PRODUCTION OF METHIONINE VIA CONDENSATION OF METHIONAL AND C1-DERIVED FORMYL-COA

TECHNICAL FIELD

[0001] The present disclosure generally relates to a method for producing methionine, in particular, a method for producing methionine via condensation of methional and C1-derived formyl-CoA. The present disclosure also relates to genetically modified microorganism used for producing methionine.

SUMMARY OF THE INVENTION

[0002] The present disclosure provides the following exemplary embodiments.

[0003] Embodiment 1. A method for the production of 2-hydroxy-4-(methylthio)butyryl-CoA comprising contacting formyl-CoA and methional with a TPP-dependent enzyme selected from 2-hydroxyacyl-CoA synthase, 2-hydroxyacyl-CoA lyase, oxalyl-CoA decarboxylase or benzaldehyde lyase.

[0004] Embodiment 2. The method of Embodiment 1, wherein the TPP-dependent enzyme is 2-hydroxyacyl-CoA synthase or 2-hydroxyacyl-CoA lyase.

[0005] Embodiment 3. The method of Embodiment 2, wherein the TPP-dependent enzyme is selected from:

HACS#	GenBank Accession Number	HACS#	GenBank Accession Number	HACS#	GenBank Accession Number
1	XP_012756082.1	47	MXY78649.1	93	TAJ19927.1
2	TMK01573.1	48	MBA01399.1	94	PKN81274.1
3	PYM26381.1	49	MXX31676.1	95	RLT34960.1
4	EEG70177.1	50	MXV80929.1	96	MBT5775398.1
5	MBH80817.1	51	MBI4083577.1	97	TMD99851.1
6	WP_030891887.1	52	MBK6319978.1	98	MSQ12864.1
7	AGK93615.1	53	MBI5948182.1	99	MBL0714078.1
8	MAX57815.1	54	PFG74273.1	100	WP_114297888.1

9	WP_068916287.1	55	WP_158065972.1	101	MAK25262.1
10	WP_062165271.1	56	MBN9492325.1	102	WP_068138361.1
11	MBB43458.1	57	MBK6663287.1	103	RMG94145.1
12	PCJ72347.1	58	MBI2766664.1	104	MBA4180234.1
13	TMQ19149.1	59	HEM18354.1	105	MBM3723043.1
14	MAX11513.1	60	GBD22648.1	106	ABF11225.1
15	HAK63664.1	61	MBF6599205.1	107	TAL98798.1
16	MBG92919.1	62	MXW00101.1	108	NNN20496.1
17	PZC46201.1	63	MYA07641.1	109	MBP1761901.1
18	MBB84818.1	64	REJ76484.1	110	PPQ43247.1
19	OGA51379.1	65	HDY15625.1	111	MSQ25793.1
20	PWB41796.1	66	MBW2231087.1	112	TMK28344.1
21	MAE93843.1	67	NRA08835.1	113	HIB12002.1
22	OGP60024.1	68	NQZ98823.1	114	WP_179589464.1
23	OWB57166.1	69	MBI3918747.1	115	MXY42918.1
24	KXN72624.1	70	MBI2761137.1	116	WP_184156128.1
25	PVU86112.1	71	MBE0608783.1	117	HET53513.1
26	ORZ16580.1	72	MYA54281.1	118	TMK22624.1
27	XP_005644825.1	73	NRA01576.1	119	MXX66290.1
28	KZV27770.1	74	MBW2623123.1	120	GIS94895.1
29	EJY87672.1	75	MBI5615765.1	121	MBN1557905.1
30	HIG47824.1	76	MSR14309.1	122	MSV30368.1
31	TMD03111.1	77	XP_004342722.2	123	MBN2179295.1
32	MBJ56818.1	78	MSP42197.1	124	TDI90456.1
33	WP_095860310.1	79	TDI61101.1	125	OGN76415.1
34	MBL8483477.1	80	MBO0741576.1	126	WP_102074055.1
35	WP_058697592.1	81	MBO0736096.1	127	PZC47999.1
36	WP_130292058.1	82	MBV9828771.1	128	HHH88785.1
37	WP_207956071.1	83	MAW55136.1	129	OLB93949.1
38	WP_132429652.1	84	MBV38827.1	130	PKB76696.1
39	WP_060575023.1	85	TMJ68231.1	131	HED24197.1
40	WP_068796145.1	86	TMJ64557.1	132	WP_066960443.1
41	OJY48151.1	87	MBV9815528.1	133	WP_169259343.1
42	WP_062397209.1	88	MYH41266.1	134	WP_201494572.1
43	WP_169186431.1	89	MPZ97997.1	135	MBN9621549.1
44	WP_133828190.1	90	MBT5774752.1	136	OZG26106.1
45	MBS0560157.1	91	XP_014714961.1	137	WP_016501746.1
46	PCJ59575.1	92	TAK78428.1		

or

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[0006] Embodiment 4. The method of Embodiment 1, further comprising generating formyl-CoA by contacting a one-carbon (C1) substrate selected from formaldehyde or formate with an enzyme.

[0007] Embodiment 5. The method of Embodiment 4, wherein the C1 substrate is formaldehyde, and the enzyme is an acylating formaldehyde dehydrogenase.

[0008] Embodiment 6. The method of Embodiment 4, wherein the C1 substrate is formate and the enzyme is selected from:

(i) an acyl-CoA transferase catalyzing the conversion of formate to formyl-CoA;

(ii) an acyl-CoA synthase catalyzing the conversion of formate to formyl-CoA; or

(iii) a formate kinase catalyzing the conversion of formate to formyl-phosphate and a phosphate formyltransferase catalyzing the conversion of formyl-phosphate to formyl-CoA.

