Supplementary Information

Record-high biotin production in *Pseudomonas mutabilis* via multi-strategy metabolic engineering and BioB redesign

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Table S1 Biotin production in engineered microorganisms

Strain	Bioreactor	Time	Yield	Reference
E. coli (pBO30A-15/9)	2 L Fed-batch	1 d	35 mg L^{-1}	[1]
E. coli (pTG3410)	10 L Fed-batch	48 h	45 mg L^{-1}	[2]
P. mutabilis (pBBR1M-bioWIAFD)	2 L Fed-batch	168 h	271.88 mg L^{-1}	[3]
B. subtilis	7 L Fed-batch	30 h	21 mg L^{-1}	[4]
Kurthia Sp.(pYK114)	50 mL Flask	120 h	39 mg L^{-1}	[5]
S. marcescens (pLGM304PA)	Fed-batch	11 d	600 mg L^{-1}	[6]
Agrobacterium/Rhizobium HK4 (pBO30- A15/9 (pBO47))	Fed-batch	20 d	110 mg L^{-1}	[1]
Sphingomonas sp *(pSP304)*	1 L Batch	120 h	66 mg L^{-1}	[7]
C. utilis	30 mL Flask	72 h	1.89 mg L^{-1}	[8]

Table S2 Strains used in this study

Strain name	Characterize	Source
P. mutabilis	Wild-type *Pseudomonas mutabilis* ATCC31014	Lab stock
E.coli BL21	-	Lab stock
E.coli DH5α	-	Lab stock
Lactobacillus plantarum ATCC8014	-	Lab stock
E.coli BL21 ΔyigM ΔbioB	*E.coli BL21 ΔyigM ΔbioB*	This study
PM-VIII	-	Lab stock
PM-IX	PM-VIII-*iscR*	This study
PM-X	PM-VIII-*iscRΔglpR*	This study
PM-XI	PM-X-*WIAFDBorf23::ΔbdhA*	This study
PM-XII	PM-X-*WIAFDBorf23WIAFDBorf23::Δmuc*	This study
PM-XIII	PM-X-*WIAFDBorf23WIAFDBorf23::ΔmucWIAFDBorf23::Δpp_1735*	This study
PM-XIV	PM-X-*WIAFDBorf23WIAFDBorf23::ΔmucWIAFDBorf23::Δpp_1735 bioZ:: ΔgcdH*	This study
PM-XV	PM-X-*WIAFDBorf23WIAFDBorf23::ΔmucWIAFDBorf23::Δpp_1735 bioZ:: ΔgcdH PA5530::Δcds1*	This study
PMX-XVI	PM-X-*WIAFDBorf23WIAFDBorf23::ΔmucWIAFDBorf23::Δpp_1735 bioZ:: ΔgcdH PA5530::Δcds1 sam2 matBC ::ΔnicA*	This study
PMX-XVII	PM-X-*WIAFDBorf23WIAFDBorf23::ΔmucWIAFDBorf23::Δpp_1735 bioZ:: ΔgcdH PA5530::Δcds1 sam2 matBC ::ΔnicA fabG pp_0067 pp_1647::ΔtesA*	This study
PMX-XVIII	PM-X-*WIAFDBorf23WIAFDBorf23::ΔmucWIAFDBorf23::Δpp_1735 bioZ:: ΔgcdH PA5530::Δcds1 sam2 matBC ::ΔnicA fabG fabA fabI::ΔtesA*	This study
PMX-XIX	PM-X-*WIAFDBorf23WIAFDBorf23::ΔmucWIAFDBorf23::Δpp_1735 bioZ:: ΔgcdH PA5530::Δcds1 sam2 matBC ::ΔnicA fabG pp_0067 pp_1647::ΔtesA aas-acpS-acpP::ΔnicP*	This study
PM-XX	PM-X-*WIAFDBorf23WIAFDBorf23::ΔmucWIAFDBorf23::Δpp_1735 bioZ:: ΔgcdH PA5530::Δcds1 sam2 matBC ::ΔnicA fabG pp_0067 pp_1647::ΔtesA aas-acpS-acpP::ΔnicPPnpt2-iscR*	This study
PMX-XX (H107Y)	PM-X-*WIAFDBorf23WIAFDBorf23::ΔmucWIAFDBorf23::Δpp_1735 bioZ:: ΔgcdH PA5530::Δcds1 sam2 matBC ::ΔnicA fabG pp_0067 pp_1647::ΔtesA aas-acpS-acpP::ΔnicPPnpt2-iscR*（H107Y）	This study
PMX-XX (L15F)	PM-X-*WIAFDBorf23WIAFDBorf23::ΔmucWIAFDBorf23::Δpp_1735 bioZ:: ΔgcdH PA5530::Δcds1 sam2 matBC ::ΔnicA fabG pp_0067 pp_1647::ΔtesA aas-acpS-acpP::ΔnicPPnpt2-iscR*（L15F）	This study
PMX-XX (C92Y)	PM-X-*WIAFDBorf23WIAFDBorf23::ΔmucWIAFDBorf23::Δpp_1735 bioZ:: ΔgcdH PA5530::Δcds1 sam2 matBC ::ΔnicA fabG pp_0067 pp_1647::ΔtesA aas-acpS-acpP::ΔnicPPnpt2-iscR*（C92Y）	This study
PM-XXI	PM-XX/pCr-bioWIorf23FADB232	This study

Table S3 Plasmids used in this study

Name	Relevant genotype	Resistance	Source
pUCPK	*E. coli–Pseudomonas* shuttle plasmid containing the *Pseudomonas* replicon pRO1600	Kan^r	Lab stock
pRK2-Cpf1	Cas12a expression	Kan^r	(Sun et al., 2018)
pSEVA-gRNA	Expression of crRNA and homology-arm sequences	Gm^r	(Sun et al., 2018)
pCrRNA-iscR	Complementation of the mutant iron-sulfur-cluster transcriptional regulator IscR	Gm^r	This study
pCrRNA-ΔglpR	Deletion of the glycerol transcriptional repressor GlpR	Gm^r	This study
pCrRNA-Δpp_1735	Deletion of the *pp_1735* gene	Gm^r	This study
pCrRNA-Δpp_0063	Deletion of the *pp_0063* gene	Gm^r	This study
pCrRNA-bio-ΔbdhA	Targeted integration of the *bioW-I-orf23-FADB* gene cluster at the *bdhA* locus	Gm^r	This study
pCrRNA-bio-Δmuc	Targeted integration of the *bioW-I-orf23-FADB* gene cluster at the *muc* locus	Gm^r	This study
pCrRNA-bio-Δpp_1735	Targeted integration of the *bioW-I-orf23-FADB* gene cluster at the *pp_1735 locus*	Gm^r	This study
pUCPK-bioA-PM	Overexpression of the *bioA* gene derived from *P. mutabilis*	Kan^r	This study
pUCPK-bioF-PM	Overexpression of the *bioF* gene derived from *P. mutabilis*	Kan^r	This study
pUCPK-bioD-PM	Overexpression of the *bioD* gene derived from *P. mutabilis*	Kan^r	This study
pUCPK-bioB-PM	Overexpression of the *bioB* gene derived from *P. mutabilis*	Kan^r	This study
pUCPK-bioA-E	Overexpression of the *bioA* gene derived from *E. coli BL21*	Kan^r	This study
pUCPK-bioF-E	Overexpression of the *bioF* gene derived from *E. coli BL21*	Kan^r	This study
pUCPK-bioD-E	Overexpression of the *bioD* gene derived from *E. coli BL21*	Kan^r	This study
pUCPK-bioB-E	Overexpression of the *bioB* gene derived from *E. coli BL21*	Kan^r	This study
pUCPK-bioA-S	Overexpression of the *bioA* gene derived from *Serratia marcescens*	Kan^r	This study
pUCPK-bioF-S	Overexpression of the *bioF* gene derived from *S. marcescens*	Kan^r	This study
pUCPK-bioD-S	Overexpression of the *bioD* gene derived from *S. marcescens*	Kan^r	This study
pUCPK-bioB-S	Overexpression of the *bioB* gene derived from *S. marcescens*	Kan^r	This study

