi-RIAD</i> , <i>TmCrtE:: TmCrtE-RIDD</i> | this study |

Supplementary Table 3. Strains and plasmids construction

| Plasmids | Fragments | Template | Primers |
|--------------------------|---------------------------|-------------------------|---|
| pETM-MenD _{RA} | MenD | <i>E. coli</i> | MenD-F MenD-R |
| pETM-MenH _{RA} | MenH | <i>E. coli</i> | MenH-F MenH-R |
| pETM-MenD _{RA2} | <u>Fragment 1*</u> | pETM-MenD _{RA} | MenD _{RA2} -1-F MenD _{RA2} -1-R |
| | <u>Fragment 2</u> | pETM-MenD _{RA} | MenD _{RA2} -2-F MenD _{RA2} -2-R |
| | Fragment 3 | pETM-MenD _{RA} | MenD _{RA2} -3-F MenD _{RA2} -31-R |
| | MenD _{RA} | Fragment 3 | MenD _{RA2} -3-F MenD _{RA2} -32-R |
| | <u>MenD_{RA2}</u> | MenD _{RA} | MenD _{RA2} -3-F MenD _{RA2} -33-R |
| pFZ82 | Idi | pFZ81 | 82-1-F 82-1-R |
| | RIAD | Synthesis [†] | 82-2-F 82-2-R |
| | <u>Idi-RIAD</u> | Idi+RIAD | 82-1-F 82-2-R |
| | <u>Fragment 1</u> | pFZ81 | 82-3-F 82-3-R |
| | <u>Fragment 2</u> | pFZ81 | 82-4-F 82-4-R |
| | pFZ1532 | CrtE | pFZ153 |
| RIDD | | Synthesis [†] | 1532-2-F 1532-2-R |
| <u>CrtE-RIDD</u> | | CrtE+RIDD | 1532-1-F 1532-2-R |
| <u>Fragment 1</u> | | pFZ153 | 1532-3-F 1532-3-R |
| <u>Fragment 2</u> | | pFZ153 | 1532-4-F 1532-4-R |
| pFZ81f | CFP | Synthesis [†] | 81f-1-F 81f-1-R |
| | <u>Linker-CFP</u> | <u>CFP</u> | 81f-11-F 81f-1-R |
| | <u>Fragment 1</u> | pFZ81 | 81f-2-F 81f-2-R |
| | <u>Fragment 2</u> | pFZ81 | 81f-3-F 81f-3-R |
| pFZ82f | <u>CFP</u> | pFZ81f | 81f-1-F |

| | | | |
|----------|------------------------|------------------------|-------------------------------|
| | <u>RIAD-Fragment 1</u> | pFZ82 | 82f-1-R 82f-2-F 82f-2-R |
| | <u>Fragment 2</u> | pFZ81f | 82f-3-F 82f-3-R |
| pFZ1532f | <u>YFP</u> | Synthesis [†] | 1532f-1-F 1532f-1-R |
| | <u>RIDD</u> | Synthesis | 1532f-2-F 1532f-2-R |
| | <u>Fragment 1</u> | pFZ1532 | 1532f-3-F 1532f-3-R |
| | <u>Fragment 2</u> | pFZ1532 | 1532f-4-F 1532f-4-R |
| pFZ1530 | F-1530 | pFZ153 | 1530-F 1530-R |
| pFZ15322 | Idi _{RA} | pFZ82 | 15322-1-F 15322-1-R |
| | F-CrtYZW | pFZ153 | 15322-2-F 15322-2-R |
| pFZ15301 | <u>F-CrtE</u> | pFZ153 | 15301-1-F 15301-1-R |
| | <u>Idi</u> | pFZ153 | 15301-2-F 15301-2-R |
| | F-CrtI | pFZ153 | 15301-3-F 15301-3-R |
| pFZ15302 | Idi | pFZ81 | 15302-1-F 15302-1-R |
| | F-CrtEIB | pFZ153 | 15302-2-F 15302-2-R |

Strains

| | | | |
|-------|------------|---------|----------------------------------|
| TM624 | CrtE left | TM606 | crtE_left-F RI-crtE R |
| | RIDD | pFZ1532 | crtE-RI F GPM RI R |
| | tGPM | TM606 | RI-GPM F crtE_RI-terminator-R |
| | HghMX | pUG75 | crtE_hygR-F crtE_hygR-R |
| | CrtE right | TM606 | crtE_right-F crtE_right-R |
| | Idi left | TM606 | idi left F RIAD idi R |
| | RIAD | pFZ82 | idi RIAD F |

| | | |
|-----------|-------|--|
| Ter | TM606 | Ter RIAD R RIAD Ter F G418 Ter R |
| KanMX | pUG6 | TER G418 F MRP G418 R |
| Idi right | TM606 | G418 MRP F MRP R |

*Underline was the fragments used in Gibson assembly.