[0009] Embodiment 7. The method of Embodiment 5, wherein the formaldehyde dehydrogenase is selected from:

ACR#	GenBank Accession Number	ACR#	GenBank Accession Number
1	ABX41556.1	26	WP_094899923.1
2	WP_185879480.1	27	WP_152887945.1
3	WP_051457024.1	28	HAR84250.1
4	WP_088269124.1	29	MBS5083929.1
5	WP_087641473.1	30	WP_027296492.1
6	WP_051541705.1	31	WP_104149274.1
7	HHY51863.1	32	WP_135035016.1
8	WP_119112248.1	33	EJO19495.1
9	WP_114642697.1	34	WP_051245790.1
10	WP_202656015.1	35	WP_125552613.1
11	WP_115130814.1	36	HCL03003.1
12	WP_052127368.1	37	WP_126792036.1
13	MBN6206692.1	38	NQJ18683.1
14	WP_051217603.1	39	WP_191507870.1
15	WP_129009770.1	40	MBR5981728.1
16	MTK10086.1	41	WP_215633330.1
17	WP_106009325.1	42	MBP3327663.1
18	HBB29922.1	43	WP_152889336.1
19	WP_216437565.1	44	WP_052356672.1
20	WP_090039956.1	LmACR	Q8Y7U1
21	WP_083963349.1	PtACDH	A0A178TYF6
22	WP_012101452.1	StEutE	P41793
23	WP_122646076.1	RpPduP	Q21A49

24	WP_070791575.1	BmACDH	I3E349	or
25	WP_010715224.1	CbAld	Q716S8	.

[0010] Embodiment 8. The method of Embodiment 6, wherein the formate kinase and the phosphate formyltransferase pair is selected from:

JGIK #	GenBank Accession Number		JGIK #	GenBank Accession Number		
	ACK	PTA		ACK	PTA	
1	AKJ38693.1	AKJ38694.1	21	MBU2064158.1	MBU2064159.1	
2	BCV24779.1	BCV24778.1	22	MBU2501086.1	MBU2501087.1	
3	EDM85332.1	EDM85331.1	23	NLA96479.1	NLA96478.1	
4	GFI65571.1	GFI65570.1	24	NLI62094.1	NLI62095.1	
5	HBG22385.1	HBG22386.1	25	NLM93282.1	NLM93283.1	
6	HFV10353.1	HFV10352.1	26	NMA59030.1	NMA59029.1	
7	HGG13858.1	HGG13857.1	27	OGI05344.1	OGI05345.1	
8	HIX51076.1	HIX51075.1	28	OHB58473.1	OHB58472.1	
9	KXK65140.1	KXK65141.1	29	PWM39673.1	PWM39672.1	
10	KXL51791.1	KXL51790.1	30	TKJ47541.1	TKJ47542.1	
11	MBE5816841.1	MBE5816840.1	31	WP_022744670.1	WP_022744669.1	
12	MBE6451999.1	MBE6451998.1	32	WP_023275423.1	WP_023275424.1	
13	MBF0205569.1	MBF0205570.1	33	WP_076546120.1	WP_076546119.1	
14	MBI4335478.1	MBI4335477.1	34	WP_078810629.1	WP_078810628.1	
15	MBN1633386.1	MBN1633385.1	35	WP_099343330.1	WP_099343331.1	
16	MBR2784446.1	MBR2784445.1	36	WP_106012460.1	WP_106012461.1	
17	MBR3082232.1	MBR3082233.1	37	AAA72042.1	AAA72041.1	
18	MBS5449595.1	MBS5449596.1	38	BAG33697.1	BAG33698.1	
19	MBS6942200.1	MBS6942201.1	39	A0A0J8DB00	A0A0J8D6J2	or
20	MBU0667180.1	MBU0667181.1	40	P0A6A3	P0A9M8	.

[0011] Embodiment 9. The method of Embodiment 6, wherein the acyl-CoA transferase is selected from:

ACT#	GenBank Accession Number	ACT#	GenBank Accession Number
1	NBW24427.1	33	MBW7889249.1
2	HBE84973.1	34	MBK7676927.1
3	WP_132821656.1	35	MBV8105822.1
4	WP_220287672.1	36	MBP7764230.1

5	WP_121965416.1	37	MSO92582.1
6	XP_014530103.1	38	MBE9574050.1
7	2OAS_1	39	MBX9950024.1
8	WP_084235291.1	40	AEK61848.1
9	MBS6366228.1	41	MBL8096225.1
10	MBR5999861.1	42	OPZ27283.1
11	3D3U_1	43	RYD04206.1
12	WP_073092544.1	44	MBQ9059638.1
13	MBR0090292.1	45	2G39_1
14	MBU4349155.1	46	2NVV_1
15	NLU48536.1	47	MBQ9530926.1
16	3EH7_1	48	4EU3_1
17	MBS0639490.1	49	KAF4531260.1
18	WP_194298948.1	50	2HJ0_1
19	3UBM_A	51	SJZ60628.1
20	WP_203555095.1	52	ALP92681.1
21	MBC7087538.1	53	GFX41336.1
22	WP_191390389.1	54	5VIT_1
23	MBR0127170.1	55	SDR52074.1
24	WP_206582952.1	56	MBF0160244.1
25	MBK5252043.1	57	MBF0445994.1
26	MBR2778738.1	58	HGX18505.1
27	MBF8291010.1	59	MBK9387745.1
28	RLI25587.1	60	MBF0310027.1
29	HHW62298.1	61	RKY20198.1
30	NWF83872.1	62	NYH33089.1
31	NUN70050.1	63	Q9RM86
32	MBU2447274.1	64	O06644

or

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[0012] Embodiment 10. The method of Embodiment 6, wherein the acyl-CoA synthase is selected from:

Gene	**GeneBank**
EcACS	P27550
BsACS	P39062
StACSstab	Q8ZKF6_PROSS
MhACS	F2NQX2
ArACS	A0A0Q7JEV7

or

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[0013] Embodiment 11. The method of Embodiment 5, further comprising generating formaldehyde from methanol by contacting methanol with a methanol dehydrogenase.

[0014] Embodiment 12. The method of Embodiment 11, wherein the methanol dehydrogenase is selected from:

Gene	GeneBank
BmMDH2	I3E2P9
CnMDH2	F8GNE5
BsMDH	P42327

or .

[0015] Embodiment 13. The method of Embodiment 11 or 12, wherein further comprising generating methanol from methane by contacting methane with a methane monooxygenases.

[0016] Embodiment 14. The method of Embodiment 13, wherein the methane monooxygenases is selected from:

Gene	GeneBank
mmoXYBZCD	P18797
pmoA1A2B1B2	G1UBD1 Q607G3

or .

[0017] Embodiment 15. The method of any one of Embodiments 1 to 14, further comprising generating methional from acrolein.

[0018] Embodiment 16. The method of Embodiment 15, further comprising generating acrolein from propene.

