US 20250101441A1

(19) **United States**

(12) **Patent Application Publication**

Donohue et al.

(10) **Pub. No.: US 2025/0101441 A1**

(43) **Pub. Date: Mar. 27, 2025**

(54) **MICROORGANISMS AND METHODS FOR PRODUCING CIS,CIS-MUCONIC ACID**

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(73) Assignee: **Wisconsin Alumni Research Foundation**, Madison, WI (US)

(21) Appl. No.: **18/891,498**

(22) Filed: **Sep. 20, 2024**

Related U.S. Application Data

(60) Provisional application No. 63/584,362, filed on Sep. 21, 2023.

Publication Classification

(51) **Int. Cl.**

C12N 15/74	(2006.01)
C12N 9/02	(2006.01)
C12N 9/10	(2006.01)
C12N 9/88	(2006.01)
C12N 9/90	(2006.01)
C12P 7/44	(2006.01)

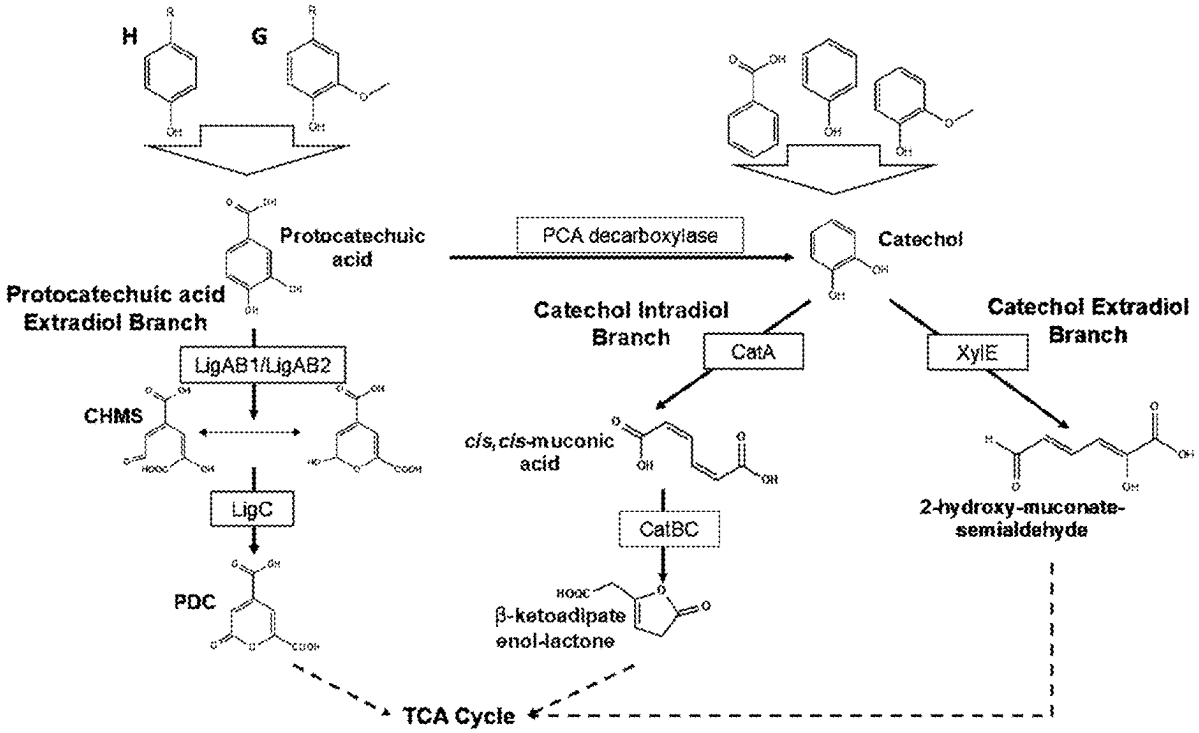
(52) **U.S. Cl.**

CPC ***C12N 15/74*** (2013.01); ***C12N 9/0069*** (2013.01); ***C12N 9/1085*** (2013.01); ***C12N 9/88*** (2013.01); ***C12N 9/90*** (2013.01); ***C12P 7/44*** (2013.01); *C12Y 113/11001* (2013.01); *C12Y 113/11002* (2013.01); *C12Y 113/11008* (2013.01); *C12Y 205/01* (2013.01); *C12Y 401/01063* (2013.01); *C12Y 503/03004* (2013.01); *C12Y 505/01001* (2013.01)

(57) **ABSTRACT**

Recombinant microorganisms configured for enhanced production of cis,cis-muconic acid and methods of using the recombinant microorganisms for the production of same.

