Supplemental Information

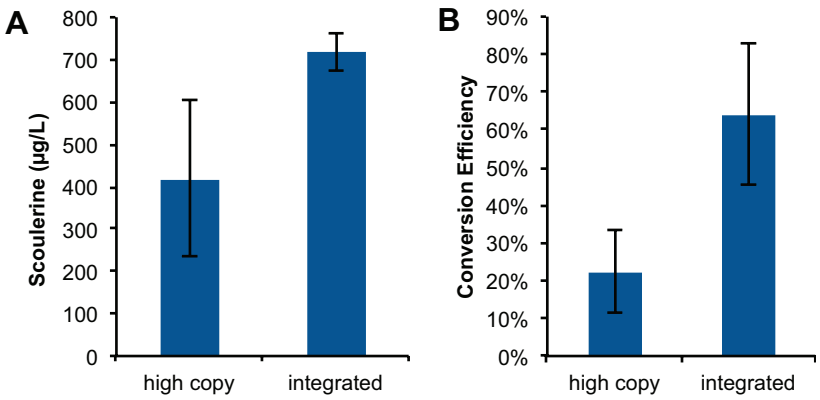

Figure S1 Expression optimization of berberine bridge enzyme (BBE). **(A)** Scoulerine production as a function of PsBBE expression method. PsBBE was expressed from either a high-copy plasmid in CSY288 or integrated into the chromosome (CSY844). **(B)** Conversion efficiency of reticuline to scoulerine as a function of PsBBE expression method. Strains are as described in A.