[0019] Embodiment 17. A method for the production of 2-hydroxy-4-(methylthio)butyric acid comprising:

(a) providing 2-hydroxy-4-(methylthio)butyryl-CoA obtained by the method of claim 8; and

(b) converting 2-hydroxy-4-(methylthio)butyryl-CoA to 2-hydroxy-4-(methylthio)butyric acid by contacting 2-hydroxy-4-(methylthio)butyryl-CoA with any one of (i) and (iii):

(i) a thioesterase or an acyl-CoA transferase catalyzing the conversion of 2-hydroxy-4-(methylthio)butyryl-CoA to 2-hydroxy-4-(methylthio)butyric acid;

(ii) a phosphotransacylase catalyzing the conversion of 2-hydroxy-4-(methylthio)butyryl-CoA to 2-hydroxy-4-(methylthio)butyryl-phosphate and a

carboxylate kinase catalyzing the conversion of 2-hydroxy-4-(methylthio)butyryl-phosphate to 2-hydroxy-4-(methylthio)butyric acid; or

(iii) an acyl-CoA reductase catalyzing the conversion of 2-hydroxy-4-(methylthio)butyryl-CoA to 4-(methylthio)-2-hydroxy-butanal and an aldehyde dehydrogenase catalyzing the conversion of 4-(methylthio)-2-hydroxy-butanal to 2-hydroxy-4-(methylthio)butyric acid.

[0020] Embodiment 18. The method of Embodiment 17, wherein the acyl-CoA reductase is selected from:

ACR#	GenBank Accession Number	ACR#	GenBank Accession Number
1	ABX41556.1	26	WP_094899923.1
2	WP_185879480.1	27	WP_152887945.1
3	WP_051457024.1	28	HAR84250.1
4	WP_088269124.1	29	MBS5083929.1
5	WP_087641473.1	30	WP_027296492.1
6	WP_051541705.1	31	WP_104149274.1
7	HHY51863.1	32	WP_135035016.1
8	WP_119112248.1	33	EJO19495.1
9	WP_114642697.1	34	WP_051245790.1
10	WP_202656015.1	35	WP_125552613.1
11	WP_115130814.1	36	HCL03003.1
12	WP_052127368.1	37	WP_126792036.1
13	MBN6206692.1	38	NQJ18683.1
14	WP_051217603.1	39	WP_191507870.1
15	WP_129009770.1	40	MBR5981728.1
16	MTK10086.1	41	WP_215633330.1
17	WP_106009325.1	42	MBP3327663.1
18	HBB29922.1	43	WP_152889336.1
19	WP_216437565.1	44	WP_052356672.1
20	WP_090039956.1	LmACR	Q8Y7U1
21	WP_083963349.1	PtACDH	A0A178TYF6
22	WP_012101452.1	StEutE	P41793
23	WP_122646076.1	RpPduP	Q21A49
24	WP_070791575.1	BmACDH	I3E349
25	WP_010715224.1	CbAld	Q716S8

or .

[0021] Embodiment 19. The method of Embodiment 17, wherein the aldehyde dehydrogenase is:

Gene	GeneBank Accession Number
yqhD	NP_417484
YahK	P75691
Ybbo	Q46856
YjgB	P27250
AKR1B1	AAA51713.1
GLD1	ABD83952.1

or

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[0022] Embodiment 20. A method for the production of 2-keto-4-(methylthio)butyric acid comprising:

(a) providing 2-hydroxy-4-(methylthio)butyric acid by the method of any one of Embodiments 17 to 19; and

(b) converting 2-hydroxy-4-(methylthio)butyric acid to 2-keto-4-(methylthio)butyric acid by contacting 2-hydroxy-4-(methylthio)butyric acid with a 2-hydroxy acid dehydrogenase.

[0023] Embodiment 21. The method of Embodiment 20, wherein the 2-hydroxy acid dehydrogenase is selected from:

Gene	GeneBank Accession Number
CtBP1	U37408
2HADH	A0A8V0XVZ4
EcMdh-5Q	P61889 (5Q)
LlLdhA(Q85C)	Q01462
RmLdh	P13491
LlPanE	Q9CFY8
LldD	P33232
GLO2	Q9LRS0
dld	P06149

or

.

[0024] Embodiment 22. A method for the production of L-methionine or D-methionine comprising:

(a) providing 2-keto-4-(methylthio)butyric acid by the method of Embodiment 20 or 21; and

(b) converting 2-keto-4-(methylthio)butyric acid to L-methionine or D-methionine by contacting 2-keto-4-(methylthio)butyric acid with an enzyme selected from an amino acid dehydrogenase or a methionine aminotransferase.

[0025] Embodiment 23. The method of Embodiment 22, wherein the amino acid dehydrogenase is selected from:

Gene	GeneBank Accession Number
Bcd	M4KZ55
dadA1	Q9HTQ0
dadA	M1K4N6
dadA	B4EXU2
MtAld	P9WQB1
BsAld	Q08352

or .

[0026] Embodiment 24. The method of Embodiment 22, wherein the methionine aminotransferase is selected from:

Gene	GeneBank Accession Number
YbdL	A0A378BX14
YbdL	P77806
Aro8	P53090
BCAT	Q9LE06

or .

[0027] Embodiment 25. The method of any one of Embodiments 1 to 24, wherein the enzyme used in the method is isolated from a microorganism.

[0028] Embodiment 26. The method of any one of Embodiments 1 to 24, wherein the enzyme used in the method is contained in a microorganism.

[0029] Embodiment 27. A genetically modified microorganism providing 2-hydroxy-4-(methylthio)butyryl-CoA by the method of any one of Embodiments 1 to 16.

[0030] Embodiment 28. The microorganism of Embodiment 27, further providing 2-hydroxy-4-(methylthio)butyric acid by the method of any one of Embodiments 17 to 19.

[0031] Embodiment 29. The microorganism of Embodiment 28, further providing 2-keto-4-(methylthio)butyric acid by the method of Embodiment 20 or 21.

[0032] Embodiment 30. The microorganism of Embodiment 28, further providing L-methionine or D-methionine by the method of any one of Embodiments 22 to 24.

[0033] Embodiment 31. The microorganism of Embodiments 27 to 30, wherein the microorganism is selected from the group consisting of bacteria, yeast and fungi.