pUCPK-bioA-C	Overexpression of the *bioA* gene derived from *S. cerevisiae*	Kan^r	This study
pUCPK-bioF-C	Overexpression of the *bioF* gene derived from *S. cerevisiae*	Kan^r	This study
pUCPK-bioD-C	Overexpression of the *bioD* gene derived from *S. cerevisiae*	Kan^r	This study
pUCPK-bioB-C	Overexpression of the *bioB* gene derived from *S. cerevisiae*	Kan^r	This study
pUCPK-bioA-B	Overexpression of the *bioA* gene derived from *B. subtilis*	Kan^r	This study
pUCPK-bioF-B	Overexpression of the *bioF* gene derived from *B. subtilis*	Kan^r	This study
pUCPK-bioD-B	Overexpression of the *bioD* gene derived from *B. subtilis*	Kan^r	This study
pUCPK-bioB-B	Overexpression of the *bioB* gene derived from *B. subtilis*	Kan^r	This study
pCrRNA-bioZ-ΔgcdH	Insertion of *Pnpt2–bioZ–gcdG* into the *PM-X III* chromosome and knockout of *gcdH*	Gm^r	This study
pCrRNA-PA5530::Δcds1	Insertion of *pA5530* into the chromosome of the PM-X IV strain	Gm^r	This study
pCrRNA-*matBC ::*Δ*nicA*	Insertion of the *matBC* gene cluster and knockout of the *tesA* gene in the *PM-XV* strain genome	Gm^r	This study
pCrRNA-*fabG-pp_0067 pp_1647::*Δ*tesA*	Insertion of the *Pnpt2-pp_0067-pp_1647* gene cluster into the genome of the *PM-XVI* strain	Gm^r	This study
pCrRNA-*fabG-fabA fabI::*Δ*tesA*	Insertion of the *Pnpt2-fabGAI* gene cluster into the genome of the *PM-XVI* strain	Gm^r	This study
pCrRNA-*Pnpt2-iscR*	Insertion of *Pnpt2* upstream of the *iscR* gene in the *PM-XVI* strain genome	Gm^r	This study
pCrRNA-*Pnpt2-iscR*（H107Y）	Mutation of the *iscR* gene to IscRH107Y in the *PM-XVI* strain genome	Gm^r	This study
pCrRNA-*Pnpt2-iscR*（L15F）	Mutation of the *iscR* gene to IscRL15F in the *PM-XVI* strain genome	Gm^r	This study
pCrRNA-*Pnpt2-iscR*（C92Y）	Mutation of the *iscR* gene to IscRC92Y in the *PM-XVI* strain genome	Gm^r	This study
pCrRNA-*aas-acpS-acpP::*Δ*nicP*	Insertion of the *Pnpt2–aas–acpP–acpS* gene cluster into the genome of the *PM-XIX* strain	Gm^r	This study
pCas	Expression of Cas9 protein	Kan^r	Lab stock
pTarget-sgRNA-yigM	Knockout of the *yigM* gene in *E. coli* BL21	Spc^r	This study
pTarget-sgRNA-bioB	Knockout of the *bioB* gene in *E. coli* BL21	Spc^r	This study
pUCPK-BioB232Ala	Mutation of the *bioB* gene at position 232 to alanine in the pUCPK-BioB plasmid	Kan^r	This study
pUCPK-BioB68Ala	Mutation of the *bioB* gene at position 68 to alanine in the pUCPK-BioB plasmid	Kan^r	This study
pUCPK-BioB159Ala	Mutation of the *bioB* gene at position 159 to alanine in	Kan^r	This study

	the pUCPK-BioB plasmid		
pUCPK-BioB228Ala	Mutation of the *bioB* gene at position 228 to alanine in the pUCPK-BioB plasmid	Kan^r	This study
pUCPK-BioB62Ala	Mutation of the *bioB* gene at position 62 to alanine in the pUCPK-BioB plasmid	Kan^r	This study
pUCPK-BioB161Ala	Mutation of the *bioB* gene at position 161 to alanine in the pUCPK-BioB plasmid	Kan^r	This study
pUCPK-BioB174Ala	Mutation of the *bioB* gene at position 174 to alanine in the pUCPK-BioB plasmid	Kan^r	This study

Table S4 Primers used in this study

Name	Sequence 5'→3'	Application
PMB-F	agctcggtacccggggatctctagatttcgacggagca	pUCPK-bioB-PM
PMB-R	cgatgggagggaacgatgaaaggtcgatcgatcg	
PMD-F	cgtagcaagactcgatggacacaaagcgctggctgc	pUCPK-bioD-PM
PMD-R	aattccgggctggctaagctagtcgatcgatg	
PMA-F	ctacgtacgtacacgtgtgatttgcag	pUCPK-bioA-PM
PMA-R	ctgatcgtagatcgatcgatcgatcga	
PMF-F	cgtacgtagctacgacacacatgctagctag	pUCPK-bioF-PM
PMF-R	cagtcagtcagtcagtcagtcagtcagtcagc	
SHA-F-F	cagacggcgatcgatcgtattagcacgatcg	pUCPK-bioF-S
SHA-F-R	cgatcgatcgatcgatcgatcgatcgatcgat	
SHA-D-F	cgatcgatcgatcgatgctagctagcatcgatc	pUCPK-bioD-S
SHA-D-R	cgatcgatcgatcgatcgatcgatgcatcgatc	
SHA-B-F	cgatcgatcgatcgatcgatcgatcgatcgatc	pUCPK-bioB-S
SHA-B-R	cgatcgatcgatcgatgcatcgatcgatcgta	
SHA-A-F	ttaacgcgcggcggcggccacggcg	pUCPK-bioA-S
SHA-A-R	atgtctgtcaccgcttccgacctggc	
C-B-F	cgatcgatcgatcgatcg	pUCPK-bioB-C
C-B-R	atcgatcgatgcatcgatcgatgc	
C-A-F	cgatcagtcgatcagtccc	pUCPK-bioA-C
C-A-R	atgtagtagctagtcgtaag	
C-D-F	cgatcgatcgatcgatagtcagtc	pUCPK-bioD-C
C-D-R	cgatcgatcgatcgatcgttacgatgctagct	
C-F-F	cgatcgtacgatcgtagctacgt	pUCPK-bioF-C
C-F-R	cgatcgatcattacgatcgctag	
BS-A-F	cgtagctagctacgatcgatcgata	pUCPK-bioA-B
BS-A-R	cgtagcattttagcgcgcgcagt	
BS-B-F	cgatcgatcgatcgatcgatgc	pUCPK-bioB-B
BS-B-R	atcgatcgatgcatgctagcta	
BS-D-F	cagtcagctagctagctagcta	pUCPK-bioD-B
BS-D-R	cgtacgatcgatcgtagctacgta	
BS-F-F	cgtagctagccgctagctacgat	pUCPK-bioF-B
BS-F-R	cgatcgatcgatcgatcgtagct	
XhoI-bioF-R	tttgctgggatctgtcaaaggataaattaactcacactatca	pCrRNA-bio-ΔbdhA
SpeI-bioF-R	tttgctgggatctgtcaaaggataaactagtattaactcacatatcat	
SpeI-orf3-F	atgatagtgtgagttaatactagtttatcctttgacagatcccagcaaa	
XbaI-bdhA-up arm-R	ctagtagcagtgtctagaccgtagttcggttagtgacgacgtgctacgt	
bioA-F-XbaI	ctgtagctgtagagtcgtacgtgtctagattaacgcgcggcggcggccacgg	
XhoI-bioD-R	cgccgtggccgccgccgcgcgttaactcgag	
bdhA-up-R-XbaI	aatgtcatggcctccccgtatgcctaaatcgtctaga	
orf3-SpeI-F	aggcatacggggaggccatgacatttgctgcgcactagtttatcctttgacagatccca	