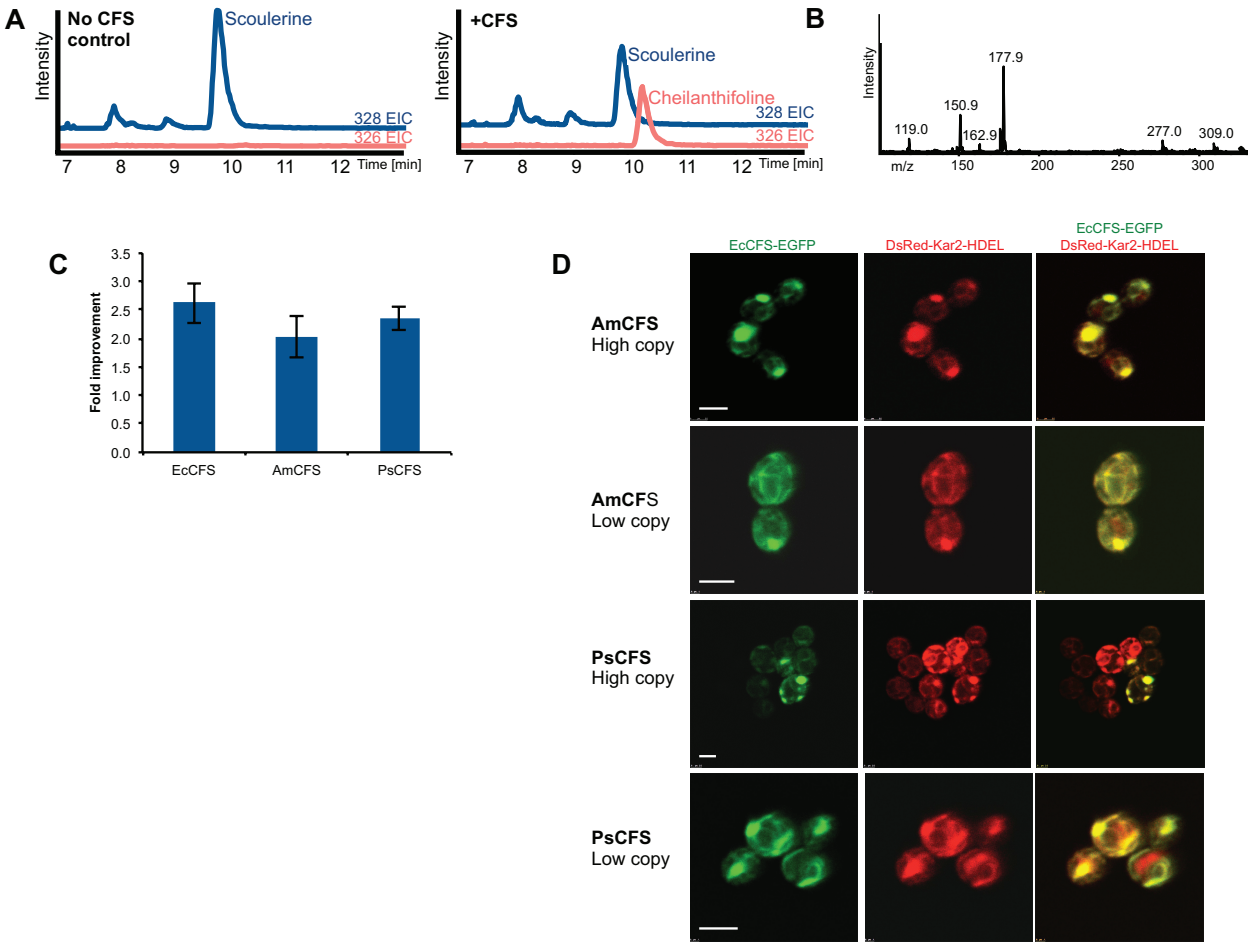

Figure S2 Expression optimization of cheilanthifoline synthase (CFS). **(A)** LC-MS analysis of growth media of CSY844 with EcCFS expressed from a low-copy plasmid. Traces are shown for a no EcCFS enzyme control strain (left) and an engineered strain expressing EcCFS (right). EICs for compounds corresponding to scoulerine (328 m/z) and cheilanthifoline (326 m/z) are shown. **(B)** Fragmentation pattern of the 326 EIC peak produced from the engineered yeast strain. Major predicted fragment: m/z = 178 (Schmidt and Raith, 2005). LC-MS traces and fragmentation patterns are representative of at least 3 independent experiments. **(C)** Fold improvement in cheilanthifoline production when CFS variants are expressed from a low-copy plasmid versus a high-copy plasmid in CSY844. **(D)** Confocal microscopy analysis of CFS variants C-terminally tagged with GFP on high- or low-copy plasmids coexpressed with ER marker DsRed-Kar2-

HDEL in CSY844. Scale bars are 4 μm. Images are representative of at least 3 independent experiments.