[0034] Embodiment 32. The microorganism of Embodiments 27 to 31, wherein the microorganismis Escherichia coli or Saccharomyces cerevisiae.

[0035] The following description of examples provides additional details, any one of which can be subject to patenting in combination with any other. The specification in its entirety is to be treated as providing a variety of details that can be used interchangeablely with other details.

[0036] The present disclosure is illustrated by the following non-limiting examples.

[0037] It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only, and are not restrictive of the invention. Further, the accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate embodiments of the invention and together with the description, serve to explain principles of the invention.

BRIEF DESCRIPTION OF DRAWINGS

[0038] The drawings referenced herein form a part of the specification. Features shown in the drawing illustrate only some embodiments of the application, and not of all embodiments of the application, unless the detailed description explicitly indicates otherwise, and readers of the specification should not make implications to the contrary.

[0039] Figure 1 is a scheme illustrating production of methionine from condensation of methional and C1-derived formyl-CoA.

[0040] The same reference numbers will be used throughout the drawings to refer to the same or like parts.

DETAILED DESCRIPTION OF THE INVENTION

[0041] The following detailed description of exemplary embodiments of the application refers to the accompanying drawings that form a part of the description. The drawings illustrate specific exemplary embodiments in which the application may be practiced. The detailed description, including the drawings, describes these embodiments in sufficient detail to enable those skilled in the art to practice the application. Those skilled in the art may further utilize other embodiments of the application, and make logical, mechanical, and other changes without departing from the spirit or scope of the application. Readers of the following detailed description should, therefore,

not interpret the description in a limiting sense, and only the appended claims define the scope of the embodiment of the application.

[0042] In this application, the use of the singular includes the plural unless specifically stated otherwise. In this application, the use of "or" means "and/or" unless stated otherwise. Furthermore, the use of the term "including" as well as other forms such as "includes" and "included" is not limiting. Additionally, the section headings used herein are for organizational purposes only, and are not to be construed as limiting the subject matter described.

[0043] EXAMPLES

[0044] EXAMPLE 1: PRODUCTION OF METHIONINE THROUGH CONDENSATION OF METHIONAL AND FORMYL-COA

[0045] This Example demonstrates the implementation of methionine production through C1+bio platform by condensation of methional and formyl-CoA and followed by derivation to methionine (**Figure 1** below). The condensation can be catalyzed by HACS (e.g., HACS set forth in **Table 1** below), including but not limited to beach sand metagenome BsmHACS (GeneBank accession: HAK63664.1), DhcHACS from *Dehalococcoidia bacterium* (GeneBank accession: PWB41796.1), CfhHACS from *Chloroflexi bacterium* (GeneBank accession: PKN81274.1), to generate 4-methylthio-2-hydroxybutyryl-CoA. 4-methylthio-2-hydroxybutyryl-CoA is further converted to 4-methylthio-2-hydroxybutyrate (desmeninol, liquid methionine) through the action of a thioesterase such as TesB from *E. coli* or *Pseudomonas putida* (McMahon, M.D. and Prather, K.L.J. Appl. Environ. Microbiol. 80:1042-1050 (2014)). Alternatively, 4-methylthio-2-hydroxybutyryl-CoA can be reduced to 4-methylthio-2-hydroxybutanal by an acyl-CoA reductase such as *Clostridium beijerinckii* ALD (Kim, S., et al. J. Ind. Microbiol. Biotechnol. 42:465-475 (2015)) (e.g., ACR set forth in **Table 2** below). 4-methylthio-2-hydroxybutanal can be further oxidized to desmeninol by a suitable aldehyde dehydrogenase such as *E. coli* AldA or AldB (Baldoma and Aguilar, J Biol Chem. 1987, 262(29):13991-6). Desmeninol is then converted to 4-methylthio-2-oxobutyrate by 2-hydroxy acid dehydrogenase (2HADH) (Matelska et al., BMC Evol Biol. 2018;18(1):199), it was then finally converted to L-methionine by amino acid dehydrogenase (AADH) (Nitta et a., J Bacteriol. 1974; 117(2): 588–592) or methionine aminotransferase (MAT) (Venos et al., BMC Microbiol, 2004; 4, 39) (e.g., enzymes set forth in **Table 3** below).

[0046] Methional can be chemically produced from propene. Propene was firstly oxidized with O_2 to generate acrolein (see, e.g., WO2005053839), and then condensed with CH_3SH to form methional (see, e.g., WO2006015684). Alternatively, methional can be chemically produced from glycerol. Glycerol was firstly dehydrated to form 3-hydroxyaldehyde, and then further dehydrated to generate acrolein (Manfro., et al., Catalysis for Sustainable Energy, 2013 (1): 60-70). As mentioned above, acrolein can be condensed with CH_3SH to form methional.

[0047] EXAMPLE 2. INTERCONVERSION OF ONE-CARBON COMPOUNDS TO GENERATE FORMYL-COA

[0048] Formyl-CoA can be provided through the interconversion of C1 compounds including methane, methanol, formaldehyde, formate (**Figure 1** below) with suitable enzymes (e.g., enzymes set forth in **Table 4** below). Methane is readily available C1 source from natural gas, landfills, and agriculture. Biological oxidation of methane to methanol is catalyzed by methane monooxygenases. Functional expression of soluble methane monooxygenase (sMMO) from *Methylococcus capsulatus* is demonstrated in *E. coli* as a host (bioRxiv 2021. 08.05.455234). Subsequent oxidation of methanol to formaldehyde can be catalyzed by NAD+-dependent methanol dehydrogenases (MDH), MDH from *Bacillus methanolicus* MGA3 (BmMDH), *Bacillus stearothermophilus* (BsMDH) (Metab. Eng. 39:49-59, 2017) and *Cupriavidus necator* (Appl. Microbiol. Biotechnol. 100:4969-4983, 2016) have been expressed and characterized in *E. coli*. Formaldehyde can be directly oxidized to formic acid catalyzed by *E. coli* formaldehyde detoxification system (*frmA*). Alternatively, formaldehyde oxidation to formyl-CoA can be catalyzed by various acylating aldehyde dehydrogenase candidates (Nat. Chem. Biol. 15:900-906, 2019). Formyl-CoA can either be fed to the C1 elongation platform or further converted to generate energy. Formyl-CoA hydrolysis to formate can be catalyzed by two different families of enzymes. Acyl-CoA transferases (ACT, e.g., set forth in **Table 5** below) can catalyze reversible CoA transfer from various CoA donor, such as 4-methylthio-2-hydroxy butyryl-CoA, 2,4-dihydroxy butyryl-CoA, acetyl-CoA or succinyl-CoA to formate. Phosphotransacylase-formate kinase (PTA-FOK, e.g., set forth in **Table 6** below) pair catalyze reversible phosphorylation of formyl-CoA to formyl-phosphate, followed by dephosphorylation to formate generating 1 ATP. While these two reactions are considered fully reversible, AMP-forming acyl-CoA synthetases are favored toward formate activation to formyl-CoA.