†Sequence of RIAD:

GGTGGTGGTGGTTCAGGTGGTGGTGGTTCAGGTGGTGGTGGTGTGGTCTGGAAC
AGTATGCAAATCAGCTGGCAGATCAGATTATCAAAGAAGCAACCGAAGGTTGC

Sequence of RIDD:

GGTGGTGGTGGTTCAGGTGGTGGTGGTTCAGGTGGTGGTGGTGTGGTAGCCTGC
GTGAATGTGAACTGTATGTTTCAGAAACATAATATTCAGGCCCTGCTGAAAGATAGC
ATTGTTTCAGCTGTGTACCGCACGTCCGGAACGTCCGATGGCATTCTGCGCGAATA
TTTTGAACGTCTGGAAAAGAAGAAGCCAAA

Sequence of CFP:

ATGGTGAGCAAGGGCGAGGAGCTGTTACCGGGGTGGTGCCCATCCTGGTTCGAG
CTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGC
GATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGC
CCGTGCCCTGGCCACCCTCGTGACCACCCTGTCCTGGGGCGTGCAGTGCTTCGC
CCGCTACCCCGACCACATGAAGCAGCAGACTTCTTCAAGTCCGCCATGCCCGAA
GGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACC
CGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAG
GGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAAC
TACTTTAGCGACAACGTCTATATCACCGCCGACAAGCAGAAGAACGGCATCAAGG
CCAACCTCAAGATCCGCCACAACATCGAGGACGGCGGCGTGCAGCTCGCCGACC
ACTACCAGCAGAACACCCCATCGGGCGACGGCCCCGTGCTGCTGCCCGACAACC
ACTACCTGAGCACCCAGTCCAAGCTGAGCAAAGACCCCAACGAGAAGCGCGATC
ACATGGTCCTGCTGGAGTTCGTGACCGCCGCGGGATCACTCTCGGCATGGACGA
GCTGTACAAGTAA

Sequence of YFP:

TCTAAAGGTGAAGAATTATTCAGTGGTGTGTCCCAATTTTGGTTGAATTAGATGG
TGATGTTAATGGTCACAAATTTTCTGTCTCCGGTGAAGGTGAAGGTGATGCTACTT
ACGGTAAATTGACCTTAAAATTGATTTGTACTACTGGTAAATTGCCAGTTCATGG
CCAACCTTAGTCACTACTTTAGGTTATGGTTTGCAATGTTTTGCTAGATACCCAGAT
CATATGAAACAACATGACTTTTTCAAGTCTGCCATGCCAGAAGGTTATGTTCAAGA
AGAACTATTTTTTTCAAAGATGACGGTAACTACAAGACCAGAGCTGAAGTCAAG
TTTGAAGGTGATACCTTAGTTAATAGAATCGAATTTAAAGGTATTGATTTTAAAGA
AGGTGGTAACATTTTAGGTCACAAATTGGAATACAACACTATACTCTCACAATGTTT
ACATCACTGCTGACAAACAAAAGAATGGTATCAAAGCTAACTTCAAATAGACA
CAACATTGAAGATGGTGGTGTTCATTAGCTGACCATTATCAACAAAATACTCCAA
TTGGTGTGATGGTCCAGTCTTGTTACCAGACAACCATTAATCTATCAATCTGCCT
TATCCAAAGATCCAAACGAAAAGAGAGATCACATGGTCTTGTTAGAATTTGTTACT
GCTGCTGGTATTACCCATGGTATGGATGAATTGTACAATAA

Sequence of MenD-MenH fusion:

ATGGCTAGCATGACTGGTGGACAGCAAATGGGTTCGCGGATCCGAATTCGAGCTCA
TGTCTTCTCTGGTGCCACGCGTCTGGATCCTCAGTAAGCGCATTAAACCGACGC
TGGGCGGCGGTCATTCTGGAAGCATTAAACGCGTCACGGCGTCAGACACATCTGTA
TCGCCCCAGGCTCGCGTTCTACACCGTTAACGTTAGCGGGCGGCGGAGAATTCCGC
ATTCATTACCCACACCCATTTTCGATGAGCGTGGGTTGGGGCATCTGGCGCTGGGG
CTGGCGAAAGTCAGCAAGCAGCCGGTGGCGGTGATTGTGACCTCCGGCACGGCG
GTGGCAAATCTCTATCCGGCACTGATTGAAGCCGGGTTAACCGGAGAAAACTGA
TTCTCTTAACCGCCGATCGCCCCGCCGGAGCTAATTGACTGCGGCGCGAATCAGGC
AATTCGCCAGCCGGGAATGTTTCGCCTCTACCCCCACGCACAGTATTTTCATTGCCGC
GCCCCAGCCAGGATATCCCCGCACGTTGGCTGGTTTCTACCATCGACCACGCTCTC
GGTACGCTTCATGCGGGGGGAGTCCATATCAACTGCCCCGTTTGCTGAACCGCTGTA
TGCGCAAATGGACGATAACCGGGCTTAGCTGGCAACAGCGTCTGGGTGACTGGTG
GCAGGACGACAAACCGTGGCTGCGTGAAGCGCCTCGTCTGGAAAGTGAAAAAC
AGCGCGACTGGTTCTTCTGGCGACAAAAGCGCGGCGTGGTGGTTGCCGGGCGCA
TGAGTGCGGAAGAGGGCAA AAAAGTTGCCCTGTGGGCGCAA ACTCTTGGCTGGC
CGCTGATTGGCGATGTGCTGTCAAAACCGGGCAGCCGCTGCCGTGTGCCGATCT
TTGGTTAGGCAATGCCAAAGCGACCAGCGAGCTGCAGCAGGCGCAAATTGTGGT
GCAACTGGGAAGCAGCCTGACGGGGAAACGGCTCCTGCAATGGCAGGCAAGCTG
TGAACCAGAAGAGTACTGGATTGTTGATGACATTGAAGGGCGACTTGATCCGGCA
CACCATCGCGGACGTCGCTTAATTGCCAATATTGCCGACTGGCTGGAGCTGCATCC
GGCAGAAAAACGCCAGCCCTGGTGC GTTGAAATCCCGCGCCTGGCGGAACAGGC
AATGCAGGCGGTTATTGCCCGCCGTGATGCGTTTGGCGAAGCGCAACTGGCGCAT
CGCATCTGCGACTATCTGCCTGAACAGGGGCAATTGTTTGTGGTAACAGCCTGGT
GGTACGCTGATTGATGCGCTTTCGCAACTTCCGGCAGGTTACCCGGTGTACAGCA
ACCGTGGGGCCAGCGGTATCGACGGGCTGCTTTCGACCGCCGCCGGCGTTCAGCG
GGCAAGCGGCAAACCGACGCTGGCGATTGTGGGCGATCTCTCCGCACTTTACGAT
CTCAACGCGCTGGCGTTATTGCGTCAGGTTTCTGCGCCGCTGGTATTAATTGTGGT
GAACAACAACGGCGGGCAAATTTCTCGCTGTTGCCAACGCCGCAAAGCGAGCG
TGAGCGTTTCTATCTGATGCCGCAAAACGTCCATTTTGAGCACGCCGCCGCGATGT
TCGAGCTGAAATATCATCGTCCGCAAAACTGGCAGGAACTTGAAACGGCATTTC
CGACGCCTGGCGCACGCCAACCACCGGTGATTGAAATGGTGGTTAACGACAC
CGATGGTGCGCAAACGCTCCAGCAACTTCTGGCGCAGGTAAGCCATTTAGTCGAC
AAGCTTGGTGGCGGCGGCGGATCAATCCTGCACGCGCAGGCAAACACGGAAAA
CCAGGTTTACCCTGGCTGGTGT TTTTGCACGGTTTTTCCGGCGATTGCCACGAATG
GCAAGAAGTGGGCGAGGCGTTTGCCGACTACTCACGGTTGTATGTTGATCTCCCA
GGTCACGGTGGTTCGGCGGCGATTAGCGTCGATGGATTTGATGATGTCACCGACTT
ACTGCGTAAAACCTTGGTTAGTTACAACATCCTTGACTTCTGGCTGGTGGGGTACT
CGCTTGGTGGACGGGTGGCGATGATGGCGGCTTGCCAGGGGCTGGCGGGGCTTT
GTGGGGTTATTGTGCAAGGCGGGCATCCGGGGCTGCAAATGCTGAACAACGTGC
GGAACGTCAGCGTTCGATCGCCAATGGGTGCAGCGTTTTCTCACAGAACCGTTA
ACGGCGGTATTTGCCGACTGGTATCAACAGCCTGTTTTTGCCTCACTCAATGACGA
TCAACGCCGGGAGCTGGTGGCGCTGCGCAGCAACAATAATGGCGCAACGCTTGC
CGCCATGCTGGAGGCGACTTCTCTCGCCGTCCAGCCTGATTTACGTGCTAACCTTA
GCGCCCGCACATTTGCGTTTTATTATTTATGTGGTGAACGTGACAGCAAATTCCGC
GCCCTGGCGGCGGAACTGGCTGCCGACTGCCATGTCATTCTCGCGCCGGACATA

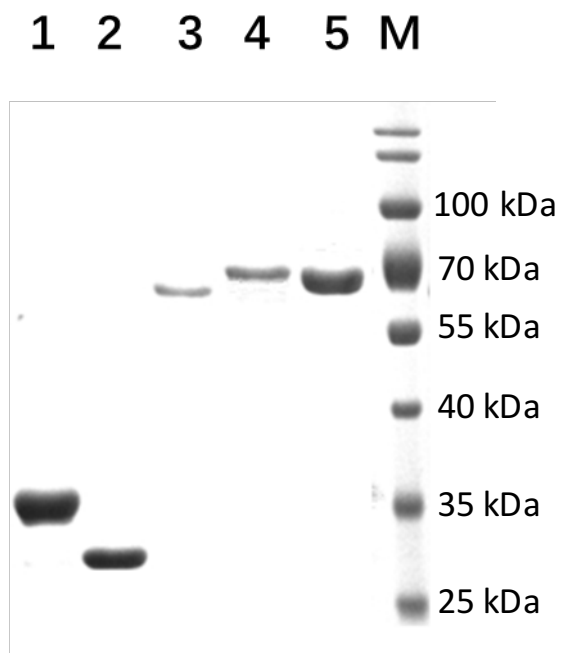
ACGCGCATCGGGAAAATCCCGCTGGCGTAATCGCAAGTCTGGCGCAGATCTTGCG
TTCCTCGAGCACCACCACCACCACCTGA