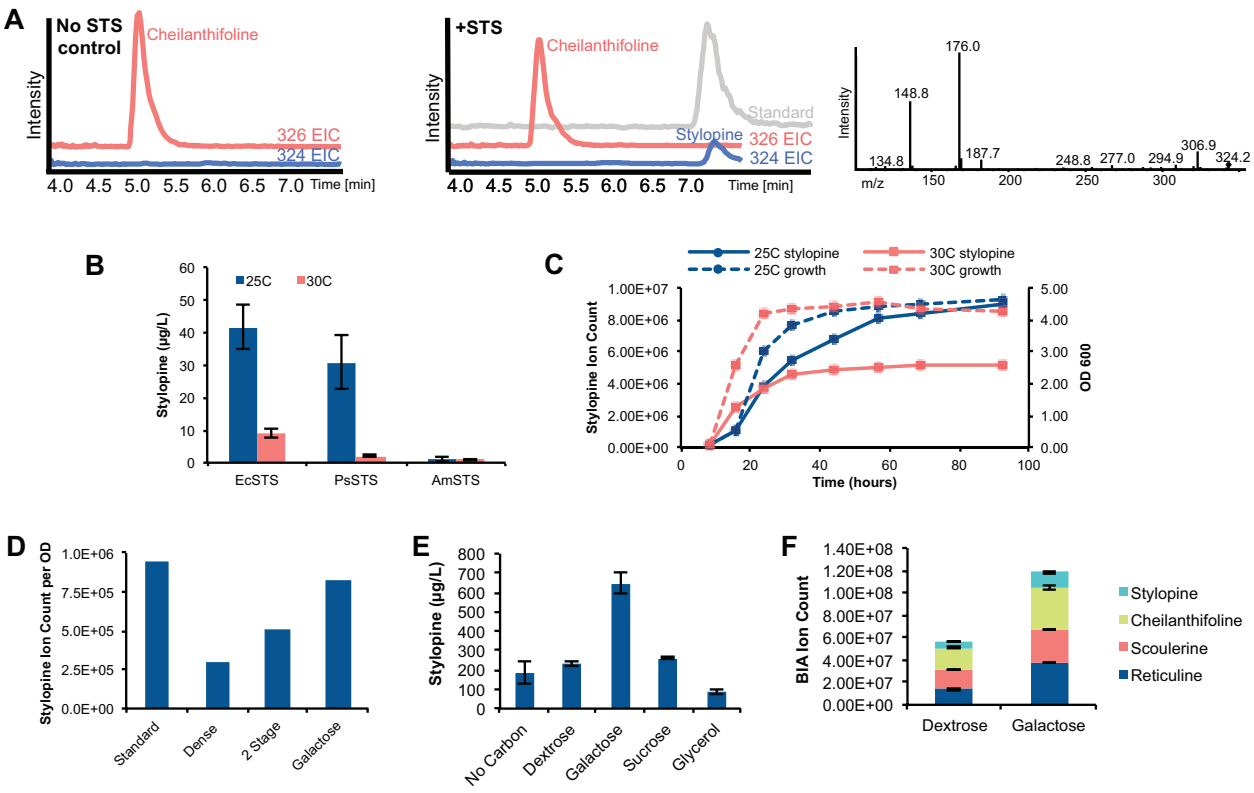

Figure S3 Optimization of stylopine production through culturing methods. **(A)** LC-MS analysis of growth media of CSY844 with EcCFS and EcSTS each on low copy plasmids. Traces are shown for a no EcSTS enzyme control strain (left) and an engineered strain expressing EcSTS (middle). EICs for compounds corresponding to cheilanthifoline (326 m/z) and stylopine (324 m/z) are shown. A 250 nM stylopine standard is included for comparison. Fragmentation pattern of the 324 EIC peak produced from the engineered yeast strain are shown (right). Major predicted fragments: m/z = 176, 149 (Hagel et al., 2012). LC-MS traces and fragmentation patterns are representative of at least 3 independent experiments. **(B)** Stylopine production as a function species variant and growth temperature. STS variants were expressed with EcCFS from low-copy plasmids in CSY844. **(C)** Stylopine production and growth over time in CSY904. Dashed lines represent the growth curves while solid lines represent the stylopine production curve. **(D)** Stylopine production per optical density unit as a function of culturing conditions in

CSY904. All cultures were grown at 25 °C. Standard: 1X YNB dropout media with 2% dextrose and 2 mM norlaudanosoline; Dense: 5X YNB dropout media with 2% dextrose and 2 mM norlaudanosoline; 2 stage: growth stage in 5X YNB dropout media with 2% dextrose, then production stage with 1X YNB dropout media with 2% dextrose and 2 mM norlaudanosoline; Galactose: As described in 2 stage but with 2% galactose during production phase. **(E)** Stylopine production as a function of carbon source during production stage. **(F)** Total BIA production as a function of carbon source during the production stage.