[0049] EXAMPLE 3: OVERVIEW OF METHODS USED FOR GENETIC MANIPULATION

[0050] The genes for overexpression are either cloned into appropriate vectors or inserted into chromosome with strong synthetic constitutive promoter, such as M1-93. When cloned into vectors, these genes are amplified through PCR using appropriate primers to append homology on each end for recombination into the vector backbone with e.g., Phusion polymerase (Thermo Scientific, Waltham, MA) to serve as the gene insert. Plasmids are linearized by the appropriate restriction enzymes (New England Biolabs, Ipswich, MA, USA) and recombined with the gene inserts using the In-Fusion HD Eco-Dry Cloning system. The mixture is subsequently transformed into Stellar competent cells. Transformants that grow on solid media (LB+Agar) supplemented with the appropriate antibiotic are isolated and screened for the gene insert by PCR. Plasmids from verified transformants are isolated and the sequence of the gene insert is further confirmed by DNA sequencing. The sequence confirmed plasmids are then introduced to host strain through electroporation.

[0051] All molecular biology techniques are performed with standard methods (Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989).) or by manufacturer protocol. Strains are stored in glycerol stocks at -80°C. Plates are prepared using LB medium containing 1.5% agar, and appropriate antibiotics are included at the following concentrations: ampicillin (100 μg/mL), kanamycin (50 μg/mL), spectinomycin (50 μg/mL) and chloramphenicol (12.5 μg/mL).

[0052] Expression of selected enzyme variants was achieved using plasmid-based gene expression by cloning the desired gene(s) into pETDuet-1 or pCDFDuet-1 (Novagen, Darmstadt, Germany) digested with appropriate restriction enzymes and by utilizing In-Fusion cloning technology (Clontech Laboratories, Inc., Mountain View, CA). Linear DNA fragments for insertion were created via PCR of the open reading frame of interest (for genes native to *E. coli*) or by gene synthesis of the codon optimized gene. Genes were synthesized by GeneArt (Life Technologies, Carlsbad, CA). Resulting In-Fusion reaction products were used to transform *E. coli* Stellar cells (Clontech Laboratories, Inc., Mountain View, CA), and clones identified by PCR screening were further confirmed by DNA sequencing. As a host for these vectors, an engineered strain of *E. coli* based on MG1655(DE3), BW25113 or ATCC8739 with knockouts for formaldehyde (Δ*frmA*),

formate (Δ*fdhF* Δ*fdnG* Δ*fdoG*) oxidation and aldehyde consumption (including *adhP*, *yjgB*, *yqhD*, *adhE*, etc) can be used, which we expected could compete or interfere with the analysis of our pathway.

[0053] EXAMPLE 4: FERMENTATION CONDITIONS USED FOR IN VIVO PRODUCTION OF METHIONINE

[0054] MOPS minimal medium (Neidhardt et al. J. Bacteriol.119736-47 (1974)) with 125 mM MOPS and Na_2HPO_4 in place of K_2HPO_4 (2.8 mM), supplemented with 20 g/L glucose, 10 g/L tryptone, 5 g/L yeast extract, 100 μM $FeSO_4$, 5 mM $(NH_4)_2SO_4$, and 30 mM NH_4Cl is used for fermentations. If required, 55 g/L of CaCO3 is also supplemented as pH buffer. 20 mM lactic acid is supplemented, if it is not synthesized intracellularly and needed for the experiment. 500 mM methanol is also supplemented. Antibiotics (50 μg/mL carbenicillin, 50 μg/mL spectinomycin and 50 μg/mL kanamycin) are included when appropriate. All chemicals are obtained from Fisher Scientific Co. (Pittsburg, PA) and Sigma-Aldrich Co. (St. Louis, MO).

[0055] Fermentations are performed in 25 mL Pyrex Erlenmeyer flasks (narrow mouth/heavy duty rim, Corning Inc., Corning, NY) or 96 deep well plates (2.2 mL, v bottom, USA scientific) filled with appropriate volume of fermentation medium and sealed with foam plugs filling the necks. For anaerobic conditions, 17.5 mL Hungate tubes are completely filled with fermentation medium and sealed with tubber septa. A single colony of the desired strain is cultivated overnight (14-16 hrs) in LB medium with appropriate antibiotics and used as the inoculum with initial OD_{600} as ~0.05. After inoculation, flasks are incubated in a NBS I24 Benchtop Incubator Shaker (New Brunswick Scientific Co., Inc., Edison, NJ) at 200 rpm and 37°C or 30°C. When optical density (550 nm, OD_{550}) reached ~0.3-0.5, appropriate concentration of isopropyl beta-D-1-thiogalactopyranoside (IPTG) (or other suitable inducer) is added for plasmid gene induction. Additional fermentations are conducted in a SixFors multi- fermentation system (Infors HT, Bottmingen, Switzerland) with an air or argon flowrate of 2 N L/hr, independent control of temperature (37 °C), pH (controlled at 7.0 with NaOH and H_2SO_4), and appropriate stirrer speed. Pre-cultures are grown in 25 mL Pyrex Erlenmeyer flasks as described above and incubated for 4 hours post-induction. An appropriate amount of this pre-culture is centrifuged, washed twice with fresh media, and used for inoculation (400 mL initial volume). The fermentations in bioreactor use described fermentation

media with 40 g/L glucose and appropriate IPTG and antibiotics. If required, lactic acid (20 mM) is added at 0, 24, and 48 hours.