XbaI-bdhA-up-R	tctagactgatcgtattcgtagtcgatcagctagctaggtagctgctcgtgtagaaacaa	
XhoI-bioA-R	ctcgagttaacgcgcggcggcggccac	
XbaI-bioD-F	tctagacctagctagctagcatcgatcgataatgtgtg	
1735-N23-R-1	atctactttttttgaggagctcggtacccggggatc	pCrRNA-bio-
1735-N23-F-1	cctcgtgctagctgatcgatcgatcgatacgtacgatcgatcgatcgatcgatcgatcg	Δpp_1735
muc-N23-3-F	ccccgtagtcgtcgcttttttttgaggagctcggtacccggggatc	pCrRNA-bio-
muc -N23-3-R	cctacgactgatcgatcgatcgatcgatcgaaagaatggctaa	Δmuc
matBC-R	attccaatttctttggtttttcccatg	
matBC-F	ttaagtataagaaggagatatacatatgtccaagagcaaaactttcttatttacctctg	pCrRNA-
nicA-npt2-up-R	cagctagcagcgtagaaaatggta	*matBC ::ΔnicA*
nicA-down-F	aaaaccaaagaaattggaatcccgagtcgtagctagctagcatcgactagc	
tesA-N23-R-1	tttttttgaggagctcggtacccggggat	pCrRNA-*fabG-*
		pp_0067
		pp_1647::ΔtesA
tesA-N23-F-1	acccctgctagcatcgatcatagcatgctagctagct	pCrRNA-*fabG-*
		fabA
		fabI::ΔtesA
gcdH-N23-6-R	ctacgatcgatcgatcgatcaaaatgctagtagctacgatcagctagctacgatcgta	pCrRNA-bioZ-
gcdH-N23-6-F	tttttttgaggagctcggtacccgggga	ΔgcdH
Kan-R	agccatattcaacgggaaacgtctt	pUCPK
Kan-F	agaaaaactcatcgagcatcaaatg	
iscR-N23-1-R	gatgtgtcagtcgatcagctagctagtcgatcgatacgtgccccgctcgtccccc	pCrRNA-iscR
iscR-N23-1-F	cgtcgtcgatcgtcgcgatcgatcgatgctagccaaaactgtagc	
cds1-F	aacgtagctagcatcaaagctcggctgcgct	pCrRNA-
cds1-F	ccgatcccgtggtagtcgtagatcgatgc	PA5530::Δcds1
68-Met-R	ccgacgagccgtacgtacagtcagctacg	pUCPK-
68-Met-F	ccgtacgatcgatcgatcaagtcgtagtcgatcgt	BioB68Ala
233-Arg-F	cctgatgtttagctagtgtcgatgctagcagtgt	pUCPK-
233-Arg-R	ccgtacgcgtagctagagagggctagctttaggg	BioB233Ala
67-Tyr-F	ggcgtttagctacgatcgatcgatcgatgctagctag	pUCPK-
67-Tyr-R	cgtagaagcgcgtgctagctagctagcatcgatcgtttcag	BioB67Ala
62-Met-R	ccgtgaaacgggcgcgcgcgtagtgtttgaggctgcg	pUCPK-
62-Met-F	ccagcgttcgggagagagcttttaagcgtagc	BioB62Ala
232-Arg-F	ccgatcggcaagcatcgatcgccgaggacgtgca	pUCPK-
232-Arg-R	cgtcgcgcgggatgcttagctgctcgctagctgctgcca	BioB232Ala

Table S5 Accuracy evaluation of bioassay-based biotin quantification

Sample	Biotin titer (mg L^{-1})		
	LC-MS	Paper disk plate assay	Liquid bioassay
PM-XXI	143.76	151.64	155.28

Notes:

1. The standard curves for biotin quantification obtained using the paper disk plate assay, liquid bioassay, and LC-MS methods are shown in Fig. S1, Fig. S2, and Fig. S3, respectively.
2. The engineered strain PM-XXI was cultivated in shake flasks containing 50 mL of IFM medium for 144 h. Biotin concentrations in the fermentation broth were determined using LC-MS, paper disk plate assay, and the liquid bioassay, as summarized in the table above. Compared with the LC-MS method, the relative deviations of the paper disk plate assay and liquid bioassays were 5.48% and 8.01%, respectively.