Supplementary Table 4. Primers used in this study

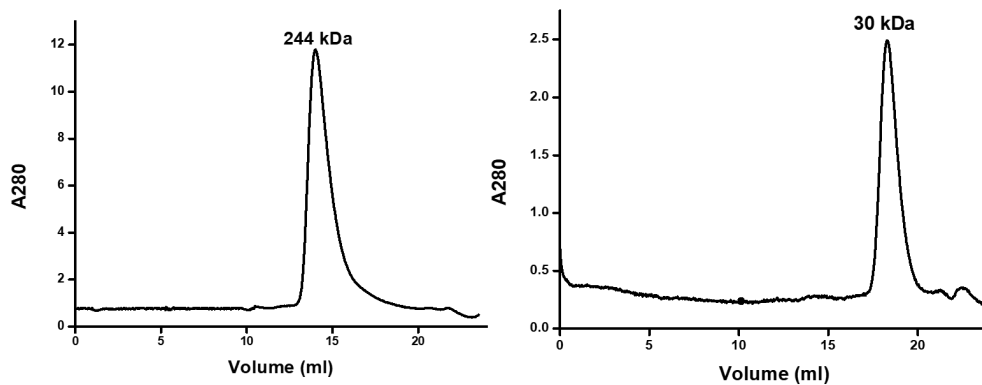
| Primers | Sequence 5'-3' (restriction sites are underlined) |
|---------------------------|--|
| MenD-F | <u>GGATCCT</u> CAGTAAGCGCATTTAACCGACGCTGGGC |
| MenD-R | <u>AAGCTT</u> GGGAGCCTAAATGGCTTACCTGCGCCAGAA |
| MenH-F | <u>GGATCCAT</u> CCTGCACGCGCAGGCAAAACACGGAAAA |
| MenH-R | <u>AAGCTT</u> GGGAGCCGAAACGCAAGATCTGCGCCAGACT |
| MenD _{RA2} -1-F | GGAAGCGACGGAAGGCTGTTAGCTCGAGGATCCGGCTGCTAACAA AGCC |
| MenD _{RA2} -1-R | AGAGAGTCAATTCAGGGTGGTGAATGTGAAACCAGTAACGTTATAC GAT |
| MenD _{RA2} -2-F | ATCGTATAACGTTACTGGTTTCACATTCACCACCCTGAATTGACTCT CT |
| MenD _{RA2} -2-R | CAGGCAGATAGTCGCAGATGCGATGCGCCAGTTGCGCTTCGCCAAA CGC |
| MenD _{RA2} -3-F | GCGTTTGGCGAAGCGCAACTGGCGCATCGCATCTGCGACTATCTGC CTG |
| MenD _{RA2} -31-R | TTGCTCCAGTCCACAGCCACCGCCTCCTGAACCTCCGCCTCCAGAT CCACCGCCACCACAACCTTCGGTTGCTTCTTT |
| MenD _{RA2} -32-R | CTAACAGCCTTCCGTCGCTTCCTTAATGATCTGGTCGGCCAGTTGGT TCGCGTATTGCTCCAGTCCACAGCCACCGCC |
| MenD _{RA2} -33-R | GGCTTTGTTAGCAGCCGGATCCTCGAGCTAACAGCCTTCCGTCGCT TCC |
| 82-1-F | TGGTGCCGCGCGGCAGCCATATGCAAACGGAACACGTCATTTTATT GA |
| 82-1-R | CCACCACCTGAACCACCACCACCTTTAAGCTGGGTAAATGCAGATA |
| 82-2-F | TATCTGCATTTACCCAGCTTAAAGGTGGTGGTGGTTCAGGTGGTGG |
| 82-2-R | TATAGGGCGAATTGGAGCTCTTAGCAACCTTCGGTTGCTTCTTTGA |
| 82-3-F | AAGCAACCGAAGGTTGCTAAGAGCTCCAATTCGCCCTATAGTGAG |
| 82-3-R | CGCTGCGAATCGGGAGCGGCGATACCGTAAAGCACGAGGAAGCGG TCAGC |
| 82-4-F | CTGACCGCTTCCCTCGTGCTTTACGGTATCGCCGCTCCCGATTTCGCAG CG |
| 82-4-R | TCAATAAAATGACGTGTTCCGTTTGCATATGGCTGCCGCGCGGCAC CA |
| 1532-1-F | ATGACCGTGTGTGCGAAAAACATGTGCATCTGACCCGTG |
| 1532-1-R | CCACCACCTGAACCACCACCACCCGACACCGCTGCCAGTTTTTTAT CG |
| 1532-2-F | CGATAAAAACTGGCAGCGGTGTCGGGTGGTGGTGGTTCAGGTGG TGG |
| 1532-2-R | TCGGTTTCATGGTTAATTCCTCCTTTATTTGGCTTCTTCTTTTCCAG |
| 1532-3-F | CTGGAAAAAGAAGAAGCCAAATAAAGGAGGAATTAACCATGAAAC CGA |
| 1532-3-R | GCTCAGCCAGGTCTGCACTGCCACGATGGCCGGCACGATC |
| 1532-4-F | GATCGTGCCGGCCATCGTGGCAGTGCAGACCTGGCTGAGC |
| 1532-4-R | CACGGGTCAGATGCACATGTTTTTTTCGCACACACGGTCAT |