Specification includes a Sequence Listing.



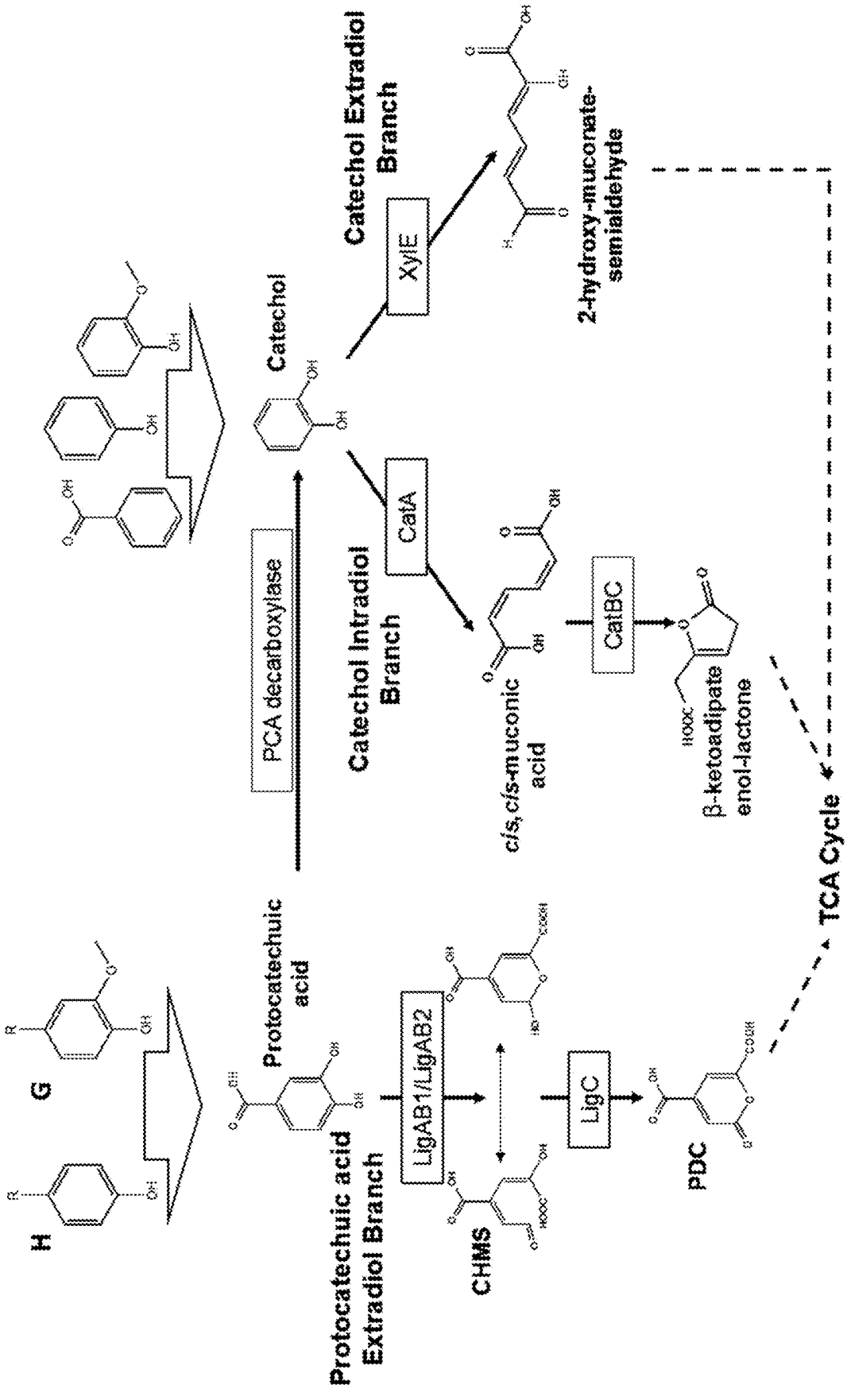

H
G
Protocatechuic acid
Extradiol Branch
Protocatechuic acid
LigAB1/LigAB2
CHMS
LigC
PDC
TCA Cycle
PCA decarboxylase
Catechol
Catechol Intradiol Branch
CatA
cis,cis-muconic acid
CatBC
β-ketoadipate enol-lactone
Catechol Extradiol Branch
XylE
2-hydroxy-muconate-semialdehyde

