

Supplementary material to

Engineering of *Corynebacterium glutamicum* for biosynthesis of the

pharmaceutically active *N*-acetyltyramine: Establishing and optimizing *de*

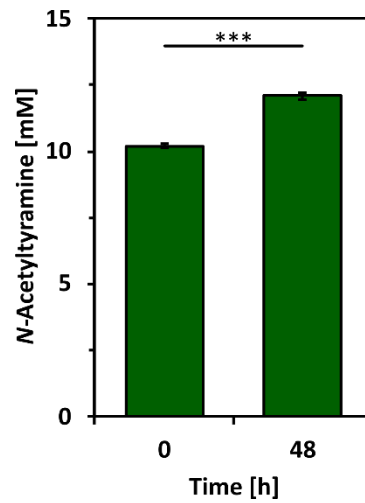
***novo* production**

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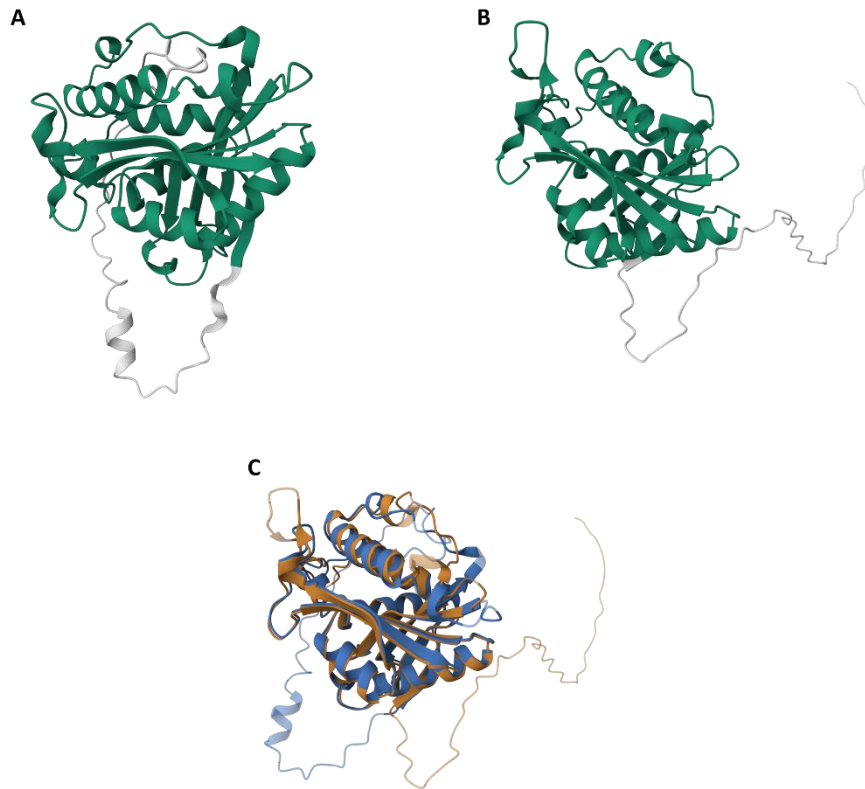
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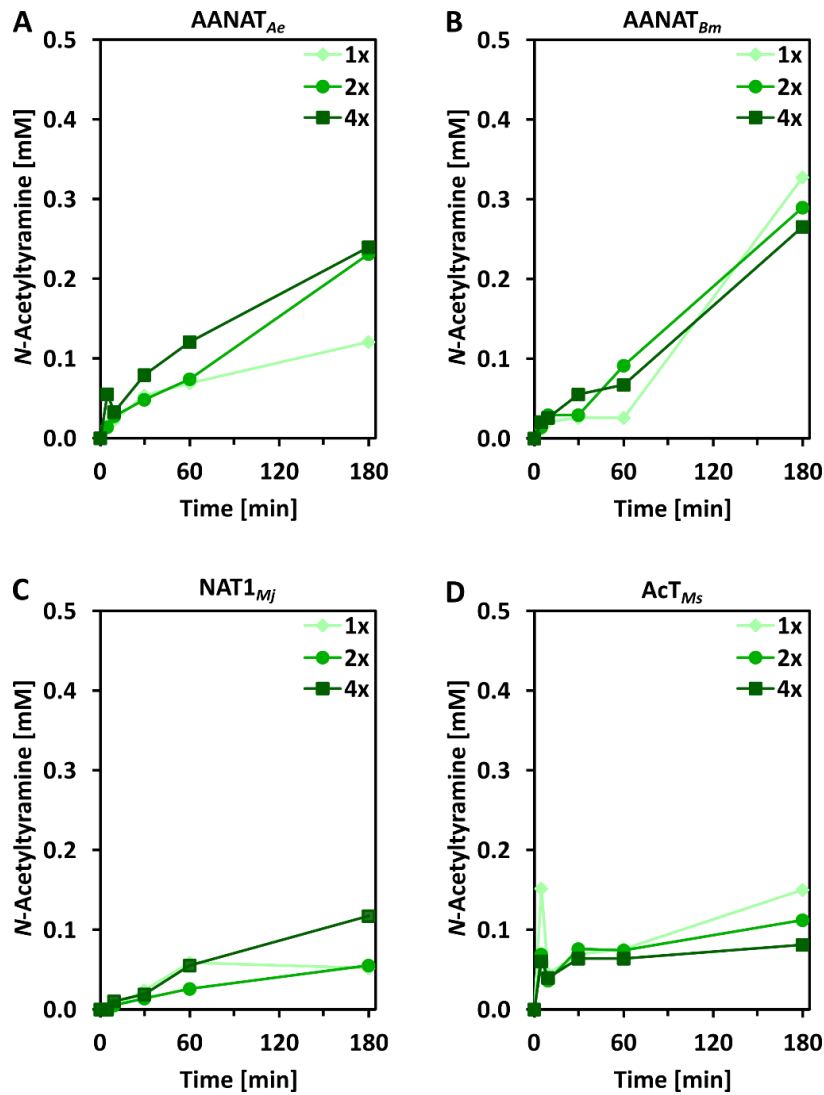


18 **Supplementary Figure S1 *N*-acetyltyramine degradation test.** Strain AROM3 was cultivated for 72 h in shake flasks in 10 mL
19 CGXII minimal medium containing 40 g L⁻¹ glucose, 0.5 mM L-phenylalanine, and 10 mM *N*-acetyltyramine. *N*-Acetyltyramine
20 concentrations were quantified by HPLC at the start and end of cultivation. Values and error bars represent means and
21 standard deviations of triplicate cultivations. Significance was calculated using a two-sided Student's *t*-test with ***: $p < 0.001$.