| | |
|-------------|--|
| 81f-1-F | GGAGGTTTCAGGTGGAGGTGGATCTATGGTGAGCAAGGGCGAGGAG CTG |
| 81f-11-F | ATCTGCATTTACCCAGCTTAAAGGAGGTGGAGGTTTCAGGTGGAGGT GGAT |
| 81f-1-R | GACTCACTATAGGGCGAATTGGAGCTCCTACTCCTTGTACAGCTCGT CCATG |
| 81f-2-F | CATGGACGAGCTGTACAAGGAGTAGGAGCTCCAATTCGCCCTATAG TGAGTC |
| 81f-2-R | CCAAAATAATAACCTTTCCCGGTGCAGAAGTTAAGAACGGTAATG AC |
| 81f-3-F | GTCATTACCGTTCTTAACTTCTGCACCGGGAAAGGTTATTATTTTTG G |
| 81f-3-R | CAGCTCCTCGCCCTTGCTCACCATAGATCCACCTCCACCTGAACCTC C |
| 82f-1-R | TTCCAGACCACAtCCtCCaCCaCCAGACTCCTTGTACAGCTCGTCCAT G |
| 82f-2-F | CATGGACGAGCTGTACAAGGAGTCTGGTGGTGGAGGATGTGGTCT GGAA |
| 82f-2-R | CCAAAATAATAACCTTTCCCGGTGCAGAAGTTAAGAACGGTAATG AC |
| 82f-3-F | GTCATTACCGTTCTTAACTTCTGCACCGGGAAAGGTTATTATTTTTG G |
| 82f-3-R | CAGCTCCTCGCCCTTGCTCACCATAGATCCACCTCCACCTGAACCTC C |
| 1532f-1-F | GTGGTTCAGGTGGaGGtGGaTCTTCTAAAGGTGAAGAATTATTCACT GGTGTG |
| 1532f-1-R | GCTTCCACAACCCCCACCGCCTTTGTACAATTCATCCATAACCATGGG TA |
| 1532f-2-F | TACCCATGGTATGGATGAATTGTACAAAGGcGGTGGgGGTTGTGGaA GC |
| 1532f-2-R | GATTATTCATGAGTATTACCTCCTTTATTTGGCTTCTTCTTTTTCCAG ACG |
| 1532f-3-F | CGTCTGGAAAAAGAAGAAGCCAAATAAAGGAGGTAATACTCATGA ATAATC |
| 1532f-3-R | AGGCCCAAGGGGTTATGCTAGTTATTGCTCAGCGGTGGCAGCAGC CTA |
| 1532f-4-F | TAGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGG CCT |
| 1532f-4-R | CAACACCAGTGAATAATTCTTCACCTTTAGAAGAtCCaCCtCCACCTG AACCAC |
| crtE_left-F | CGCATTGTTATCTTTTCGCTTTTGAA |
| RI-crtE R | CCACCACCTGAACCACCACCACCGTTTTGCCTGAAAGCGATGTA AT |
| crtE-RI F | ATTACATCGCTTTCAGGCAAAACGGTGGTGGTGGTTCAGGTGGTG G |
| GPM RI R | ATCAAATCATTCACTTCAGACTTATTTGGCTTCTTCTTTTTCCA |

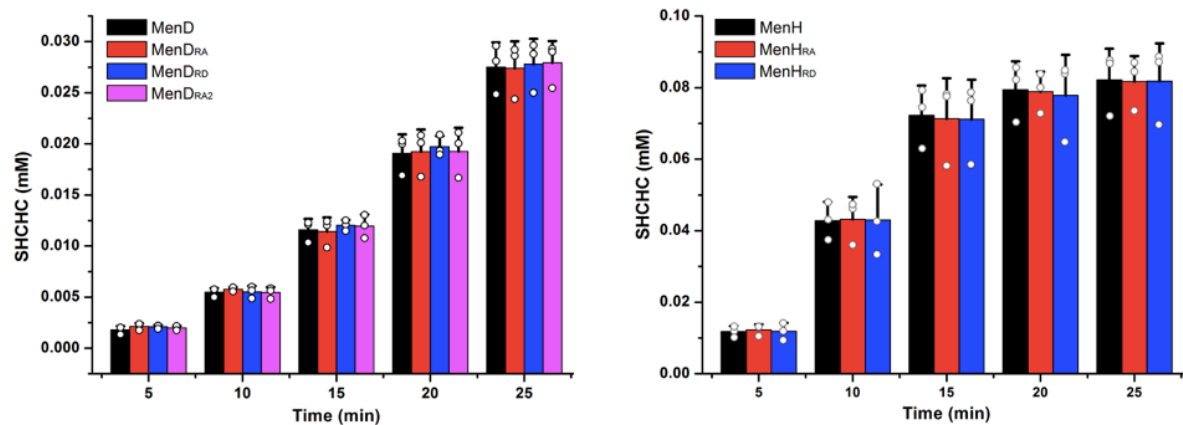
| | |
|----------------------|---|
| RI-GPM F | TGAAAAAGAAGAAGCCAAATAAGTCTGAAGAATGAATGATTG AT |
| crtE_RI-terminator-R | ATTAAGGGTTGTCGACCTGCAGCTATTCGAACTGCCCATTC |
| crtE_hygR-F | GAATGGGCAGTTCGAATAGCTGCAGGTCGACAACCCTTAAT |
| crtE_hygR-R | CATAAAAAAAGAGAATCTTCCACTAGTGGATCTGATATC |
| crtE_right-F | GATATCAGATCCACTAGTGGAAAGATTCTCTTTTTTTATG |
| crtE_right-R | TCTTCCATCAGATTTGGTATTGGTA |
| idi left F | ACAAAGAGCCACTGAAAAAATAA |
| RIAD idi R | CCACCACCTGAACCACCACCACCTAGCATTCTATGAATTTGCCTG T |
| idi RIAD F | ACAGGCAAATTCATAGAATGCTAGGTGGTGGTGGTTCAGGTGGTG G |
| Ter RIAD R | GTAGCCTATATTATTGACGCGTTGTTAGCAACCTTCGGTTGCTTC |
| RIAD Ter F | GAAGCAACCGAAGGTTGCTAACAACGCGTCAATAATATAGGCTAC |
| G418 Ter R | TAAGGGTTGTCGACCTGCAGCGTGAAGAGAAAAAAAAAATGTG AAC |
| TER G418 F | G TTCACATTTTTTTTTCTCTTCACGCTGCAGGTCGACAACCCTTA |
| MRP G418 R | GAATATAATCAAGGGAAATTGACCACTAGTGGATCTGATATCACC |
| G418 MRP F | GGTGATATCAGATCCACTAGTGGTCAATTTCCCTTGATTATATTC |
| MRP R | ATGTAGTGAAGTTTCTAAAC |
| 1530-F | GTATAAGAAGGAGATATACATAGGAGGATTACACTATGCCGCGGT A |
| 1530-R | CGCAGCAGCGGTTTCTTTACCAGACTCGAGTTAGCTCTCACCACGC CATA |
| 15322-1-F | GTTAAGTATAAGAAGGAGATATACATATGCAAACGGAACACGTCATT |
| 15322-1-R | CGGCATAGTGTAATCCTCCTTTAGCAACCTTCGGTTGCT |
| 15322-2-F | AGCAACCGAAGGTTGCTAAAGGAGGATTACACTATGCCG |
| 15322-2-R | CAGCGGTTTCTTTACCAGACTCGAGTTAGCTCTCACCACGCCATA AGCG |
| 15301-1-F | GCTGTCGAAATGGTTCATGCGGCTAGCCTGATCCTGGATGACATGC |
| 15301-1-R | TTGCATGCCTCCGCCGCTGCCGCCGACACCGCTGCCAGTTTTTT ATCG |
| 15301-2-F | TGTCGGGCGGCAGCGGCGGAGGCATGCAAACGGAACACGTCATT TTATTG |
| 15301-2-R | GGTTTCATGGTTAATTCCTCCTTTATTTAAGCTGGGTAAATGCAG |
| 15301-3-F | CTGCATTTACCAGCTTAAATAAAGGAGGAATTAACCATGAAACC |
| 15301-3-R | CAGGTGTTTCATCTTCGATGTAGCTAGCAACTTTAGAATACACGCTA CGCC |
| 15302-1-F | TTGTGAGCGGATAACAATCCCTCTAGACCATCTTAGTATATTAGTTA |
| 15302-1-R | TCATGCCTCCGCCGCTGCCGCCTTTAAGCTGGGTAAATGCAG |
| 15302-2-F | CAGCTTAAAGGCGGCAGCGGCGGAGGCATGACCGTGTGTGCGAAAAA AC |
| 15302-2-R | GCGGCCTGCATGGGCGTCATCCACGATATCACGTGCAATATTCGTCAGC T |