FIG. 1

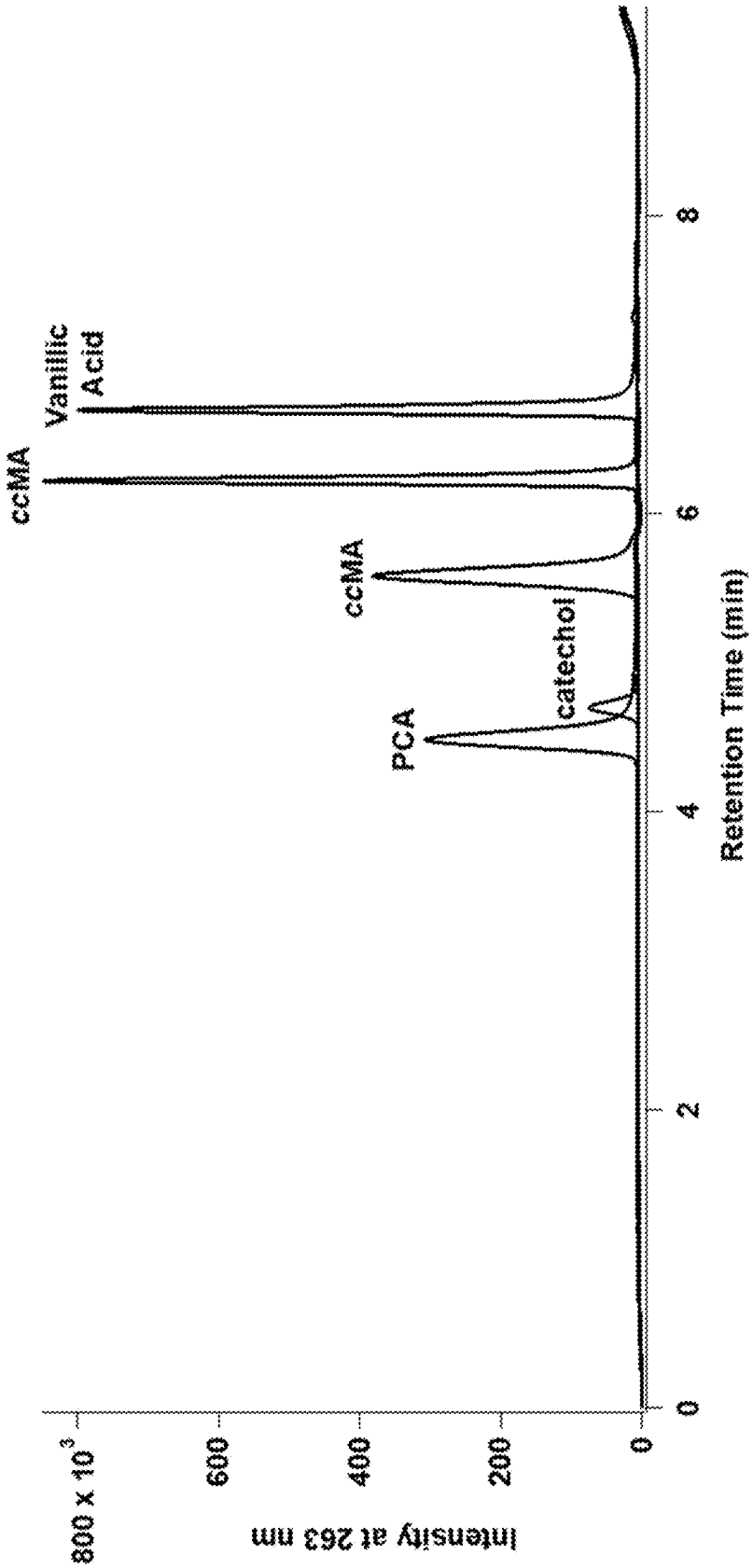

Intensity at 263 nm
800 x 10^3
600
400
200
0
0
2
4
6
8
Retention Time (min)
PCA
catechol
ccMA
ccMA
Vanillic Acid