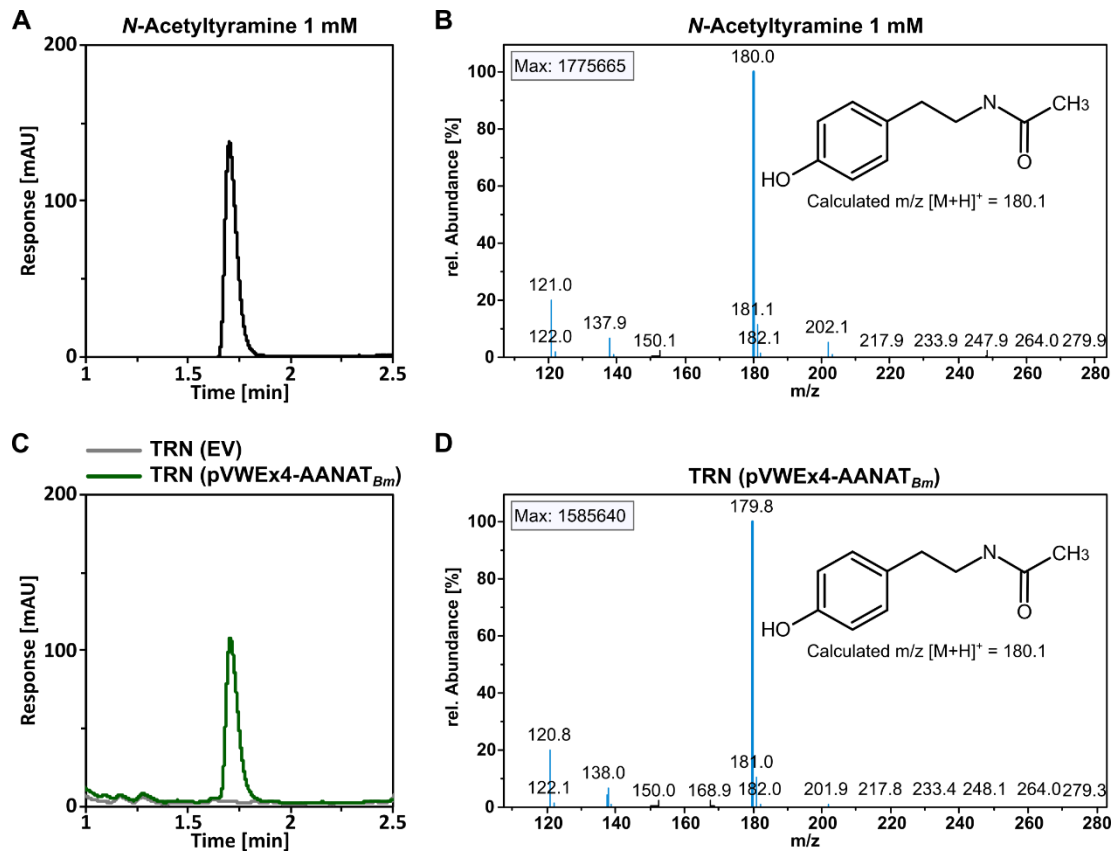
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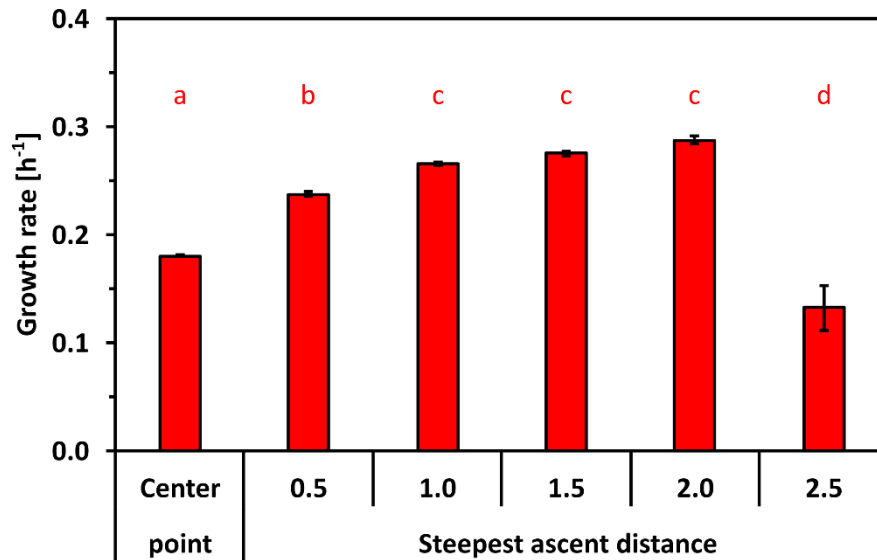
Supplementary Figure S2 Predicted protein structures and alignments of insect arylalkylamine *N*-acetyltransferases. 3D structures of AANAT_{Bm} (A) and AANAT_{Ae} (B) were predicted with AlphaFold [1]. The predicted arylalkylamine *N*-acetyltransferase domains of both AANAT proteins, are illustrated in green. A pairwise structure alignment (C) using the RCSB.org alignment tool [2] illustrates the structural similarity between AANAT_{Bm} (blue) and AANAT_{Ae} (orange).



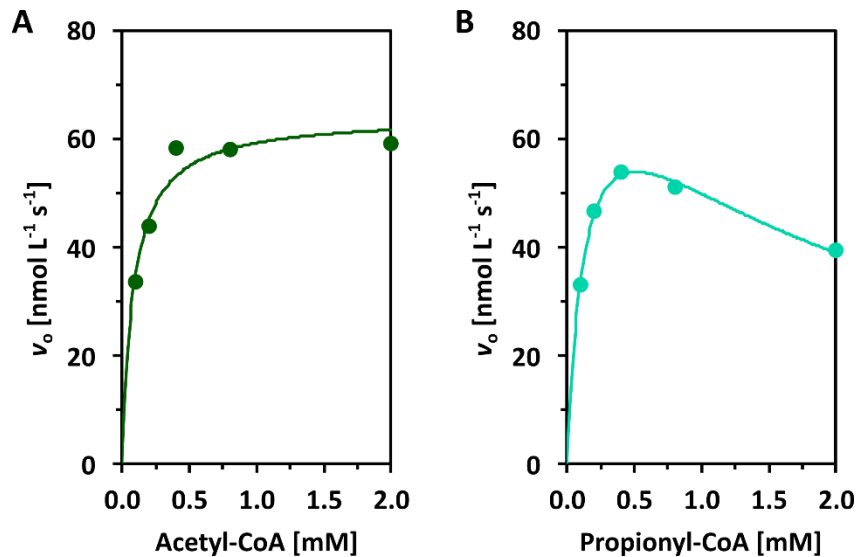
Supplementary Figure S3 Assaying bacterial and insect enzymes for tyramine acetylation. Formation of *N*-acetyltyramine within the first 3 h of an *in vitro* assay using a crude extract of *E. coli* DH5 α strains carrying plasmids encoding AANAT_{Ae} (A), AANAT_{Bm} (B), NAT1_{Mj} (C), or AcT_{Ms} (D). Darker colors indicate multiples of the crude extract concentrations (1x concentrations of crude extracts were: 0.26 mg mL⁻¹ for AANAT_{Ae}, 0.17 mg mL⁻¹ for AANAT_{Bm}, 0.66 mg mL⁻¹ for NAT1_{Mj}, and 0.78 mg mL⁻¹ for AcT_{Ms}). No *N*-acetyltyramine was detected for an assay performed with crude extract from an *E. coli* DH5 α empty vector control strain.