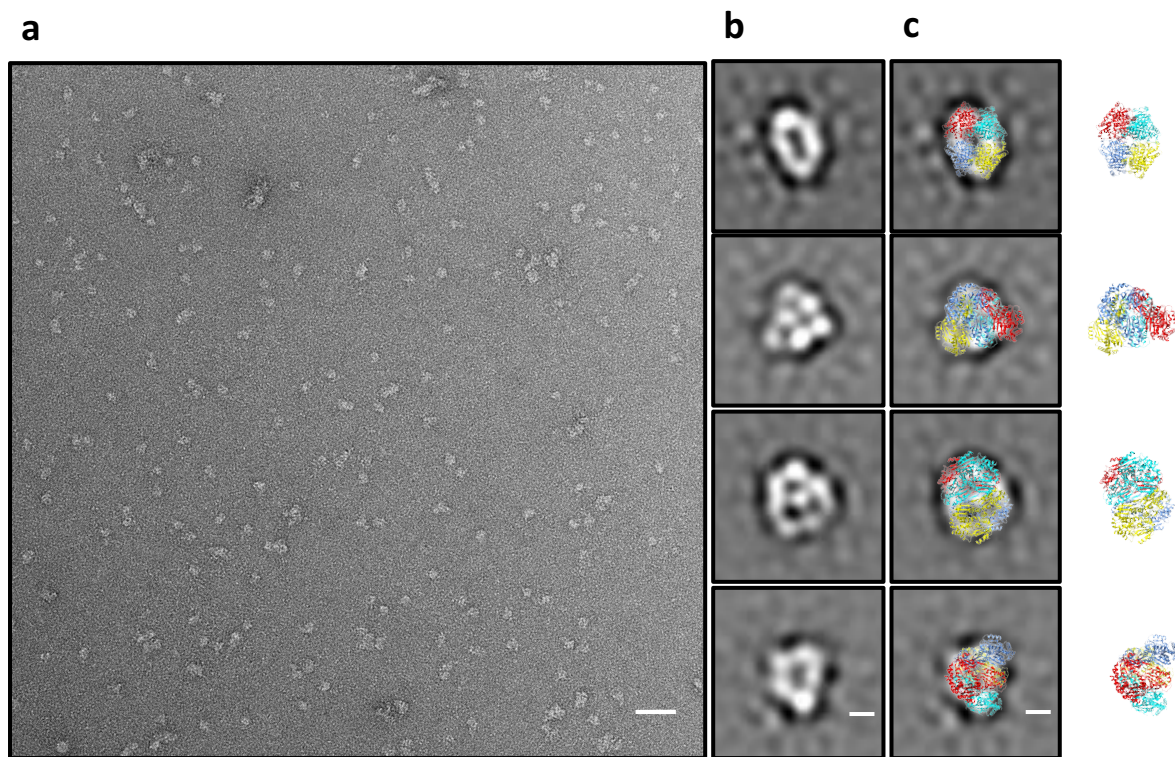
Supplementary Figure 1. Purified MenH_{RD} (lane 1), MenH_{RA} (lane 2), MenD_{RA} (lane3), MenD_{RA2} (lane 4) and MenD_{RD} (lane 5) analyzed by SDS-PAGE. M, marker. Source data are provided as a Source Data file.