[0056] After the fermentation, the supernatant obtained through 5000 g, 5 min centrifuge in an Optima L-80XP Ultracentrifuge (Beckman-Coulter, Schaumburg, IL) of 2 mL culture is prepared for HPLC analysis.

[0057] EXAMPLE 5. HPLC ANALYSIS OF METHIONINE

[0058] The supernatant aliquots of 0.1 mL transferred to 15 mL tube, mixed with 0.2 mL of acetonitrile, 0.1 mL of 0.5 mM $NaHCO_3$ and 0.1 mL of 2,4-Dinitrofluorobenzene (DNFB) solution (0.2 mL DNFB add acetonitrile to 10 mL), tighten the cap and vigorously vortex for 30 seconds before doing derivatization. The derivatization reaction was carried out on a water bath (60 °C) for 20 min in the dark. After cooling in ice-cold water, 1 mL of 0.5 M phosphate buffer solution was added to terminate the reaction. Another 1 mL acetonitrile and 7.5 mL MiniQ water was added. The resulting solution of DNFB derivatives was then briefly vortexed again and filtered through a 0.22 μm nylon filter into auto-sampler vials for HPLC analysis.

[0059] HPLC analysis is conducted on a Waters 2695 system equipped with 996 photodiode array detector and a Phenomenex Luna C18 column (250mm*4.6mm*5μm). Column temperature was maintained at 30 °C, detector was set at 360 nm, and the injection volume was 10 μL. The mobile phase consisting of 0.1% acetic acid and 0.1% trimethylamine in MiniQ water (A), and acetonitrile (B). The separation was achieved using the following gradient conditions with a flow rate of 0.8 mL/min: 0 min, 30% acetonitrile (B); 15 min, 85% acetonitrile (B); 18 min, 85% acetonitrile (B); 18.01 min, 30% acetonitrile (B); 30 min, 30% acetonitrile (B).

[0060] Table 1. List of 2-hydroxyacyl-CoA synthase (HACS) used for the condensation of methional and formyl-CoA

HACS#	GenBank Accession Number	HACS#	GenBank Accession Number	HACS#	GenBank Accession Number
1	XP_012756082.1	47	MXY78649.1	93	TAJ19927.1
2	TMK01573.1	48	MBA01399.1	94	PKN81274.1
3	PYM26381.1	49	MXX31676.1	95	RLT34960.1
4	EEG70177.1	50	MXV80929.1	96	MBT5775398.1
5	MBH80817.1	51	MBI4083577.1	97	TMD99851.1

6	WP_030891887.1	52	MBK6319978.1	98	MSQ12864.1
7	AGK93615.1	53	MBI5948182.1	99	MBL0714078.1
8	MAX57815.1	54	PFG74273.1	100	WP_114297888.1
9	WP_068916287.1	55	WP_158065972.1	101	MAK25262.1
10	WP_062165271.1	56	MBN9492325.1	102	WP_068138361.1
11	MBB43458.1	57	MBK6663287.1	103	RMG94145.1
12	PCJ72347.1	58	MBI2766664.1	104	MBA4180234.1
13	TMQ19149.1	59	HEM18354.1	105	MBM3723043.1
14	MAX11513.1	60	GBD22648.1	106	ABF11225.1
15	HAK63664.1	61	MBF6599205.1	107	TAL98798.1
16	MBG92919.1	62	MXW00101.1	108	NNN20496.1
17	PZC46201.1	63	MYA07641.1	109	MBP1761901.1
18	MBB84818.1	64	REJ76484.1	110	PPQ43247.1
19	OGA51379.1	65	HDY15625.1	111	MSQ25793.1
20	PWB41796.1	66	MBW2231087.1	112	TMK28344.1
21	MAE93843.1	67	NRA08835.1	113	HIB12002.1
22	OGP60024.1	68	NQZ98823.1	114	WP_179589464.1
23	OWB57166.1	69	MBI3918747.1	115	MXY42918.1
24	KXN72624.1	70	MBI2761137.1	116	WP_184156128.1
25	PVU86112.1	71	MBE0608783.1	117	HET53513.1
26	ORZ16580.1	72	MYA54281.1	118	TMK22624.1
27	XP_005644825.1	73	NRA01576.1	119	MXX66290.1
28	KZV27770.1	74	MBW2623123.1	120	GIS94895.1
29	EJY87672.1	75	MBI5615765.1	121	MBN1557905.1
30	HIG47824.1	76	MSR14309.1	122	MSV30368.1
31	TMD03111.1	77	XP_004342722.2	123	MBN2179295.1
32	MBJ56818.1	78	MSP42197.1	124	TDI90456.1
33	WP_095860310.1	79	TDI61101.1	125	OGN76415.1
34	MBL8483477.1	80	MBO0741576.1	126	WP_102074055.1
35	WP_058697592.1	81	MBO0736096.1	127	PZC47999.1
36	WP_130292058.1	82	MBV9828771.1	128	HHH88785.1
37	WP_207956071.1	83	MAW55136.1	129	OLB93949.1
38	WP_132429652.1	84	MBV38827.1	130	PKB76696.1
39	WP_060575023.1	85	TMJ68231.1	131	HED24197.1
40	WP_068796145.1	86	TMJ64557.1	132	WP_066960443.1
41	OJY48151.1	87	MBV9815528.1	133	WP_169259343.1
42	WP_062397209.1	88	MYH41266.1	134	WP_201494572.1
43	WP_169186431.1	89	MPZ97997.1	135	MBN9621549.1
44	WP_133828190.1	90	MBT5774752.1	136	OZG26106.1
45	MBS0560157.1	91	XP_014714961.1	137	WP_016501746.1

46	PCJ59575.1	92	TAK78428.1		

[0061] Table 2. List of acyl-CoA reductase (ACR)