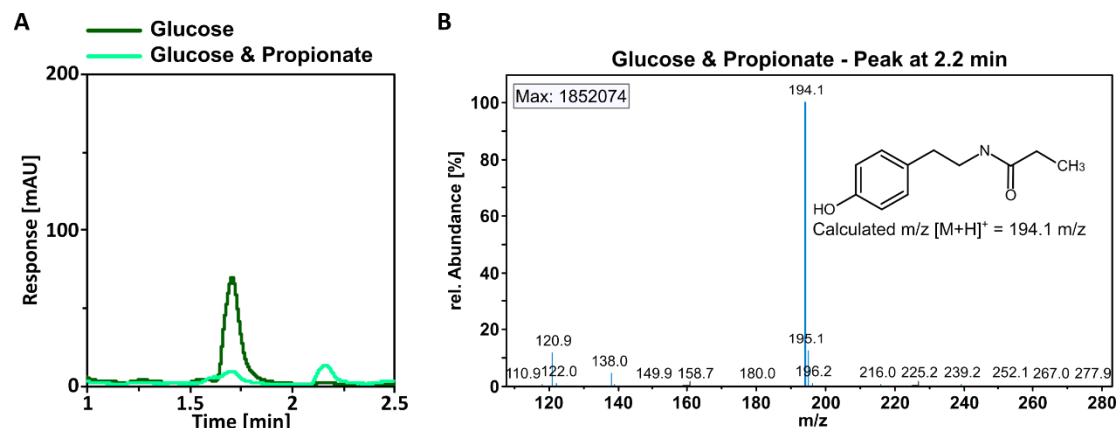
Supplementary Figure S4 LC/MS analysis of TRN (EV) and TRN (pVWEx4-AANAT_{Bm}) culture supernatant. Compared to the chromatogram of the TRN (EV) supernatant (C, grey), an additional peak at 1.7 min was observed in the DAD chromatogram of the TRN (pVWEx4-AANAT_{Bm}) culture supernatant (C, green), recorded at a wavelength of 280 nm, which corresponds to the retention time of the 1 mM *N*-acetyltyramine standard (A). The mass spectrum of the additional peak in the TRN (pVWEx4-AANAT_{Bm}) culture supernatant (D) matched that of the *N*-acetyltyramine standard (B), with the most intense signal at m/z 180.1 corresponding to the value of the protonated *N*-acetyltyramine.



Supplementary Figure S5 Growth rates of strain ATRN for the medium compositions according to the steepest ascent analysis. Strain ATRN was cultivated for 120 h in the BioLector cultivation system in 1 mL CGXII minimal medium containing 40 g L⁻¹ glucose. The medium composition at the center point corresponds to the standard composition of the CGXII minimal medium. Glucose was depleted at the end of cultivation for all cultivations. Values and error bars represent means and standard deviations from triplicate cultivations. Significance was calculated with an ANOVA followed by a Tukey's honestly significant difference (HSD) test with $\alpha = 0.05$; $p = 2.5 \times 10^{-10}$.



Supplementary Figure S6 Apparent kinetics of AANAT_{Bm} for acetyl-CoA (A) and propionyl-CoA (B). AANAT_{Bm} activity was measured in an *in vitro* assay with crude extract from an *E. coli* DH5 α strain overproducing AANAT_{Bm}. Reactions were performed in 0.1 M phosphate buffer (pH 8.0) containing 10 mM tyramine. Initial velocities v_0 measured for the different acyl-CoA concentrations are indicated as points, theoretical curves were calculated with the determined $V_{\max}$, K_M , and K_I values and are indicated as lines.



Supplementary Fig. S7 LC/MS analysis of ATRN culture supernatants after cultivation on a glucose-propionate mixture. An additional peak was observed at 2.2 min in the DAD chromatogram, recorded at a wavelength of 280 nm, in the supernatant of strain ATRN culture containing a mixture of glucose and propionate (A, light green) compared to strain ATRN cultivated on glucose as sole carbon source (A, dark green). For both culture supernatants, an *N*-acetyltyramine peak was detected after 1.7 min. In the mass spectrum of the additional peak at 2.2 min in the propionate-containing culture supernatant (B), the most intense signal at *m/z* 194.1 corresponded to the value calculated for protonated *N*-propionyltyramine [M+H]⁺.

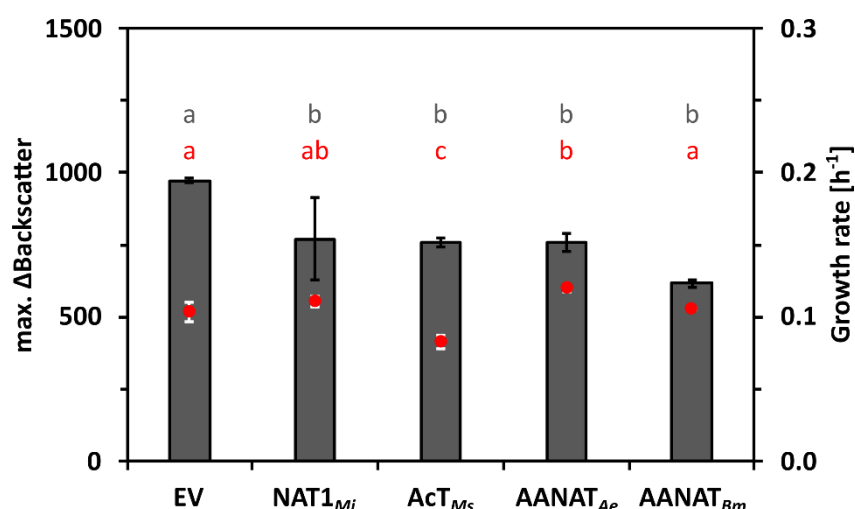
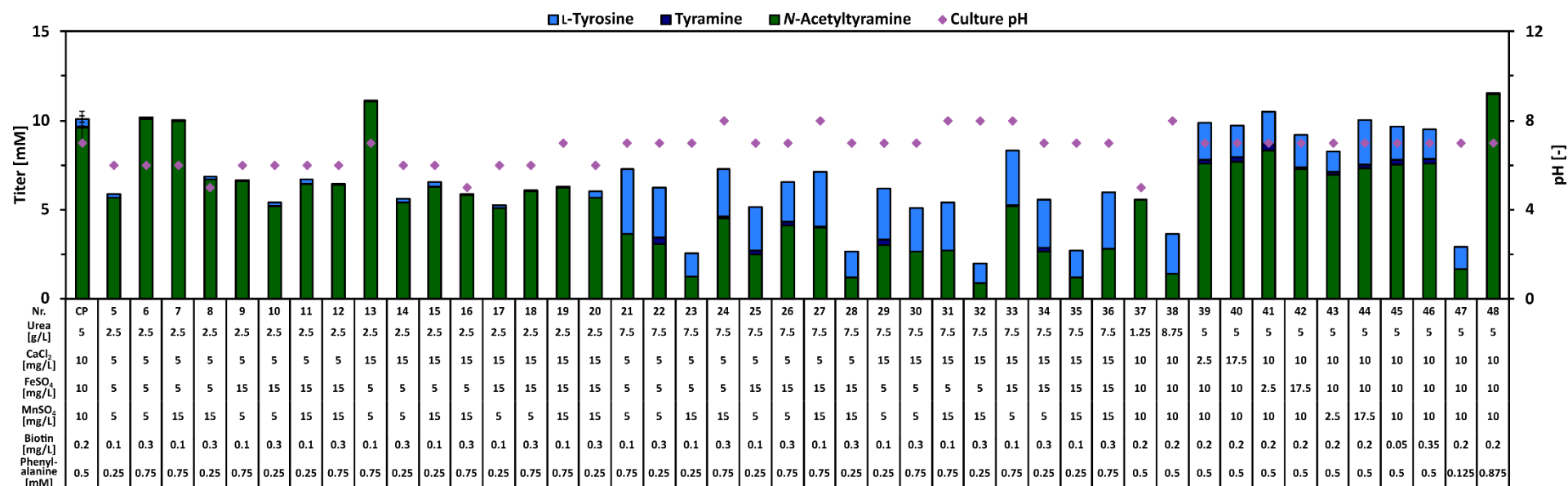