Table S6 Interaction between BioB mutant K232R and substrate

Hydrogen Bond	Distance（Å）
ASN159	3.8
ASN228	3.0，2.9
SER268	4.1
THR298	4.0
LEU51	3.6
ASN157	2.9
ASN159	3.8

Table S7 Interaction between wild-type BioB and substrate

Hydrogen Bond	**Distance（Å）**
ASN159	3.9
ASN228	3.0
SER268	3.0
THR298	4.1
GLN68	3.9
ASN157	3.6，3.0

Table S8 Interaction between BioB and substrate

Protein	(-)CDDOCKER Energy	(-)CDDOCKER Interaction Energy
WT	43.5	47.7
PK232R	53.0	57.9
PD62N	42.5	47.0
PD62G	46.5	51.8

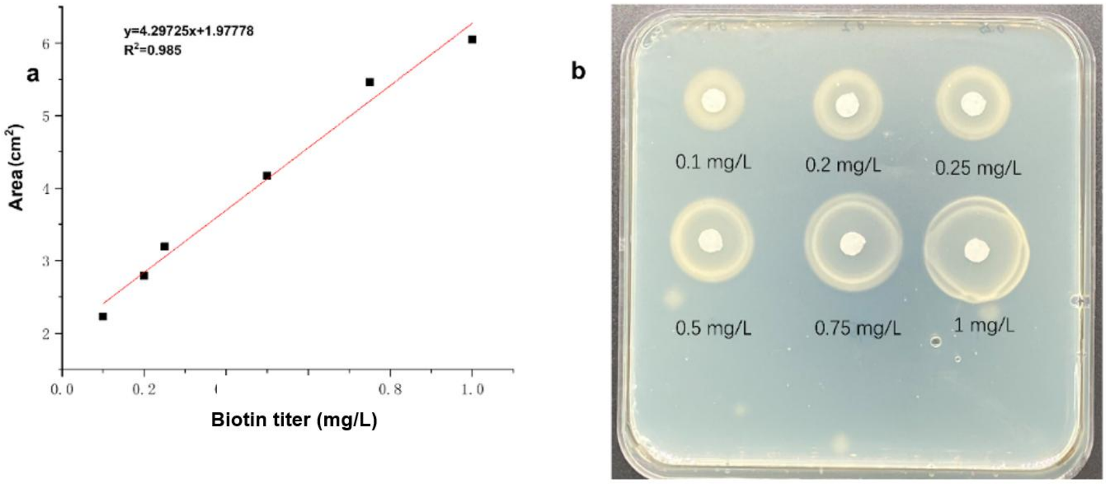


Fig. S1 Standard curve of biotin yield corresponding to the area of the biosphere.

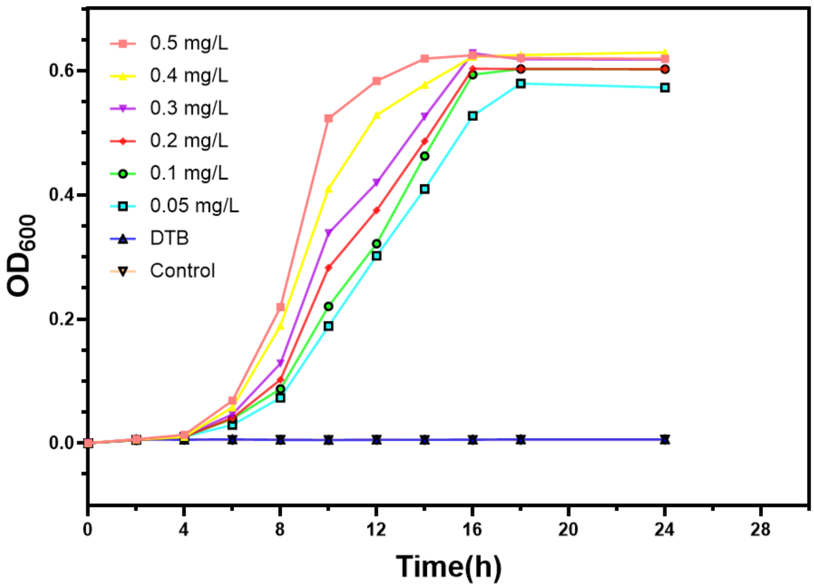


Fig. S2 Growth of *L. plantarum* in detection media containing different biotin concentrations, with OD_{600} measured at 10, 12, and 14 h to determine biotin content.

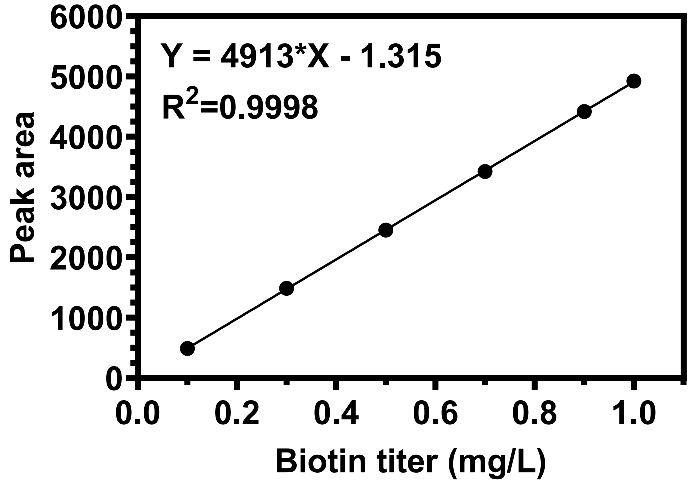


Fig. S3 Standard curve for biotin quantification by LC–MS analysis. Standard solutions of biotin ranging from 0.1 mg/L to 1.0 mg/L were analyzed, and a linear relationship between peak area and biotin concentration was obtained ($Y = 4913 \times X - 1.315$, $R^2 = 0.9998$). The strong linearity indicates high reliability and accuracy of the quantification method.

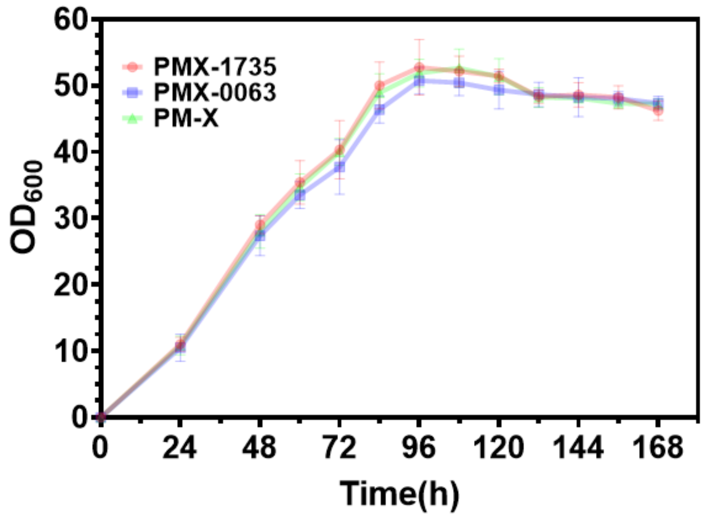


Fig.S4 Growth curves of strains PM-X, PMX-1735, and PMX-0063.