ACR#	GenBank Accession Number	ACR#	GenBank Accession Number
1	ABX41556.1	26	WP_094899923.1
2	WP_185879480.1	27	WP_152887945.1
3	WP_051457024.1	28	HAR84250.1
4	WP_088269124.1	29	MBS5083929.1
5	WP_087641473.1	30	WP_027296492.1
6	WP_051541705.1	31	WP_104149274.1
7	HHY51863.1	32	WP_135035016.1
8	WP_119112248.1	33	EJO19495.1
9	WP_114642697.1	34	WP_051245790.1
10	WP_202656015.1	35	WP_125552613.1
11	WP_115130814.1	36	HCL03003.1
12	WP_052127368.1	37	WP_126792036.1
13	MBN6206692.1	38	NQJ18683.1
14	WP_051217603.1	39	WP_191507870.1
15	WP_129009770.1	40	MBR5981728.1
16	MTK10086.1	41	WP_215633330.1
17	WP_106009325.1	42	MBP3327663.1
18	HBB29922.1	43	WP_152889336.1
19	WP_216437565.1	44	WP_052356672.1
20	WP_090039956.1	LmACR	Q8Y7U1
21	WP_083963349.1	PtACDH	A0A178TYF6
22	WP_012101452.1	StEutE	P41793
23	WP_122646076.1	RpPduP	Q21A49
24	WP_070791575.1	BmACDH	I3E349
25	WP_010715224.1	CbAld	Q716S8

[0062] Table 3. List of enzyme used for the conversion of intermediate to methionine

RX/Enz	Gene	GeneBank Accession Number
2HADH	CtBP1	U37408
2HADH	2HADH	A0A8V0XVZ4
2HADH	EcMdh-5Q	P61889 (5Q)
2HADH	LlLdhA(Q85C)	Q01462
2HADH	RmLdh	P13491

2HADH	LlPanE	Q9CFY8
2HADH	LldD	P33232
2HADH	GLO2	Q9LRS0
2HADH	dld	P06149
ALDH	yqhD	NP_417484
ALDH	YahK	P75691
ALDH	Ybbo	Q46856
ALDH	YjgB	P27250
ALDH	AKR1B1	AAA51713.1
ALDH	GLD1	ABD83952.1
AADH	Bcd	M4KZ55
AADH	dadA1	Q9HTQ0
AADH	dadA	M1K4N6
AADH	dadA	B4EXU2
AADH	MtAld	P9WQB1
AADH	BsAld	Q08352
MAT	YbdL	A0A378BX14
MAT	YbdL	P77806
MAT	Aro8	P53090
MAT	BCAT	Q9LE06

[0063] Note: 2HADH: 2-hydroxyacid dehydrogenase; ALDH: aldehyde dehydrogenase; AADH: amino acid dehydrogenase; MAT: methionine aminotransferase

[0064] Table 4. List of enzymes used for the interconversion of one-carbon compounds to generate formyl-CoA

Reaction	Gene	GeneBank Accession Number
sMMO	mmoXYBZCD	P18797
pMMO	pmoA1A2B1B2	G1UBD1 Q607G3
MDH	BmMDH2	I3E2P9
	CnMDH2	F8GNE5
	BsMDH	P42327
ALDH	EcFrmA	P25437
	PpFdhA	P46154
ACS	EcACS	P27550
	BsACS	P39062
	StACSstab	Q8ZKF6_PROSS

	MhACS	F2NQX2
	ArACS	A0A0Q7JEV7

[0065] Note: sMMO: soluble methane monooxygenase; pMMO: Particulate methane monooxygenase; MDH: methanol dehydrogenase; ALDH: aldehyde dehydrogenase; ACS: acyl-CoA synthase.

[0066] Table 5. List of acyl-CoA transferases (ACT) variants used for the interconversion of one-carbon compounds to generate formyl-CoA

ACT#	GenBank Accession Number	ACT#	GenBank Accession Number
1	NBW24427.1	33	MBW7889249.1
2	HBE84973.1	34	MBK7676927.1
3	WP_132821656.1	35	MBV8105822.1
4	WP_220287672.1	36	MBP7764230.1
5	WP_121965416.1	37	MSO92582.1
6	XP_014530103.1	38	MBE9574050.1
7	2OAS_1	39	MBX9950024.1
8	WP_084235291.1	40	AEK61848.1
9	MBS6366228.1	41	MBL8096225.1
10	MBR5999861.1	42	OPZ27283.1
11	3D3U_1	43	RYD04206.1
12	WP_073092544.1	44	MBQ9059638.1
13	MBR0090292.1	45	2G39_1
14	MBU4349155.1	46	2NVV_1
15	NLU48536.1	47	MBQ9530926.1
16	3EH7_1	48	4EU3_1
17	MBS0639490.1	49	KAF4531260.1
18	WP_194298948.1	50	2HJ0_1
19	3UBM_A	51	SJZ60628.1
20	WP_203555095.1	52	ALP92681.1
21	MBC7087538.1	53	GFX41336.1
22	WP_191390389.1	54	5VIT_1
23	MBR0127170.1	55	SDR52074.1
24	WP_206582952.1	56	MBF0160244.1
25	MBK5252043.1	57	MBF0445994.1
26	MBR2778738.1	58	HGX18505.1
27	MBF8291010.1	59	MBK9387745.1
28	RLI25587.1	60	MBF0310027.1
29	HHW62298.1	61	RKY20198.1

30	NWF83872.1	62	NYH33089.1
31	NUN70050.1	63	Q9RM86
32	MBU2447274.1	64	O06644

[0067] Table 6. List of List of acyl-CoA kinases (ACK) and phosphoacyltransferases (PTA) variants (JGIK)variants used for the interconversion of one-carbon compounds to generate formyl-CoA

JGIK#	GenBank Accession Number		JGIK#	GenBank Accession Number	
	ACK	PTA		ACK	PTA
1	AKJ38693.1	AKJ38694.1	21	MBU2064158.1	MBU2064159.1
2	BCV24779.1	BCV24778.1	22	MBU2501086.1	MBU2501087.1
3	EDM85332.1	EDM85331.1	23	NLA96479.1	NLA96478.1
4	GFI65571.1	GFI65570.1	24	NLI62094.1	NLI62095.1
5	HBG22385.1	HBG22386.1	25	NLM93282.1	NLM93283.1
6	HFV10353.1	HFV10352.1	26	NMA59030.1	NMA59029.1
7	HGG13858.1	HGG13857.1	27	OGI05344.1	OGI05345.1
8	HIX51076.1	HIX51075.1	28	OHB58473.1	OHB58472.1
9	KXK65140.1	KXK65141.1	29	PWM39673.1	PWM39672.1
10	KXL51791.1	KXL51790.1	30	TKJ47541.1	TKJ47542.1
11	MBE5816841.1	MBE5816840.1	31	WP_022744670.1	WP_022744669.1
12	MBE6451999.1	MBE6451998.1	32	WP_023275423.1	WP_023275424.1
13	MBF0205569.1	MBF0205570.1	33	WP_076546120.1	WP_076546119.1
14	MBI4335478.1	MBI4335477.1	34	WP_078810629.1	WP_078810628.1
15	MBN1633386.1	MBN1633385.1	35	WP_099343330.1	WP_099343331.1
16	MBR2784446.1	MBR2784445.1	36	WP_106012460.1	WP_106012461.1
17	MBR3082232.1	MBR3082233.1	37	AAA72042.1	AAA72041.1
18	MBS5449595.1	MBS5449596.1	38	BAG33697.1	BAG33698.1
19	MBS6942200.1	MBS6942201.1	39	A0A0J8DB00	A0A0J8D6J2
20	MBU0667180.1	MBU0667181.1	40	P0A6A3	P0A9M8