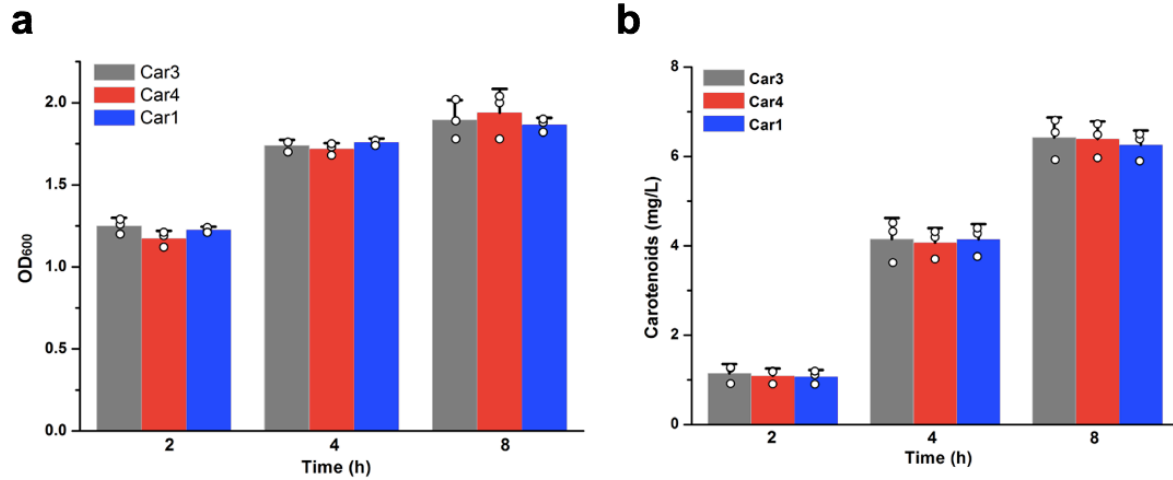
Supplementary Figure 2. Purified MenD (left) elutes as a tetramer, and MenH (right) as a monomer on Superdex 200 10/300 GL in SEC. Source data are provided as a Source Data file.



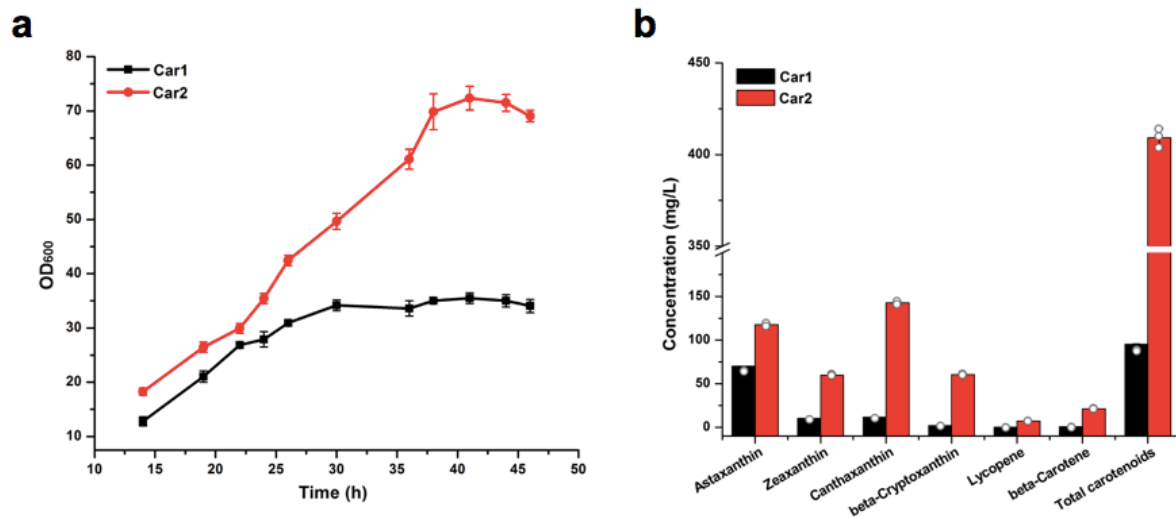
Supplementary Figure 3. Peptide tagging did not change the activity of MenD (left) and MenH (right). In the left, black column: MenD; red column: MenD_{RA}; blue column: MenD_{RD}; pink column: MenD_{RA2}. In the right, black column: MenH; red column: MenH_{RA}; blue column: MenH_{RD}. Error bars indicate the standard deviations of three biological replicates. Source data are provided as a Source Data file.



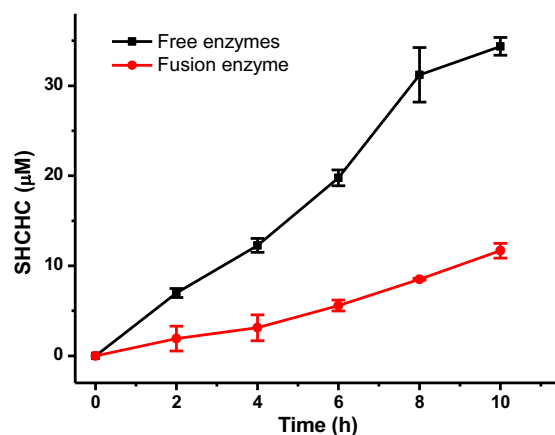
Supplementary Figure 4. TEM images of the Assembly B. **a** Raw electron micrograph. Scale bar, 50 nm. **b** 2D classification performed using RELION 2.0. Scale bar, 5 nm. **c** Superimposition of the crystal structure of MenD tetramer (PDB ID 2JLA) to the 2D classes averages. Scale bar, 5 nm. Source data are provided as a Source Data file.