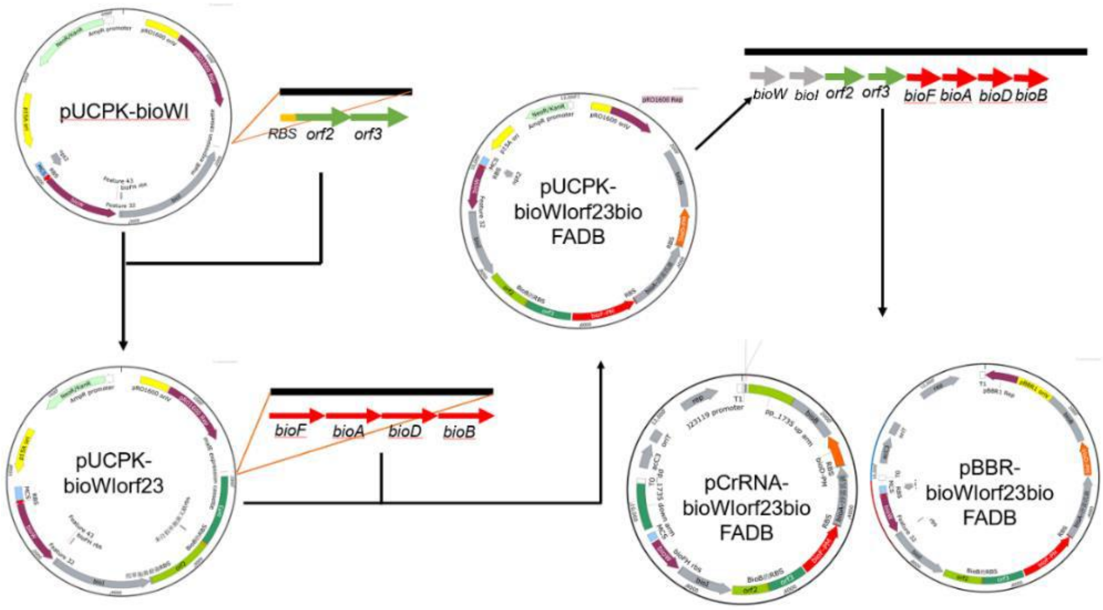


Fig. S5 Construction of plasmids pCr-bioWIorf23bioFADB and pBBR-bioWIorf23bioFADB for synthesizing biotin using pimelic acid as substrate.

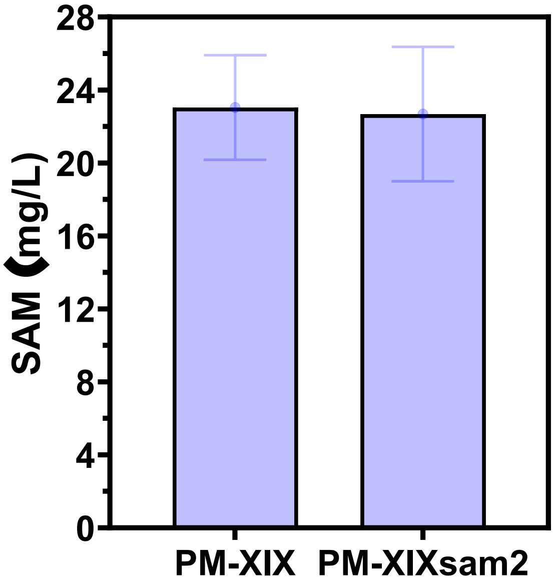


Fig. S6 SAM content of strains PM-XIX and PM-XIXsam2.

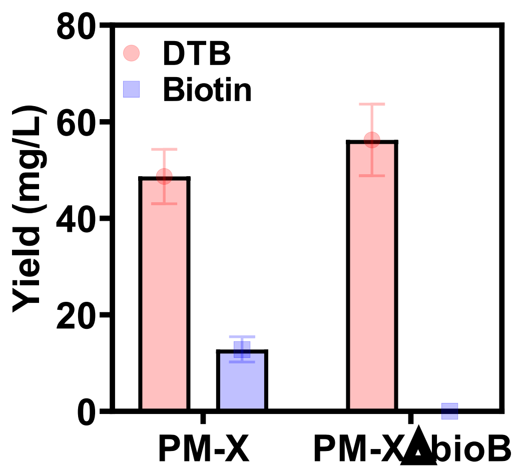
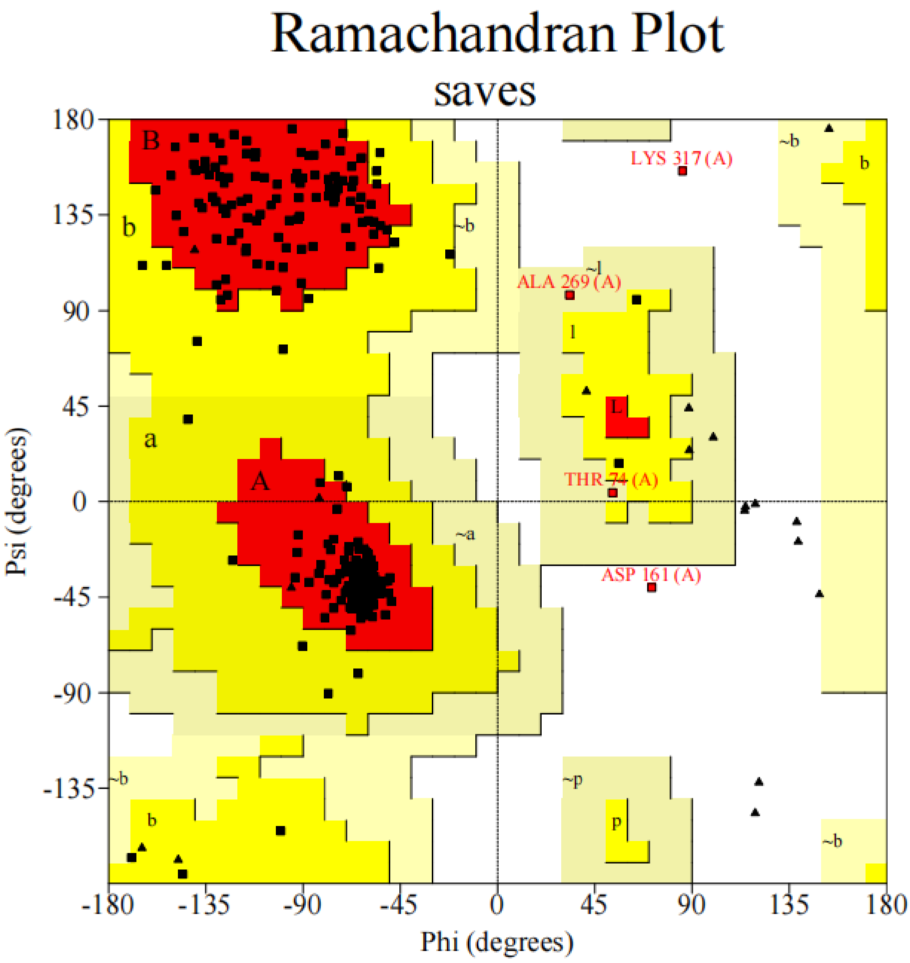


Fig. S7 Biotin and desthiobiotin production profiles of the engineered strain PM-X and its *bioB* knockout mutant during shake-flask fermentation.



Plot statistics

Residues in most favoured regions [A,B,L]	246	90.1%
Residues in additional allowed regions [a,b,l,p]	23	8.4%
Residues in generously allowed regions [~a,~b,~l,~p]	2	0.7%
Residues in disallowed regions	2	0.7%
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Number of non-glycine and non-proline residues	273	100.0%

Fig. S8 Laplace plot analysis of the BioB model reliability.