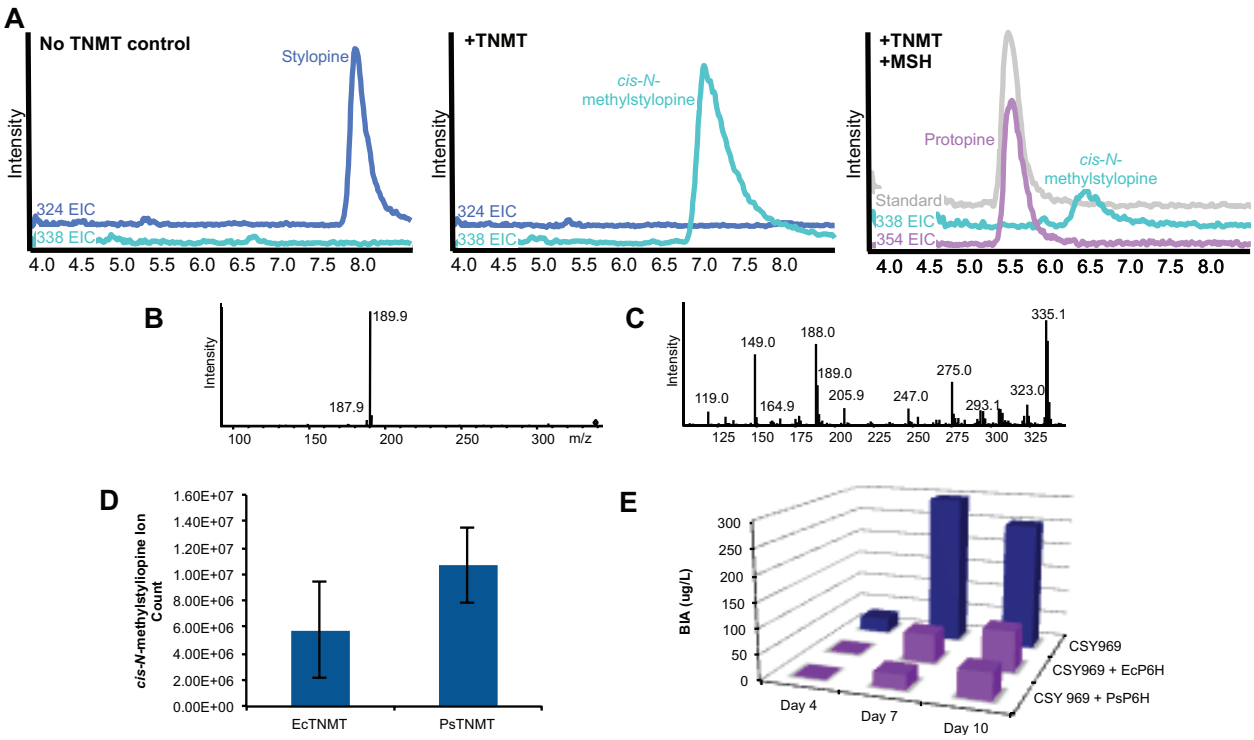

Figure S4 *cis*-*N*-methylstylopine, protopine, and sanguinarine production in engineered yeast strains. **(A)** LC-MS analysis of growth media of CSY904 with PsTNMT and PsMSH. Traces are shown for a no PsTNMT enzyme control strain (left), an engineered strain expressing PsTNMT from a low-copy plasmid (middle), and an engineered strain expressing PsTNMT and PsMSH each from a low-copy plasmid (right). A protopine standard is included for comparison (right). EICs for compounds corresponding to stylopine (324 m/z), *cis*-*N*-methylstylopine (338 m/z), and protopine (354 m/z) are shown. **(B)** Fragmentation pattern of the 338 EIC peak produced from the engineered yeast strain shown in A, middle. Major predicted fragment: m/z = 190 (Schmidt and Raith, 2005). **(C)** Fragmentation pattern of the 354 EIC peak produced from the engineered yeast strain shown in A, right. Major predicted fragments: m/z = 189, 188, 149, 206, 275, 165 (Schmidt and Raith, 2005). LC-MS traces and fragmentation patterns are representative of at least 3 independent experiments. **(D)** *cis*-*N*-methylstylopine production as a function of TNMT

species variant. **(E)** Production of protopine and sanguinarine as a function of time. Blue bars indicate protopine production and purple bars indicate sanguinarine production.

Table 1 Yeast strains used in this study.

Strain	Genotype	Reference
CSY288	W303 *his3* ::P_{TEF1} -Ps6OMT , *leu2* ::P_{TEF1} -PsCNMT , *ura3* ::P_{TEF1} -Ps4'OMT	(Hawkins and Smolke, 2008)
CSY953	*his3* ::P_{TEF1} -Ps6OMT , *leu2* ::P_{TEF1} -PsCNMT , *ura3* ::P_{TEF1} -Ps4'OMT, lys2 :: P_{TEF1}-PsBBE-loxP-KanMX-loxP	This work
CSY844	*his3* ::P_{TEF1} -Ps6OMT , *leu2* ::P_{TEF1} -PsCNMT , *ura3* ::P_{TEF1} -Ps4'OMT trp1 :: P_{TEF1}-ATR1, lys2 :: P_{TEF1}-PsBBE	This work
CSY985	*his3* ::P_{TEF1} -Ps6OMT , *leu2* ::P_{TEF1} -PsCNMT , *ura3* ::P_{TEF1} -Ps4'OMT trp1 :: P_{TEF1}-PsCPR-loxP-LEU2-loxP, lys2 :: P_{TEF1}-PsBBE-loxP-KanMX-loxP	This work
CSY850	*his3* ::P_{TEF1} -Ps6OMT , *leu2* ::P_{TEF1} -PsCNMT , *ura3* ::P_{TEF1} -Ps4'OMT trp1 :: P_{TEF}1-EcCPR, lys2 :: P_{TEF1}-PsBBE-loxP-KanMX-loxP	This work
CSY903	*his3* ::P_{TEF1} -Ps6OMT , *leu2* ::P_{TEF1} -PsCNMT , *ura3* ::P_{TEF1} -Ps4'OMT, trp1 :: P_{TEF1}-ATR1, lys2 :: P_{TEF1}-PsBBE, met15 ::P_{GPD}-EcCFS	This work
CSY904	*his3* ::P_{TEF1} -Ps6OMT , *leu2* ::P_{TEF1} -PsCNMT , *ura3* ::P_{TEF1} -Ps4'OMT, trp1 :: P_{TEF1}-ATR1, lys2 :: P_{TEF1}-PsBBE, met15 ::P_{GPD}-EcCFS-loxP-KanMX-loxP, cin5 :: P_{GPD}-EcSTS-loxP-LEU2-loxP	This work
CSY968	*his3* ::P_{TEF1} -Ps6OMT, *leu2* ::P_{TEF1} –PsCNMT, *ura3* ::P_{TEF1} -Ps4'OMT, *trp1* :: P_{TEF1}- ATR1, *lys2* :: P_{TEF1}-PsBBE-, *met15* :: P_{GPD}-EcCFS-, *cin5* :: P_{GPD}-EcSTS-, *XI-3* :: P_{GPD}-PsTNMT-loxP-LEU2-loxP	This work
CSY969	*his3* ::P_{TEF1} -Ps6OMT, *leu2* ::P_{TEF1} –PsCNMT, *ura3* ::P_{TEF1} -Ps4'OMT, *trp1* :: P_{TEF1}- ATR1, *lys2* :: P_{TEF1}-PsBBE-, *met15* :: P_{GPD}-EcCFS-, *cin5* :: P_{GPD}-EcSTS-, *XI-3* :: P_{GPD}-PsTNMT-loxP-LEU2-loxP, *XI-4* :: P_{GPD}-PsMSH-loxP-KanMX-loxP	This work