[0068] Various embodiments have been described herein with reference to the accompanying drawings. It will, however, be evident that various modifications and changes may be made thereto, and additional embodiments may be implemented, without departing from the broader scope of the invention as set forth in the claims that follow. Further, other embodiments will be apparent to those skilled in the art from consideration of the specification and practice of one or more embodiments of the invention disclosed herein. It is intended, therefore, that this application

and the examples herein be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following listing of exemplary claims.

Claims

1. A genetically modified microorganism for converting methional and formyl-CoA to 2-hydroxy-4-(methylthio)butyryl-CoA, comprising an overexpressed TPP-dependent enzyme selected from a 2-hydroxyacyl-CoA synthase, a 2-hydroxyacyl-CoA lyase, an oxalyl-CoA decarboxylase or a benzaldehyde lyase.
2. The genetically modified microorganism of claim 1, further comprising an overexpressed enzyme catalyzing the conversion of 2-hydroxy-4-(methylthio)butyryl-CoA to 2-hydroxy-4-(methylthio)butyric acid, wherein the overexpressed enzyme is selected from thioesterase, acyl-CoA transferase, a phosphotransacylase and a carboxylate kinase or an acyl-CoA reductase and an aldehyde dehydrogenase.
3. The genetically modified microorganism of claim 2, further comprising an overexpressed 2-hydroxy acid dehydrogenase catalyzing the conversion of 2-hydroxy-4-(methylthio)butyric acid to 2-keto-4-(methylthio)butyric acid.
4. The genetically modified microorganism of claim 3, further comprising an overexpressed amino acid dehydrogenase catalyzing the conversion of 2-keto-4-(methylthio)butyric acid to L-methionine or D-methionine.
5. The genetically modified microorganism of claim 3, further comprising an overexpressed methionine aminotransferase catalyzing the conversion of 2-keto-4-(methylthio)butyric acid to L-methionine or D-methionine.
6. The genetically modified microorganisms of any one of claims 1-5, further comprising an overexpressed enzyme selected from the group consisting of:

   (i) an acylating formaldehyde dehydrogenase converting formaldehyde to formyl-CoA;

   (ii) a formate kinase converting formate to formyl-phosphate and a phosphate formyltransferase converting formyl-phosphate to formyl-CoA;

   (iii) an acyl-CoA transferase converting formate to formyl-CoA; and

   (iv) an acyl-CoA synthase converting formate to formyl-CoA.

7. The genetically modified microorganisms of any one of claims 1-6, capable of converting methional and a C1 substrate selected from methane, methanol, formaldehyde or formate to a product selected from desmeninol, 2-keto-4-(methylthio)butyric acid, L-methionine or D-methionine.

8. The genetically modified microorganisms of any one of claims 1-7, wherein the microorganisms are selected from the group consisting of bacteria, yeast and fungi.

9. The genetically modified microorganisms of any one of claims 1-8, wherein the microorganisms are Escherichia coli or Saccharomyces cerevisiae.

10. A method for the production of 2-hydroxy-4-(methylthio)butyryl-CoA comprising contacting formyl-CoA and methional with a TPP-dependent enzyme selected from 2-hydroxyacyl-CoA synthase, 2-hydroxyacyl-CoA lyase, oxalyl-CoA decarboxylase or benzaldehyde lyase.

11. A method for the production of 2-hydroxy-4-(methylthio)butyric acid comprising:

(a) providing 2-hydroxy-4-(methylthio)butyryl-CoA obtained by the method of claim 10; and

(b) converting 2-hydroxy-4-(methylthio)butyryl-CoA to 2-hydroxy-4-(methylthio)butyric acid by contacting 2-hydroxy-4-(methylthio)butyryl-CoA with any one of (i) and (iii):

(i) a thioesterase or an acyl-CoA transferase catalyzing the conversion of 2-hydroxy-4-(methylthio)butyryl-CoA to 2-hydroxy-4-(methylthio)butyric acid;

(ii) a phosphotransacylase catalyzing the conversion of 2-hydroxy-4-(methylthio)butyryl-CoA to 2-hydroxy-4-(methylthio)butyryl-phosphate and a carboxylate kinase catalyzing the conversion of 2-hydroxy-4-(methylthio)butyryl-phosphate to 2-hydroxy-4-(methylthio)butyric acid; or

(iii) an acyl-CoA reductase catalyzing the conversion of 2-hydroxy-4-(methylthio)butyryl-CoA to 4-(methylthio)-2-hydroxy-butanal and an aldehyde dehydrogenase catalyzing the conversion of 4-(methylthio)-2-hydroxy-butanal to 2-hydroxy-4-(methylthio)butyric acid.

12. A method for the production of 2-keto-4-(methylthio)butyric acid comprising:

(a) providing 2-hydroxy-4-(methylthio)butyric acid obtained by the method of claim 11; and

(b) converting 2-hydroxy-4-(methylthio)butyric acid to 2-keto-4-(methylthio)butyric acid by contacting 2-hydroxy-4-(methylthio)butyric acid with a 2-hydroxy acid dehydrogenase.

13. A method for the production of L-methionine or D-methionine comprising:

(a) providing 2-keto-4-(methylthio)butyric acid obtained by the method of claim 12; and
(b) converting 2-keto-4-(methylthio)butyric acid to L-methionine or D-methionine by contacting 2-keto-4-(methylthio)butyric acid with an enzyme selected from an amino acid dehydrogenase or a methionine aminotransferase.