Figure S8 Growth parameters of strain TRN carrying a pVWEx4-plasmid for the overproduction of different acetyltransferases. The max. Δbackscatter (grey bars) as a measure of biomass formation and the growth rate (red circles) are indicated. The strains were cultivated for 120 h in the BioLector cultivation system in 1 mL CGXII minimal medium containing 40 g L⁻¹ glucose and 0.5 mM L-phenylalanine. Glucose was depleted at the end of cultivation for all cultivations except for strains TRN (pVWEx4-AcT_{Ms}) and TRN (pVWEx4-AANAT_{Bm}). Values and error bars represent means and standard deviations from triplicate cultivations. Significance was calculated for the max. Δbackscatter (grey letters) and growth rate (red letters) with an ANOVA followed by a Tukey's honestly significant difference (HSD) test with $\alpha = 0.05$; p (max. Δbackscatter) = 0.001, p (growth rate) = 9.8×10^{-6} .



70 **Supplementary Figure S9** Titters of L-tyrosine and its derivatives for each condition tested in the RSM. Titters of L-tyrosine (light blue), tyramine (dark blue), and N-acetyltyramine (green), as well as
 71 pH values determine in culture supernatants at the end of cultivation using pH test strips (purple diamonds). are indicated. Strain ATRN was cultivated for 120 h in the Biolector cultivation system in
 72 1 mL CGXII minimal medium containing 40 g L⁻¹ glucose. The medium composition at the center point corresponds to the standard composition of the CGXII minimal medium. Nr. refers to the media
 73 compositions listed in Supplementary Table S3. Values and error bars of the center point represent means and standard deviations of quadruplicate cultivations.

Gene name and UniProt ID	Sequence
<i>AANAT_{Ae}</i> F4WZQ5	CTTAGAAAAATATAATTAATTTTAAAAGGAGGTATTCT ATGG GAGAATATTCGAGCAAACCGGG TTAACGCTATCCATATTGATAATGACAAAACCTATTACTACTAACTTCGTGAAGGATGCACAAA AATCACTCGCTATTCTCTGGCGGATCTGGCAGACGGTTCATCTAATTCATGGATTACCACATT GAGATTATCAACAAAGATGATAAGCTTCGCGTGCTTAAGTTTCTCCGCCGCTTTTTTTTTTCGC GACGAGCCTCTTAACCAAAATATCCAGCTGATCCCTGAAGGCGAAGATTCCACTTGCCTGA ACTTGAGGAATATTGCTGTAAACGCGAGCTTCGAAAACAATATGTCCTAATGGCAGTTTCAAC TTCCGGCACCATCGTTGGTGTCTTCTTAACGGTAAGATGGATAACCTTGTTAGCAACGAAGA ACCAGAATACATTGATCTTGCAAGAACGCTAAGTTCAAAAAAATTCTGCGCCTCCTTCACTA TCTGGATAAGTCCGTTAATATGGGCGGTCTTTCCGTGATTCAAAGATCCTTGAGATTGCGATT ATCTCTGTGGACACCAACTGGCGCGGTGCGCGGCTTGGGACTACTCTTATGGGTAAAGACCGC TGAAATGGCAAAGAACAGGGTTACCACTACCTTCGAGCAGACTGCACTTCAATCTTCTCAG CAAAAATGTGTGAACGTCTTGGCTATGATCAAATTTACAAGATCAACTACAAAGACTATGTGG ATGAGGACGGTAAGCCAATTTTCAGCCCTGTTAGCCACATATTGCTGCAGTTAGCTACGTTA AAAAGCTCT TAA
<i>AANAT_{Bm}</i> A0EM56	CGGATAATTCTACAAAAGGAGGTTTTCT ATGG CCGTTACCAGCACCCGCGGCATTGTGAATC TTAAAGAGAAAGGCCATTTTGAGCGAGAAATGCACGAGTGAAAAGTACACTCTTGGTGA TGAAGTCCAGCACAGCTATCCTCTCTTGAGATTGACCTCGTAAGATGAGCGTTCCTGCCTA CACTATCCAGCGACTTACTTACAACGACCGAGATCTAGTTCTGAAGTTCTTCGCCGCTTCTTC TTTCGTGACGAGCCTATGAATCTTGCAGTTAATCTGCTAGAAACCCCTGAGAGCCGCTGCACT GAGCTTGACGATTATGCAGCGGCCACTCTTTCTGACGGTGTAGCGTTGCAGCCGTTGACGA GAACGGCGATTACGTTGGTGTATTATTAAACGGAATTGTGCGACGCGAGGAAGTTGATTACA CTGATAAAAGCGAAGATTGCCCTAATCCTAAGTTCCGACGAATTCTGAAGTTCTGGGACAC CTTGATCGTGAAGCCCGTATTTGGGATAAGCTGCCAGAGACTTGTGATAGCGTTCTTGAGATT CGAATCGCAAGCACCCACTCCAGCTGGCGTGGCCGCGGTCTGATGCGAGTGCTGTGTGAGG AAGCAGAACGACTGGCAAAGGCAATGGGTGCAGGTGCCCTACGCATGGACACCACTAGCGC CTTCAGCGCAGCAGCCGAGAGCGACTTAATTACAAAATGGCATTGAGTTGATATGCCG ACCTTCCATACGCGCCTCAACCTGAAGCGCCTCACCTAGAAGCACGAGTTACATTAAGAG CTG TAA
<i>act_{Ms}</i> A0R5U7	AACGAGACTTACGTAAATATTATATCATAAAAAGGAGGTAATTT ATGG CCAAGCGAATTCTGT GTTTCGGTGATTCCCTGACCTGGGGCTGGGTCCCCGTCGAAGACGGGGCACCCACCGAGCG GTTCCGCCCCGACGTGCGCTGGACCGGTGTGCTGGCCAGCAGCTCGGAGCGGACTTCGA GGTGATCGAGGAGGACTGAGCGCGCGCACCACCAACATCGACGACCCACCGATCCGCG GCTCAACGGCGCGAGCTACCTGCCGTGCTGCCTCGCGACGCACCTGCCGCTCGACCTGGTG ATCATCATGCTGGGCACCAACGACACCAAGGCCTACTTCCGGCGCACCCCGCTCGACATCGC GCTGGGCATGTGGTGCTCGTCACGCAGGTGCTCACCAGCGCGGGCGGCGTGGCACCAC GTACCCGGCACCAAGGTGCTGGTGGTCTCGCCGCCACCGCTGGCGCCCATGCCGACCCCT GGTTCAGTTGATCTTCGAGGGCGGCGAGCAGAAGACCACTGAGCTCGCCGCGTGTACAG CGCGCTCGCGTCTTCATGAAGGTGCCGTTCTTCGACGCGGGTTCGGTGATCAGCACCGAC GGCGTCGACGGAATCCAATTCACCGAGGCCAACAATCGCGATCTCGGGGTGGCCCTCGCGG AACAGGTGCGGAGCCTGCTG TAA
<i>NAT1_{Mj}</i> Q98D42	AGTTACAAGACAGGGAGACCCCTGACCGACCCTAAAAAGGAGGTTTTG ATGA ACGACGCG CCCCCTTTCGATCTGGATGCATACCTGGCGCGCATCGGGTATACCGGGCCACGCAACGCATCC CTGGACACTCTGAAGGCACTGCACTTCGCACACCCACAGGCAATCCCTTTCGAAAACATCGA CCCATTCTAGGGCGCCAGTGCGTCTGGATCTAGCAGCACTGCAGGATAAGATCGTGCTAG GCGGGCGCGGGGGCTATTGCTTCGAGCACAACCTGCTGTTTCATGCACGCACTGAAGGCACT AGGGTTCGAAGTGGGCGGGCTAGCAGCACGAGTGCTGTGGGGCCAGTCCGAAGATGCAAT CACCGCACGCTCCCATATGCTACTACGCGTGGAAGTGGATGGCCGTACCTATATCGCAGACGT