Table 2 Plasmids used in this study.

Plasmid Name	Genotype	Reference
pAG416GPD-ccdB	*Centromeric URA,* P_{GPD}-ccdB recombination cassette	(Alberti et al., 2007)
pAG414GPD-ccdB	*Centromeric TRP,* P_{GPD}-ccdB recombination cassette	(Alberti et al., 2007)
pAG413GPD-ccdB	*Centromeric HIS,* P_{GPD}-ccdB recombination cassette	(Alberti et al., 2007)
pAG424GPF-ccdB	*2u TRP,* P_{GPD}-ccdB recombination cassette	(Alberti et al., 2007)
pAG416GPD-ccdB-EGFP	*Centromeric URA,* P_{GPD}-ccdB-EGFP recombination cassette, for C-terminal GFP tag	(Alberti et al., 2007)
pAG426GPD-ccdB-EGFP	*2u URA,* P_{GPD}-ccdB-EGFP recombination cassette, for C-terminal GFP tag	(Alberti et al., 2007)
pCS2238	*pAG416-* P_{GPD} *-EcCFS*	This work
pCS2219	*pAG416-* P_{GPD} *-AmCFS*	This work
pCS2237	*pAG416-* P_{GPD} *-PsCFS*	This work
pCS2402	*pAG414-* P_{GPD} *-EcSTS*	This work
pCS2403	*pAG414-* P_{GPD} *-PsSTS*	This work
pCS2329	*pAG414-* P_{GPD} *-AmSTS*	This work
pCS3044	*pAG414-* P_{GPD} *-EcCFS*	This work
pCS3045	*pAG414-* P_{GPD} *-AmCFS*	This work
pCS3046	*pAG414-* P_{GPD} *-PsCFS*	This work
pCS3047	*pAG424-* P_{GPD} *-EcCFS*	This work
pCS2438	*pAG416-*P_{TEF1}*-EcCFS*	This work
pCS2439	*pAG416-*P_{PGK1}*-EcCFS*	This work
pCS2440	*pAG416-*P_{HXT7}*-EcCFS*	This work
pCS3048	*pAG416-*P_{TPI1}*-EcCFS*	This work
pCS2230	*pAG416-* P_{GPD} *–EcCFS-EGFP*	This work
pCS2331	*pAG416-* P_{GPD} *–AmCFS-EGFP*	This work
pCS2303	*pAG416-* P_{GPD} *–PsCFS-EGFP*	This work
pCS2149	*pAG426-* P_{GPD} *–EcCFS-EGFP*	This work
pCS2221	*pAG426-* P_{GPD} *–AmCFS-EGFP*	This work
pCS2118	*pAG426-* P_{GPD} *–PsCFS-EGFP*	This work
pCS1970	*2u TRP, DsRED-KAR2-HDEL*	(Thodey et al., 2014)
pCS3052	*pAG413-* P_{GPD}-PsTNMT	This work
pCS3053	*pAG413-* P_{GPD}-EcTNMT	This work
pCS3054	*pAG414-* P_{GPD}-EcP6H	This work
pCS3055	*pAG414-* P_{GPD}-PsP6H	This work

Table 3 Integration Primers used in this study.