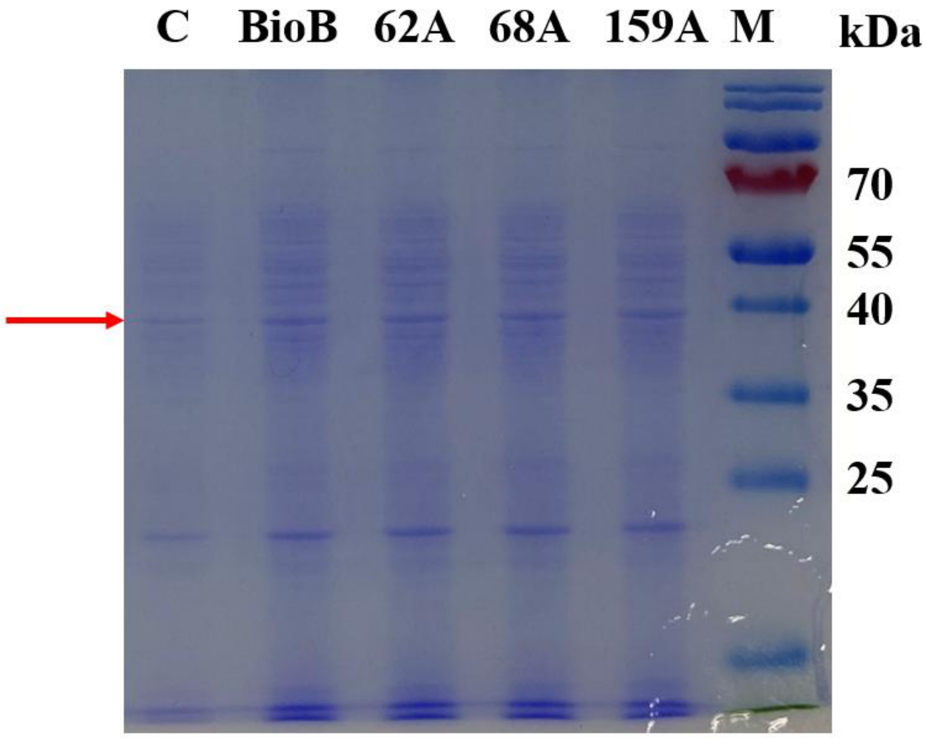

Fig. S9 Expression of BioB and alanine-substitution variants in *E. coli* BL21ΔbioBΔyigM. Whole-cell lysates were analyzed by SDS–PAGE. Lane M, protein marker; Lane C, chassis strain (*E. coli* BL21ΔbioBΔyigM); Lane BioB, BL21ΔbioBΔyigM/pUCPK-BioB; Lanes 62A, 68A, and 159A, BL21ΔbioBΔyigM carrying pUCPK-BioB62A (alanine substitution at residue 62), pUCPK-BioB68A (alanine substitution at residue 68), and pUCPK-BioB159A (alanine substitution at residue 159), respectively. The calculated molecular mass of BioB is ~38.9 kDa.

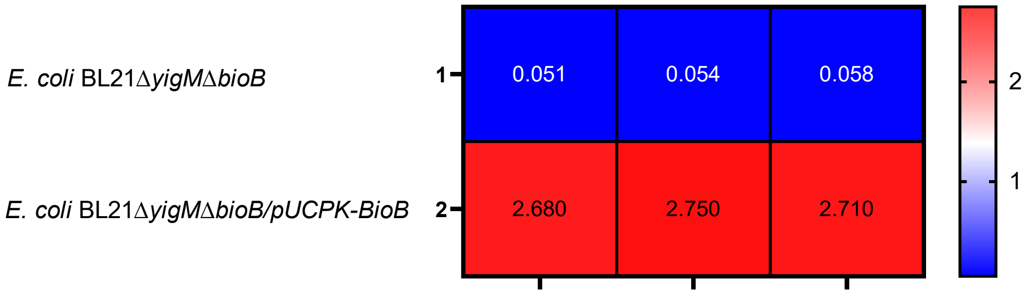

Fig. S10 Growth of *E. coli* BL21ΔyigMΔbioB and BL21ΔyigMΔbioB/pUCPK-BioB.

Numbers in the heatmap indicate the OD_{600} values of *L. plantarum* in the liquid bioassay, reflecting biotin concentration.

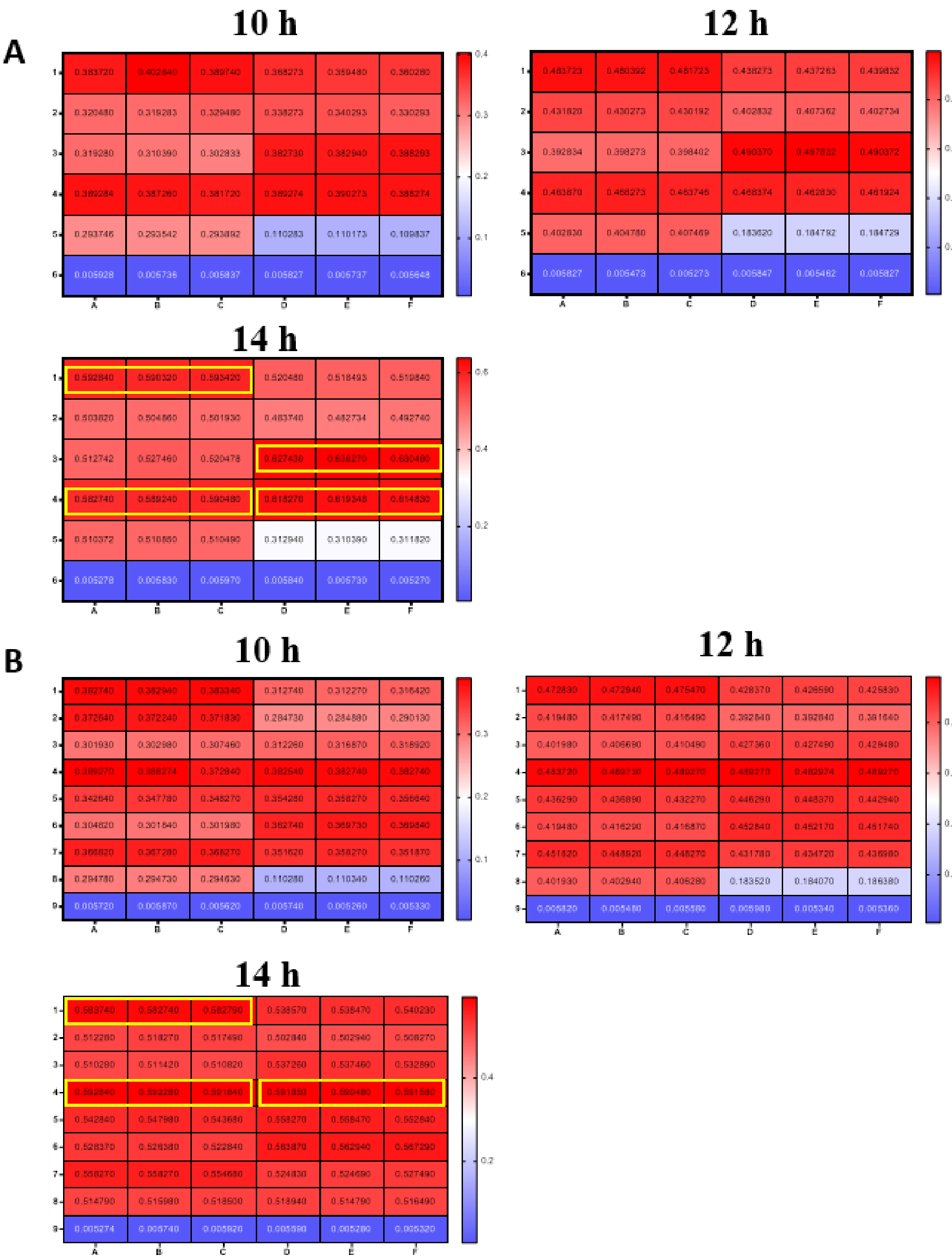

Fig. S11 Screening of BioB site-saturation mutagenesis libraries. A second round of screening identified seven top-performing mutants, which are highlighted within the yellow boxes. Numbers in the heatmap indicate the OD_{600} values of *L. plantarum* in the liquid bioassay, reflecting biotin concentration.