GGGCTTCGGCGGCCTGACCCTGACCGCACCACTGCTGCTGGAGCCAGGCCGCGAGCAGAA
AACCCACATGAACCCCTCCGCATCGTGGAAGCAGATGACCACTCCGCCTGCAGGCAGCAA
TCGGCGGCGATTGGCGCTCCCTGTATCGCTTCGATCTGCAGCCACAATATGAGGTGGACTATT
CTGTGACCAATTATTTCTGTCCACCTCCCCAACATCCCACTTCCTGTCTTCTGTGATCGCAGC
GCGCGCAGCACCAAGATCGTCGCTATGCACTGCGTGGGAACCGCCTATCCATCCACCATCTAG
GCGGCCGCACCGAACAGACAGAAATAGCAACCGCAGCAGATCTAGCAGATACTCTGCAAGG
CTGCTGGGCATCATCATACCTGATCGCACTGCGTTCTGAAGCAAAGGTGCGAGAACTAAGA
TCGTGGAAACCAACGCG**TAA**

75 Optimized RBS are underlined. Translational start and stop codons are indicated in bold. Genes encoding AANAT_{Ae}, AANAT_{Bm},
76 and NAT1_{Mj} were codon-harmonized for *C. glutamicum*. The gene sequence of *act_{Ms}* is the native sequence.

77

Purpose	Primer name	Sequence (5' → 3')
Verification of genes integrated into pSJEx3, pVWEx1, and pVWEx4	1143	TTTGCGCCGACATCATAACGGTTCTG
	1144	CTACGGCGTTTCACTTCTGAGTTCGG
Gibson assembly cloning of AANAT _{Ae} gene into pVWEx4	Fw_aanatAe_pVWEx4	CCTGCAGGTCGACTCTAGAGCTTAGAAAAATATAAT
	Rv_aanatAe_pVWEx4	TAATTTTAAAAGGAGGTATTCTATGGAGAATATTTCG CGAGCTCGGTACCCGGGGATCTTAGAGCTTTTAA CGTAGCTAACTGCAGC
Sequencing of AANAT _{Ae} gene	Rv_aanatAe_Seq1	GTTGGTGTCCACAGAGATAATGCG
	Fw_aanatAe_Seq2	CCTTCACTATCTGGATAAGTCCG
Gibson assembly cloning of AANAT _{Bm} gene into pSJEx3, pVWEx1, and pVWEx4	Fw_aanatBm_pVWEx4	GCTTGCATGCCTGCAGGTCGACTCTAGAGCGGATA
	Rv_aanatBm_pVWEx4	ATTTCTACAAAAGGAGGTTTTCTATGGC CGGCCAGTGAATTCGAGCTCGGTACCCGGGGATCT TACAGCTCTTTAATGTAAACTCGTGCTTCTAGG
Sequencing of AANAT _{Bm} gene	Rv_aanatBm_Seq1	GTACCCGGGGATCTTACAGC
	Fw_aanatBm_Seq2	GGCATTGGAGTTCGATATG
Gibson assembly cloning of Act _{Ms} gene into pVWEx4	Fw_actMs_pVWEx4_1	CGACTCTAGAGAACGAGACTTACGTAAATATTATATC ATAAAAAGGAGGTAATTTATGGCCAAGCGAATTCT GTGTTTCG
	Fw_actMs_pVWEx4_2	GCTTGCATGCCTGCAGGTCGACTCTAGAGAACGAG ACTTACGTAAATATTATATC
	Rv_actMs_pVWEx4	CGAGCTCGGTACCCGGGGATCTTACAGCAGGCTCC GCACC
Sequencing of Act _{Ms} gene	Rv_actMs_Seq1	CGAAGATCAACTGGAACCAGGGG
	Fw_actMs_Seq2	CGGCACCACGTACCCGGCACCC
Gibson assembly cloning of NAT1 _{Mj} gene into pVWEx4	Fw_NAT1Mj_pVWEx4	GCCTGCAGGTCGACTCTAGAGAGTTACAAGACAG GGAGACCCC
	Rv_NAT1Mj_pVWEx4	CGAGCTCGGTACCCGGGGATCTTACGCGTTGGTTT CCACGATCTTAG
Sequencing of NAT1 _{Mj} gene	Rv_NAT1Mj_Seq1	GTGCATAGCGACGATCTGGTGC
	Fw_NAT1Mj_Seq2	GACCAATTATTCCTGTCCACCTCCCC