Primer Name	Sequence
LYS2.fwd	5′-CAGAGAGAACCTGTGTTGTGGAGACTCCAACACTAAATTCCGACAAGTCCCGTTCTTTCACTTATCGCGACATCAACCGCAGATTGTACTGAGAGTGCAC-3′
LYS2.rev	5′-GTAAGTAATTGACCCATGGCAGGTGTTAAATGGGTAACTGTGCAACCATACTTACTCATCCATTCCGCTAAACGGCCCGGCGACTCACTATAGGGAGACC-3′
MET15.fwd	5′-GCTCACAGATCCAGAGCTGTACCAATTTACGCCACCACTTCTTATGTTTTCGAAAACTCTAAGCATGGTTCGCAATTGTTAGATTGTACTGAGAGTGCAC-3′
MET15.rev	5′-GACACCACCGAAACCGTTAGATAGATACTTCTTAGCATTTTCATGATGAGAATGAGATGCTAAACCAGGGTATGAAACCCCGACTCACTATAGGGAGACC-3′
CIN5.fwd	5′-GAATAACAGCTTGGAACAAGAAGGAAAACCAAAAACCTACTCAAATGTTAATGCAAATAAAAATGGACAATCATCCTTTTAATTTTCAACCTATTTTAGCTTCAGATTGTACTGAGAGTGCAC-3′
CIN5.rev	5′-GCCATGTGCTTTGAAAACTTTTAAGATGTTACTAGTACTAATAATTATTCATTATTTTATTCTTTTAATTTCGACTTTAATGATTCAATCATTTTTTTCAATTCACCGACTCACTATAGGGAGACC-3′
XI-3.fwd*	5′-CCAATCAAAGAAGCATCGGTTCAGATCGAGCAAACTGTAGGGAGAAAGGAAAGTAGAAATGCAGAGTGTGCTATATGTCCAGATTGTACTGAGAGTGCAC-3′
XI-3.rev*	5′-GCAGGAGCCAATAGTAGTCGGAAAGATGAGTGTATAGATTTTTCGTATTTTATTTCGAGTAAAAATATCACGCTCTTAGGCGACTCACTATAGGGAGACC-3′
XI-4.fwd*	5′-CCTTTCTATACCAAGTAATGAATGTCTTGAGGGCCCGTATGGCCGCGCGAAGGCTTAGTTAAGATGTTTCAGCAAACGGCAGATTGTACTGAGAGTGCAC-3′
XI-4.rev*	5′-GTAATGTAAGTGATAACAAGTATGAAAAGGCCCAATGTACTTTTTTATATTTTTCCCTTGGTTCTTTTTCCTTATCAATCCGACTCACTATAGGGAGACC-3′

*Integration loci were adapted from (Mikkelsen et al., 2012).

References

Alberti, S., Gitler, A.D., Lindquist, S., 2007. A suite of Gateway cloning vectors for high-throughput genetic analysis in Saccharomyces cerevisiae 913–919. doi:10.1002/yea

Hagel, J.M., Beaudoin, G. a W., Fossati, E., Ekins, A., Martin, V.J.J., Facchini, P.J., 2012. Characterization of a flavoprotein oxidase from opium poppy catalyzing the final steps in sanguinarine and papaverine biosynthesis. J. Biol. Chem. 287, 42972–83. doi:10.1074/jbc.M112.420414

Hawkins, K., Smolke, C., 2008. Production of benzylisoquinoline alkaloids in Saccharomyces cerevisiae. Nat. Chem. Biol. 4, 564–573. doi:10.1038/nchembio.105

Mikkelsen, M.D., Buron, L.D., Salomonsen, B., Olsen, C.E., Hansen, B.G., Mortensen, U.H., Halkier, B.A., 2012. Microbial production of indolylglucosinolate through engineering of a multi-gene pathway in a versatile yeast expression platform. Metab. Eng. 14, 104–11. doi:10.1016/j.ymben.2012.01.006

Schmidt, J., Raith, K., 2005. Analysis of benzylisoquinoline-type alkaloids by electrospray tandem mass spectrometry and atmospheric pressure photoionization. Eur. J. Mass Spectrom. 11, 325–333.

Thodey, K., Galanie, S., Smolke, C.D., 2014. A microbial biomanufacturing platform for natural and semisynthetic opioids. Nat. Chem. Biol. 10, 837–44. doi:10.1038/nchembio.1613