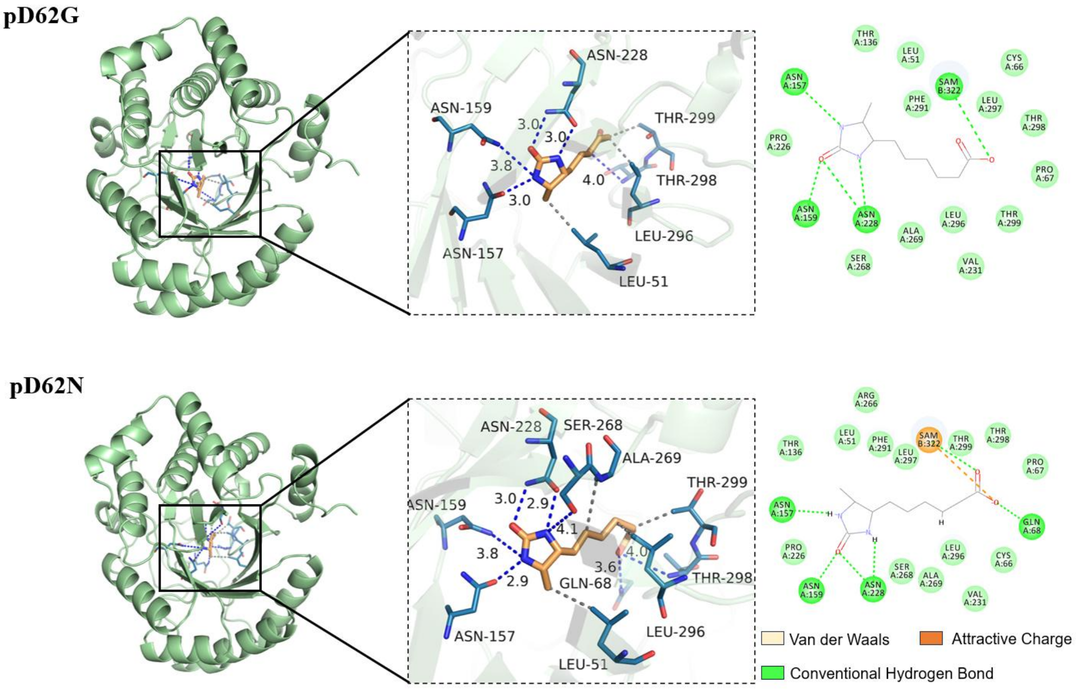

Fig. S12 Molecular docking simulations of substrate interactions with BioB mutants D62G and D62N. Structural models illustrate the overall protein conformation (left), detailed active-site interactions (center), and two-dimensional interaction networks (right)

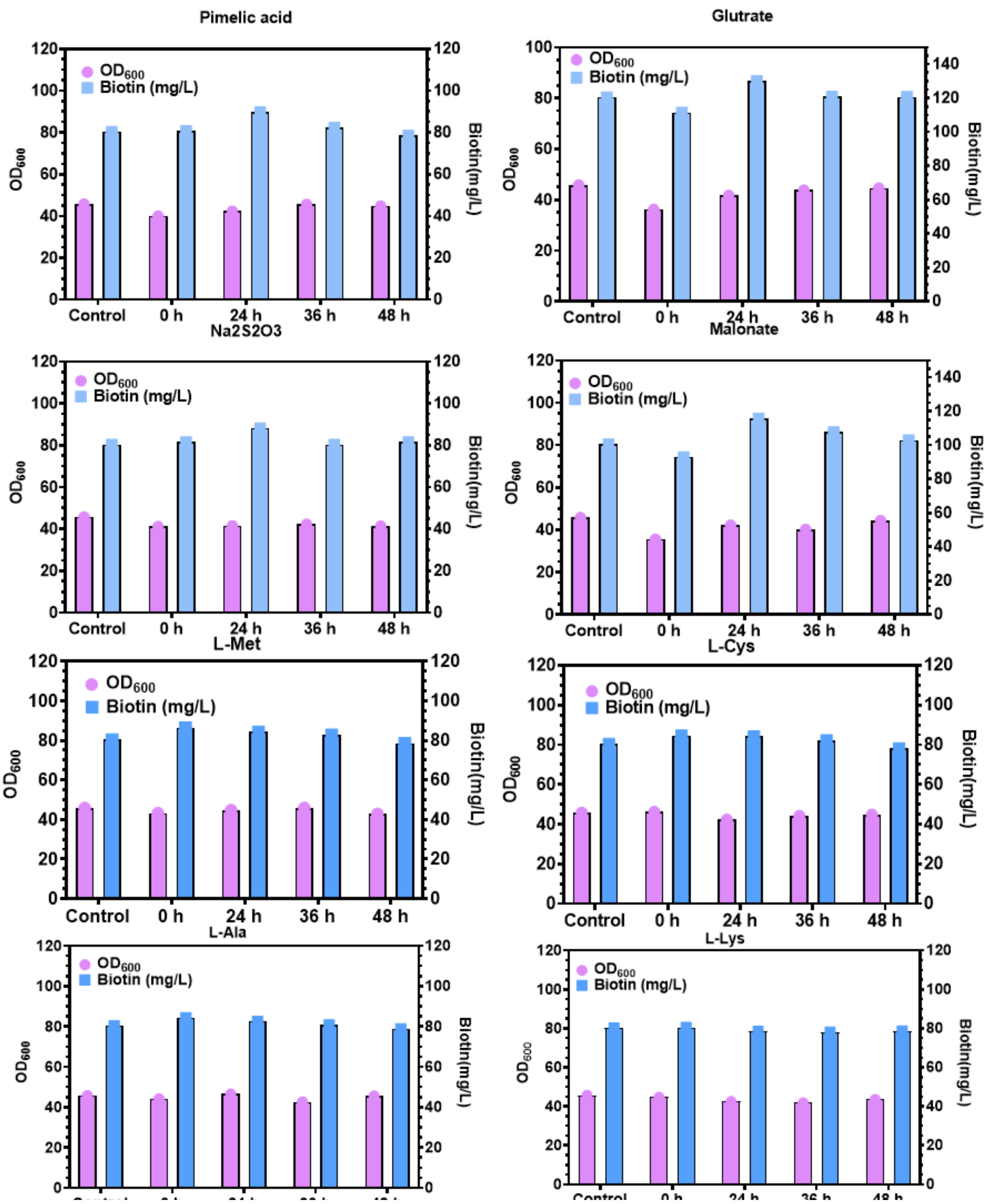

Fig. S13 Effects of different substrate addition times on biotin production and cell mass of engineered bacteria PM-XX/pCr-bioWIorf23FADB.