Nr.	Block	Ammonium	Urea	Phosphate	MOPS	Glucose	CaCl ₂	MgSO ₄	FeSO ₄	MnSO ₄	ZnSO ₄	CuSO ₄	NiCl ₂	PCA	Biotin	Phenyl- alanine	Titer
		sulfate [g L ⁻¹]	[g L ⁻¹]	[g L ⁻¹]	[g L ⁻¹]	[g L ⁻¹]	[mg/L]	x 7 H ₂ O [mg L ⁻¹]	x 7 H ₂ O [mg L ⁻¹]	x H ₂ O [mg L ⁻¹]	x 7 H ₂ O [mg L ⁻¹]	[mg L ⁻¹]	x 6 H ₂ O [mg L ⁻¹]	[mg L ⁻¹]	[mg L ⁻¹]	[mM]	[mM]
1	1	20.0	5.00	2.0	42.0	40.0	10.0	250.0	10.0	10.0	1.00	0.20	0.020	30.0	0.20	0.500	9.0
2	1	20.0	5.00	2.0	42.0	40.0	10.0	250.0	10.0	10.0	1.00	0.20	0.020	30.0	0.20	0.500	9.1
3	1	20.0	5.00	2.0	42.0	40.0	10.0	250.0	10.0	10.0	1.00	0.20	0.020	30.0	0.20	0.500	8.5
4	1	20.0	5.00	2.0	42.0	40.0	10.0	250.0	10.0	10.0	1.00	0.20	0.020	30.0	0.20	0.500	8.0
5	1	10.0	2.50	1.0	21.0	20.0	5.0	125.0	5.0	5.0	0.50	0.10	0.010	15.0	0.10	0.250	4.6
6	1	10.0	2.50	1.0	21.0	60.0	5.0	125.0	5.0	5.0	1.50	0.30	0.030	45.0	0.30	0.750	8.3
7	1	10.0	2.50	1.0	63.0	20.0	5.0	375.0	15.0	15.0	0.50	0.10	0.010	45.0	0.30	0.750	6.1
8	1	10.0	2.50	1.0	63.0	60.0	5.0	375.0	15.0	15.0	1.50	0.30	0.030	15.0	0.10	0.250	12.3
9	1	10.0	2.50	3.0	21.0	20.0	15.0	125.0	15.0	15.0	0.50	0.30	0.030	15.0	0.10	0.750	4.1
10	1	10.0	2.50	3.0	21.0	60.0	15.0	125.0	15.0	15.0	1.50	0.10	0.010	45.0	0.30	0.250	5.0
11	1	10.0	2.50	3.0	63.0	20.0	15.0	375.0	5.0	5.0	0.50	0.30	0.030	45.0	0.30	0.250	4.3
12	1	10.0	2.50	3.0	63.0	60.0	15.0	375.0	5.0	5.0	1.50	0.10	0.010	15.0	0.10	0.750	11.7
13	1	10.0	7.50	1.0	21.0	20.0	15.0	375.0	5.0	15.0	1.50	0.10	0.030	15.0	0.30	0.250	0.0
14	1	10.0	7.50	1.0	21.0	60.0	15.0	375.0	5.0	15.0	0.50	0.30	0.010	45.0	0.10	0.750	7.2
15	1	10.0	7.50	1.0	63.0	20.0	15.0	125.0	15.0	5.0	1.50	0.10	0.030	45.0	0.10	0.750	1.6
16	1	10.0	7.50	1.0	63.0	60.0	15.0	125.0	15.0	5.0	0.50	0.30	0.010	15.0	0.30	0.250	2.3
17	1	10.0	7.50	3.0	21.0	20.0	5.0	375.0	15.0	5.0	1.50	0.30	0.010	15.0	0.30	0.750	0.0
18	1	10.0	7.50	3.0	21.0	60.0	5.0	375.0	15.0	5.0	0.50	0.10	0.030	45.0	0.10	0.250	6.8
19	1	10.0	7.50	3.0	63.0	20.0	5.0	125.0	5.0	15.0	1.50	0.30	0.010	45.0	0.10	0.250	1.2
20	1	10.0	7.50	3.0	63.0	60.0	5.0	125.0	5.0	15.0	0.50	0.10	0.030	15.0	0.30	0.750	7.2
21	1	30.0	2.50	1.0	21.0	20.0	15.0	375.0	15.0	5.0	1.50	0.30	0.010	45.0	0.10	0.250	3.3
22	1	30.0	2.50	1.0	21.0	60.0	15.0	375.0	15.0	5.0	0.50	0.10	0.030	15.0	0.30	0.750	4.8
23	1	30.0	2.50	1.0	63.0	20.0	15.0	125.0	5.0	15.0	1.50	0.30	0.010	15.0	0.30	0.750	4.7
24	1	30.0	2.50	1.0	63.0	60.0	15.0	125.0	5.0	15.0	0.50	0.10	0.030	45.0	0.10	0.250	8.1
25	1	30.0	2.50	3.0	21.0	20.0	5.0	375.0	5.0	15.0	1.50	0.10	0.030	45.0	0.10	0.750	5.4
26	1	30.0	2.50	3.0	21.0	60.0	5.0	375.0	5.0	15.0	0.50	0.30	0.010	15.0	0.30	0.250	4.9