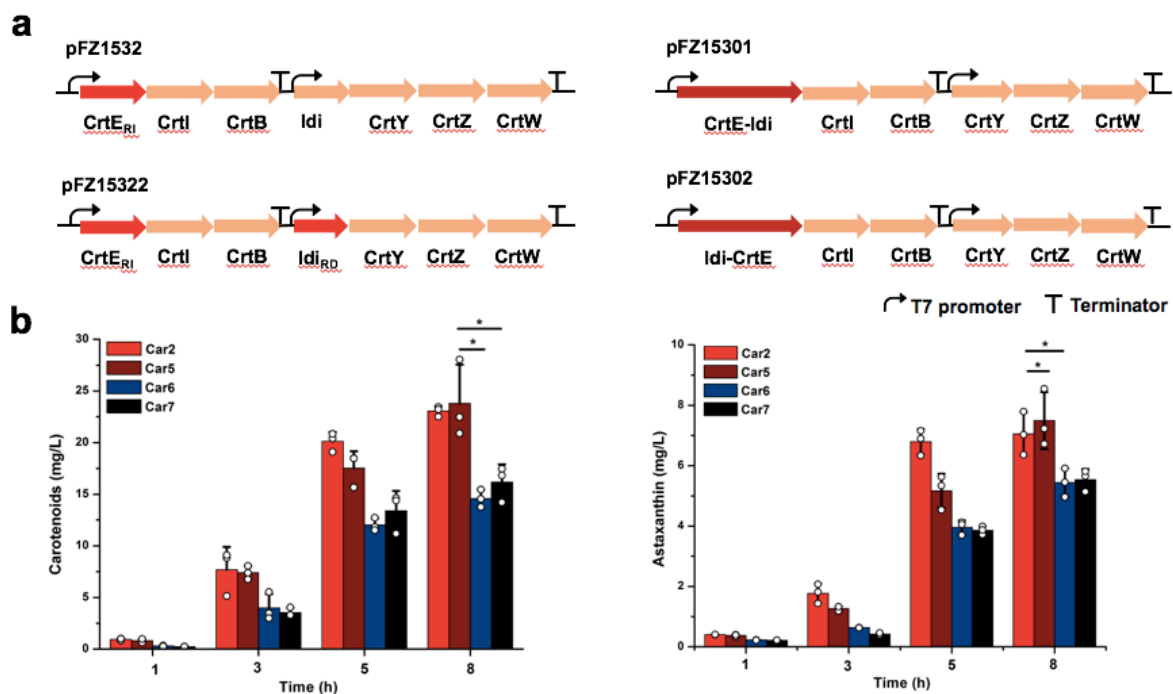
Supplementary Figure 5. Strains Car3 and Car4 have identical carotenoids production rates and growth rates as the untagged control Car1 in shake-flask fermentation. **a** The growth of strains. **b** The accumulation of carotenoids. Grey column: Car3; red column: Car4; blue column: Car1. Error bars indicate the standard deviations of three biological replicates. Source data are provided as a Source Data file.



Supplementary Figure 6. Independent fed-batch fermentations showing the same trend of carotenoid production. **a** Comparison of the growth curve and the yield of overall carotenoids of Car2 and Car1 in fed-batch fermentation. Black line: Car1; red line: Car2. **b** Comparison of the product of main carotenoids. Black column: Car1; red column: Car2. Error bars indicate the standard deviations of three replicates. Source data are provided as a Source Data file.



Supplementary Figure 7. Comparison of enzyme activity of the MenD-MenH fusion enzyme and free MenD and MenH in 1:1 ratio, showing the enzyme fusion has significantly reduced activity. The activity of converting isochorismate to SHCHC by MenD and MenH sequentially was compared, and the generation rate of SHCHC was recorded. Black line: Free enzymes; red line: Fusion enzyme. Error bars indicate the standard deviations of two replicates. Source data are provided as a Source Data file.



Supplementary Figure 8. Comparison of the assembly and fusion strains, showing that strains with enzyme assembly have a higher product titer during shake-flask fermentation. **a** Construction of Car5-7 strains. Car5 is an assembly strain with both Idi and CrtE under T7 promoter control in the same plasmid. Car6 and Car7 are fusion strains containing CrtE-Idi or Idi-CrtE fusion enzymes respectively. Car2 contains plasmids pMH1, pFZ82, pFZ1532. Car5 contains plasmids pMH1, pFZ81, pFZ15322. Car6 contains plasmids pMH1, pFZ81, pFZ15301. Car7 contains plasmids pMH1, pFZ81, pFZ15302. **b** The comparison of astaxanthin and total carotenoids production titer between the four strains. The two assembly strains Car2 and Car5 produce significantly higher (about 40% higher) product titer than the two fusion trains Car6 and Car7. Red column: Car2; wine column: Car5; blue column: Car6; black column: Car7. $*P < 0.05$ (Student's t-test: three-tailed, three-sample equal variance). Error bars indicate the standard deviations of three biological replicates. Source data are provided as a Source Data file.

Supplementary References

1. Zhu, F. et al. *In vitro* reconstitution of mevalonate pathway and targeted engineering of farnesene overproduction in *Escherichia coli*. *Biotechnol. Bioeng.* **111**, 1396-1405 (2014).
2. Ma, T. et al. Genome mining of astaxanthin biosynthetic genes from *Sphingomonas* sp. ATCC 55669 for heterologous overproduction in *Escherichia coli*. *Biotechnol. J.* **11**, 228-237 (2016).
3. Ma, T. et al. Lipid engineering combined with systematic metabolic engineering of *Saccharomyces cerevisiae* for high-yield production of lycopene. *Metab. Eng.* **52**, 134-142 (2019).