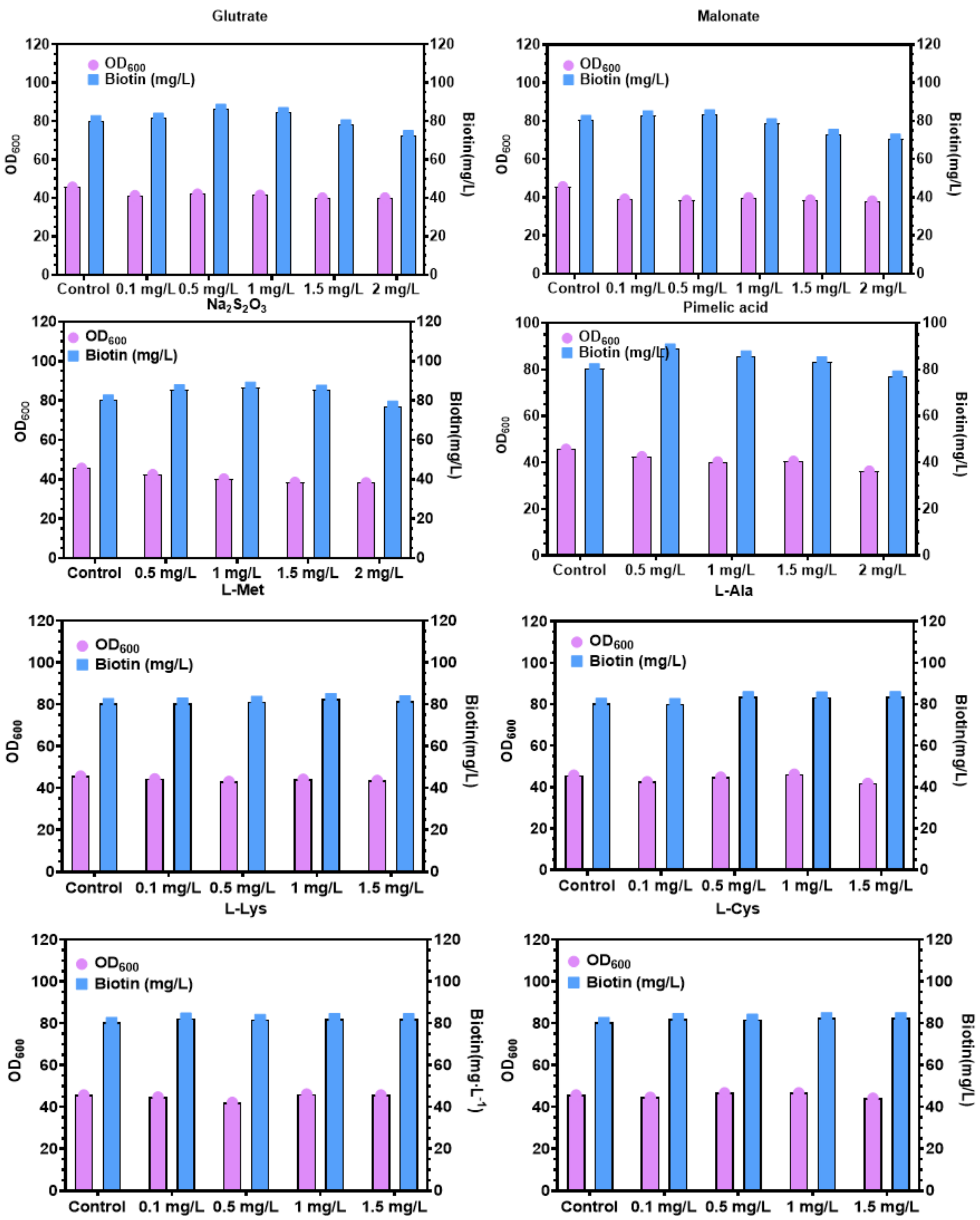

Fig. S14 Effects of different substrate addition concentration on biotin production and cell mass of engineered bacteria PM-XX/pCr-bioWIorf23FADB.

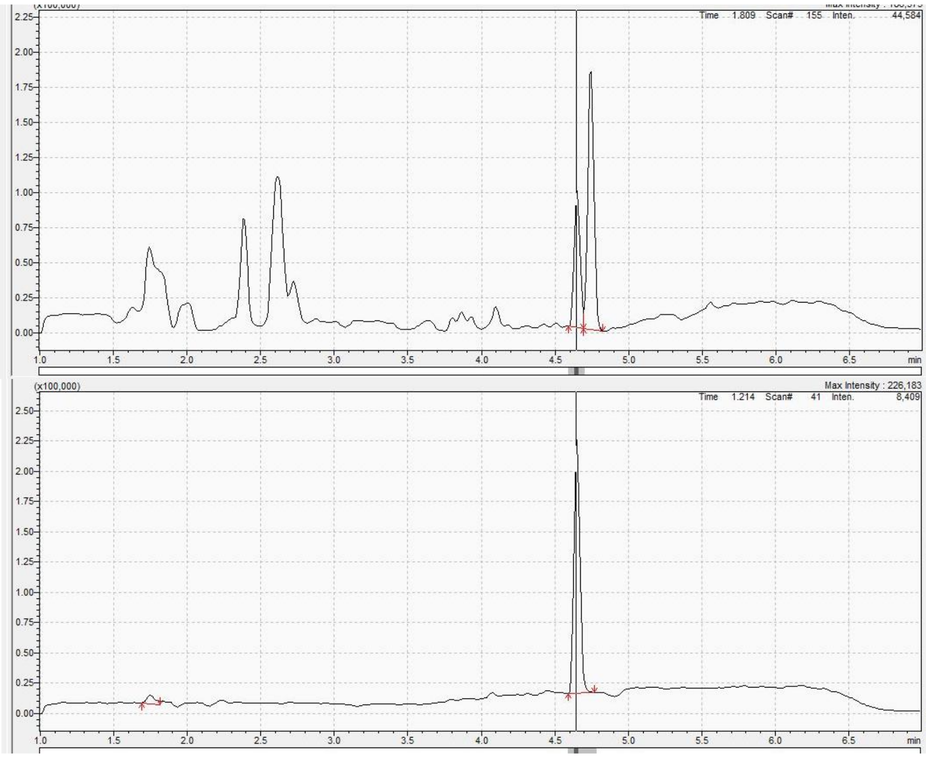

Fig. S15 LC–MS analysis of biotin. The upper panel shows biotin detected in fed-batch fermentation broth, while the lower panel presents the corresponding standard samples.

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