27	1	30.0	2.50	3.0	63.0	20.0	5.0	125.0	15.0	5.0	1.50	0.10	0.030	15.0	0.30	0.250	4.7
28	1	30.0	2.50	3.0	63.0	60.0	5.0	125.0	15.0	5.0	0.50	0.30	0.010	45.0	0.10	0.750	11.2
29	1	30.0	7.50	1.0	21.0	20.0	5.0	125.0	15.0	15.0	0.50	0.30	0.030	45.0	0.30	0.250	0.0
30	1	30.0	7.50	1.0	21.0	60.0	5.0	125.0	15.0	15.0	1.50	0.10	0.010	15.0	0.10	0.750	13.2
31	1	30.0	7.50	1.0	63.0	20.0	5.0	375.0	5.0	5.0	0.50	0.30	0.030	15.0	0.10	0.750	1.5
32	1	30.0	7.50	1.0	63.0	60.0	5.0	375.0	5.0	5.0	1.50	0.10	0.010	45.0	0.30	0.250	2.4
33	1	30.0	7.50	3.0	21.0	20.0	15.0	125.0	5.0	5.0	0.50	0.10	0.010	45.0	0.30	0.750	0.0
34	1	30.0	7.50	3.0	21.0	60.0	15.0	125.0	5.0	5.0	1.50	0.30	0.030	15.0	0.10	0.250	0.7
35	1	30.0	7.50	3.0	63.0	20.0	15.0	375.0	15.0	15.0	0.50	0.10	0.010	15.0	0.10	0.250	1.1
36	1	30.0	7.50	3.0	63.0	60.0	15.0	375.0	15.0	15.0	1.50	0.30	0.030	45.0	0.30	0.750	13.8
37	2	5.0	5.00	2.0	42.0	40.0	10.0	250.0	10.0	10.0	1.00	0.20	0.020	30.0	0.20	0.500	8.5
38	2	35.0	5.00	2.0	42.0	40.0	10.0	250.0	10.0	10.0	1.00	0.20	0.020	30.0	0.20	0.500	5.7
39	2	20.0	1.25	2.0	42.0	40.0	10.0	250.0	10.0	10.0	1.00	0.20	0.020	30.0	0.20	0.500	6.4
40	2	20.0	8.75	2.0	42.0	40.0	10.0	250.0	10.0	10.0	1.00	0.20	0.020	30.0	0.20	0.500	4.5
41	2	20.0	5.00	0.5	42.0	40.0	10.0	250.0	10.0	10.0	1.00	0.20	0.020	30.0	0.20	0.500	0.8
42	2	20.0	5.00	3.5	42.0	40.0	10.0	250.0	10.0	10.0	1.00	0.20	0.020	30.0	0.20	0.500	6.6
43	2	20.0	5.00	2.0	10.5	40.0	10.0	250.0	10.0	10.0	1.00	0.20	0.020	30.0	0.20	0.500	1.9
44	2	20.0	5.00	2.0	73.5	40.0	10.0	250.0	10.0	10.0	1.00	0.20	0.020	30.0	0.20	0.500	7.7
45	2	20.0	5.00	2.0	42.0	10.0	10.0	250.0	10.0	10.0	1.00	0.20	0.020	30.0	0.20	0.500	8.5
46	2	20.0	5.00	2.0	42.0	70.0	10.0	250.0	10.0	10.0	1.00	0.20	0.020	30.0	0.20	0.500	10.6
47	2	20.0	5.00	2.0	42.0	40.0	2.5	250.0	10.0	10.0	1.00	0.20	0.020	30.0	0.20	0.500	6.6
48	2	20.0	5.00	2.0	42.0	40.0	17.5	250.0	10.0	10.0	1.00	0.20	0.020	30.0	0.20	0.500	3.5
49	2	20.0	5.00	2.0	42.0	40.0	10.0	62.5	10.0	10.0	1.00	0.20	0.020	30.0	0.20	0.500	5.9
50	2	20.0	5.00	2.0	42.0	40.0	10.0	437.5	10.0	10.0	1.00	0.20	0.020	30.0	0.20	0.500	6.2
51	2	20.0	5.00	2.0	42.0	40.0	10.0	250.0	2.5	10.0	1.00	0.20	0.020	30.0	0.20	0.500	6.0
52	2	20.0	5.00	2.0	42.0	40.0	10.0	250.0	17.5	10.0	1.00	0.20	0.020	30.0	0.20	0.500	6.4
53	2	20.0	5.00	2.0	42.0	40.0	10.0	250.0	10.0	2.5	1.00	0.20	0.020	30.0	0.20	0.500	6.2
54	2	20.0	5.00	2.0	42.0	40.0	10.0	250.0	10.0	17.5	1.00	0.20	0.020	30.0	0.20	0.500	6.1
55	2	20.0	5.00	2.0	42.0	40.0	10.0	250.0	10.0	10.0	0.25	0.20	0.020	30.0	0.20	0.500	5.8
56	2	20.0	5.00	2.0	42.0	40.0	10.0	250.0	10.0	10.0	1.75	0.20	0.020	30.0	0.20	0.500	5.5
57	2	20.0	5.00	2.0	42.0	40.0	10.0	250.0	10.0	10.0	1.00	0.05	0.020	30.0	0.20	0.500	6.9

58	2	20.0	5.00	2.0	42.0	40.0	10.0	250.0	10.0	10.0	1.00	0.35	0.020	30.0	0.20	0.500	6.6
59	2	20.0	5.00	2.0	42.0	40.0	10.0	250.0	10.0	10.0	1.00	0.20	0.005	30.0	0.20	0.500	6.8
60	2	20.0	5.00	2.0	42.0	40.0	10.0	250.0	10.0	10.0	1.00	0.20	0.035	30.0	0.20	0.500	6.3
61	2	20.0	5.00	2.0	42.0	40.0	10.0	250.0	10.0	10.0	1.00	0.20	0.020	7.5	0.20	0.500	1.9
62	2	20.0	5.00	2.0	42.0	40.0	10.0	250.0	10.0	10.0	1.00	0.20	0.020	52.5	0.20	0.500	7.6
63	2	20.0	5.00	2.0	42.0	40.0	10.0	250.0	10.0	10.0	1.00	0.20	0.020	30.0	0.05	0.500	6.6
64	2	20.0	5.00	2.0	42.0	40.0	10.0	250.0	10.0	10.0	1.00	0.20	0.020	30.0	0.35	0.500	6.4
65	2	20.0	5.00	2.0	42.0	40.0	10.0	250.0	10.0	10.0	1.00	0.20	0.020	30.0	0.20	0.125	6.4
66	2	20.0	5.00	2.0	42.0	40.0	10.0	250.0	10.0	10.0	1.00	0.20	0.020	30.0	0.20	0.875	10.2
67	2	20.0	5.00	2.0	42.0	40.0	10.0	250.0	10.0	10.0	1.00	0.20	0.020	30.0	0.20	0.500	6.9
68	2	20.0	5.00	2.0	42.0	40.0	10.0	250.0	10.0	10.0	1.00	0.20	0.020	30.0	0.20	0.500	6.3
69	2	20.0	5.00	2.0	42.0	40.0	10.0	250.0	10.0	10.0	1.00	0.20	0.020	30.0	0.20	0.500	6.4

Nr.	Urea	CaCl ₂	FeSO ₄	MnSO ₄	Biotin	Phenyl-	Titer
	[g L ⁻¹]	[mg L ⁻¹]	x 7 H ₂ O [mg L ⁻¹]	x H ₂ O [mg L ⁻¹]	[mg L ⁻¹]	alanine [mM]	[mM]
1	5.00	10.0	10.0	10.0	0.20	0.500	10.6
2	5.00	10.0	10.0	10.0	0.20	0.500	10.0
3	5.00	10.0	10.0	10.0	0.20	0.500	9.2
4	5.00	10.0	10.0	10.0	0.20	0.500	8.5
5	2.50	5.0	5.0	5.0	0.10	0.250	5.7
6	2.50	5.0	5.0	5.0	0.30	0.750	10.1
7	2.50	5.0	5.0	15.0	0.10	0.750	10.0
8	2.50	5.0	5.0	15.0	0.30	0.250	6.7
9	2.50	5.0	15.0	5.0	0.10	0.750	6.6
10	2.50	5.0	15.0	5.0	0.30	0.250	5.2
11	2.50	5.0	15.0	15.0	0.10	0.250	6.5
12	2.50	5.0	15.0	15.0	0.30	0.750	6.4
13	2.50	15.0	5.0	5.0	0.10	0.750	11.1
14	2.50	15.0	5.0	5.0	0.30	0.250	5.4
15	2.50	15.0	5.0	15.0	0.10	0.250	6.3
16	2.50	15.0	5.0	15.0	0.30	0.750	5.8
17	2.50	15.0	15.0	5.0	0.10	0.250	5.1
18	2.50	15.0	15.0	5.0	0.30	0.750	6.0
19	2.50	15.0	15.0	15.0	0.10	0.750	6.2
20	2.50	15.0	15.0	15.0	0.30	0.250	5.7
21	7.50	5.0	5.0	5.0	0.10	0.750	3.6
22	7.50	5.0	5.0	5.0	0.30	0.250	3.1
23	7.50	5.0	5.0	15.0	0.10	0.250	1.2
24	7.50	5.0	5.0	15.0	0.30	0.750	4.5
25	7.50	5.0	15.0	5.0	0.10	0.250	2.5
26	7.50	5.0	15.0	5.0	0.30	0.750	4.1
27	7.50	5.0	15.0	15.0	0.10	0.750	4.0
28	7.50	5.0	15.0	15.0	0.30	0.250	1.2
29	7.50	15.0	5.0	5.0	0.10	0.250	3.0
30	7.50	15.0	5.0	5.0	0.30	0.750	2.7
31	7.50	15.0	5.0	15.0	0.10	0.750	2.7
32	7.50	15.0	5.0	15.0	0.30	0.250	0.9
33	7.50	15.0	15.0	5.0	0.10	0.750	5.2
34	7.50	15.0	15.0	5.0	0.30	0.250	2.6
35	7.50	15.0	15.0	15.0	0.10	0.250	1.2
36	7.50	15.0	15.0	15.0	0.30	0.750	2.8
37	1.25	10.0	10.0	10.0	0.20	0.500	5.6
38	8.75	10.0	10.0	10.0	0.20	0.500	1.4
39	5.00	2.5	10.0	10.0	0.20	0.500	7.6
40	5.00	17.5	10.0	10.0	0.20	0.500	7.7
41	5.00	10.0	2.5	10.0	0.20	0.500	8.3
42	5.00	10.0	17.5	10.0	0.20	0.500	7.3
43	5.00	10.0	10.0	2.5	0.20	0.500	7.0
44	5.00	10.0	10.0	17.5	0.20	0.500	7.3

45	5.00	10.0	10.0	10.0	0.05	0.500	7.5
46	5.00	10.0	10.0	10.0	0.35	0.500	7.6
47	5.00	10.0	10.0	10.0	0.20	0.125	1.6
48	5.00	10.0	10.0	10.0	0.20	0.875	11.5

83

84 **Supplementary Table S5 Results of the steepest ascent analysis and *N*-acetyltyramine titers obtained for the respective**
85 **conditions.**

Distance	Urea [g L⁻¹]	CaCl₂ [mg L⁻¹]	FeSO₄ x 7 H₂O [mg L⁻¹]	MnSO₄ x H₂O [mg L⁻¹]	Biotin [mg L⁻¹]	Phenyl- alanine [mM]	Titer [mM]
0.0	5.00	10.0	10.0	10.0	0.20	0.50	7.4 ± 0.5
0.5	4.26	9.5	9.0	9.7	0.19	0.58	9.7 ± 0.5
1.0	3.92	8.8	7.2	9.4	0.18	0.65	10.7 ± 0.3
1.5	3.69	7.9	5.0	9.3	0.17	0.70	10.5 ± 0.5
2.0	3.50	6.8	2.8	9.2	0.16	0.75	10.1 ± 1.0
2.5	3.32	5.7	0.6	9.2	0.16	0.78	6.4 ± 2.0

86 The concentrations of urea, CaCl₂, FeSO₄, MnSO₄, biotin, and L-phenylalanine were determined performing a steepest ascent
87 analysis based on the previously generated response surface model. Distances up to 2.5 from the center point were tested,
88 since predicted CaCl₂- and FeSO₄-concentrations became negative for higher distances, which is biologically unfeasible.
89 Obtained *N*-acetyltyramine titers of triplicate cultivations are indicated as mean ± standard deviation.

90

91 **Supplementary Table S6 ANOVA analysis results of the screening design predicting the effects on the *N*-acetyltyramine**
92 **substrate yield.**

Factor	t-value	Prob > t
Intercept	7.84	< 0.001
Block	-3.01	0.006
Ammonium sulfate	-0.92	0.366
Urea	-8.62	< 0.001
Phosphate	0.50	0.621
MOPS	4.13	< 0.001
Glucose	-1.00	0.330
CaCl ₂	-2.53	0.019
MgSO ₄	0.81	0.425
FeSO ₄	1.38	0.182
MnSO ₄	2.34	0.029
ZnSO ₄	0.88	0.390
CuSO ₄	-0.94	0.355
NiCl ₂	0.37	0.714
PCA	1.68	0.107
Biotin	-2.44	0.023
L-Phenylalanine	4.30	< 0.001
Ammonium sulfate ²	-1.17	0.254
Urea ²	-1.67	0.108
Phosphate ²	-1.83	0.081
MOPS ²	0.66	0.518
Glucose ²	-1.58	0.127
CaCl ₂ ²	-0.58	0.567
MgSO ₄ ²	-0.43	0.669
FeSO ₄ ²	-0.45	0.660
MnSO ₄ ²	-0.96	0.347
ZnSO ₄ ²	0.12	0.908
CuSO ₄ ²	-0.09	0.927
NiCl ₂ ²	-1.87	0.075
PCA ²	-0.17	0.870
Biotin ²	1.68	0.106
L-Phenylalanine ²	-1.17	0.254
	F-value	Prob > F
First order	9.13	< 0.001
Quadratic effects	2.32	0.036
Lack of fit	13.08	0.005

93 The effect of each factor and its probability, as well as the F-values and probabilities of the model are given. Squared factors
94 indicate quadratic effects. Two-factor interactions were excluded since the strong confounding among the media components
95 did not allow for reliable predictions.

96

Factor	t-value	Prob > t
Intercept	15.90	< 0.001
Urea	-7.28	< 0.001
CaCl ₂	-0.90	0.377
FeSO ₄	-1.36	0.189
MnSO ₄	-0.98	0.338
Biotin	-0.79	0.440
L-Phenylalanine	4.65	< 0.001
Urea:CaCl ₂	0.25	0.802
Urea:FeSO ₄	1.70	0.104
Urea:MnSO ₄	-0.74	0.467
Urea:Biotin	0.51	0.615
Urea:Phe	-0.19	0.851
CaCl ₂ :FeSO ₄	0.63	0.535
CaCl ₂ :MnSO ₄	-1.02	0.319
CaCl ₂ :Biotin	-1.13	0.272
CaCl ₂ :Phenylalanine	-0.56	0.583
FeSO ₄ :MnSO ₄	0.35	0.727
FeSO ₄ :Biotin	0.13	0.900
FeSO ₄ :Phenylalanine	-0.75	0.459
MnSO ₄ :Biotin	-0.06	0.950
MnSO ₄ :Phenylalanine	-0.44	0.664
Biotin:Phenylalanine	-0.70	0.489
Urea ²	-4.46	< 0.001
CaCl ₂ ²	-0.51	0.616
FeSO ₄ ²	-0.36	0.720
MnSO ₄ ²	-0.98	0.341
Biotin ²	-0.58	0.571
L-Phenylalanine ²	-1.52	0.145
	F-value	Prob > F
First order	13.15	<0.001
Quadratic effects	7.84	<0.001
Lack of fit	3.21	0.183

98 RSM for the effects of urea, CaCl₂, FeSO₄, MnSO₄, biotin, and phenylalanine on the *N*-acetyltyramine titer produced by strain
99 ATRN. The effect of each factor and its probability, as well as the F-values and probabilities of the model are given. A colon
100 between factors indicates two-factor interactions. Squared factors indicate quadratic effects.

102 **References**

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