FIG. 2

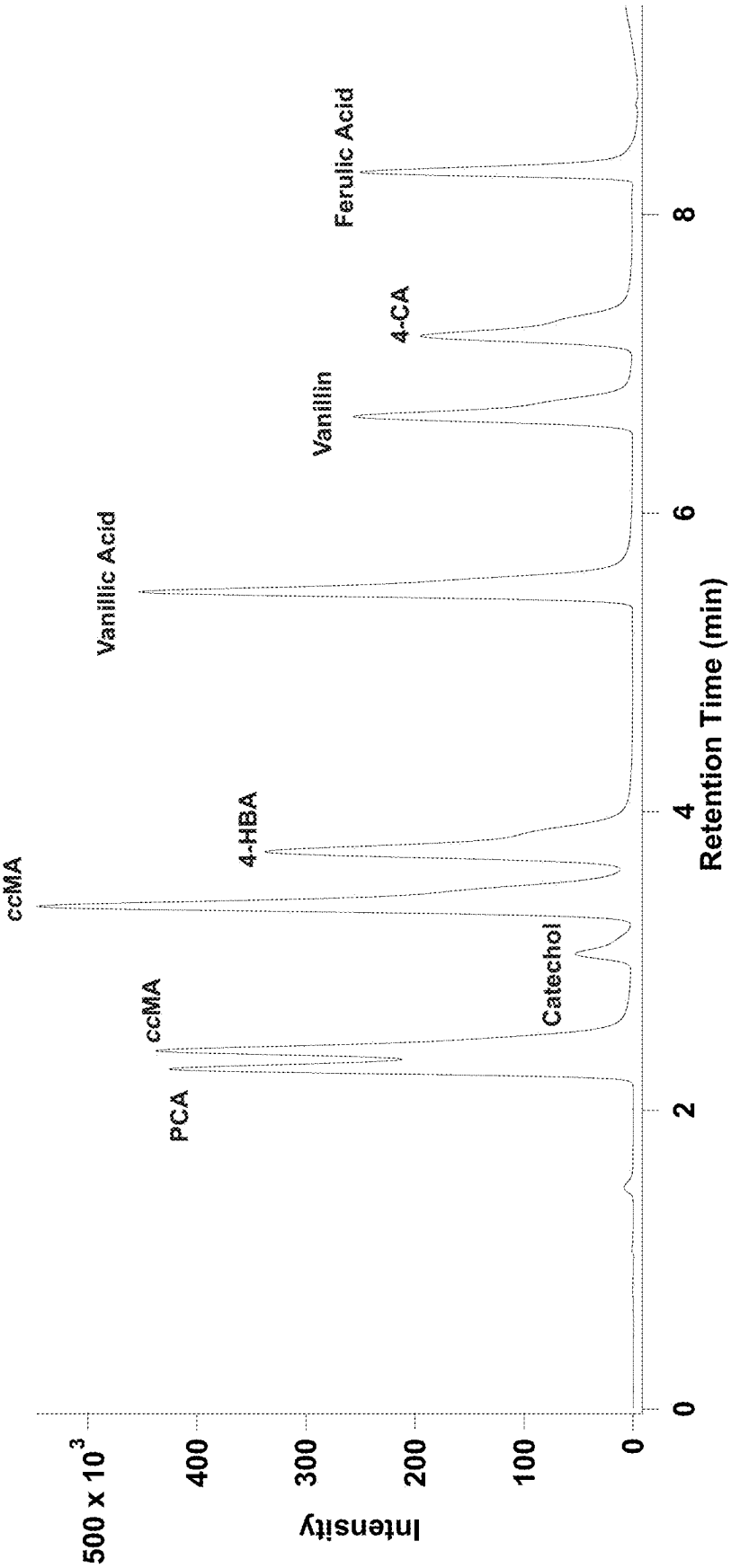

Intensity
500 x 10^3
400
300
200
100
0
0
2
4
6
8
Retention Time (min)
PCA
ccMA
ccMA
Catechol
4-HBA
Vanillic Acid
Vanillin
4-CA
Ferulic Acid

FIG. 3

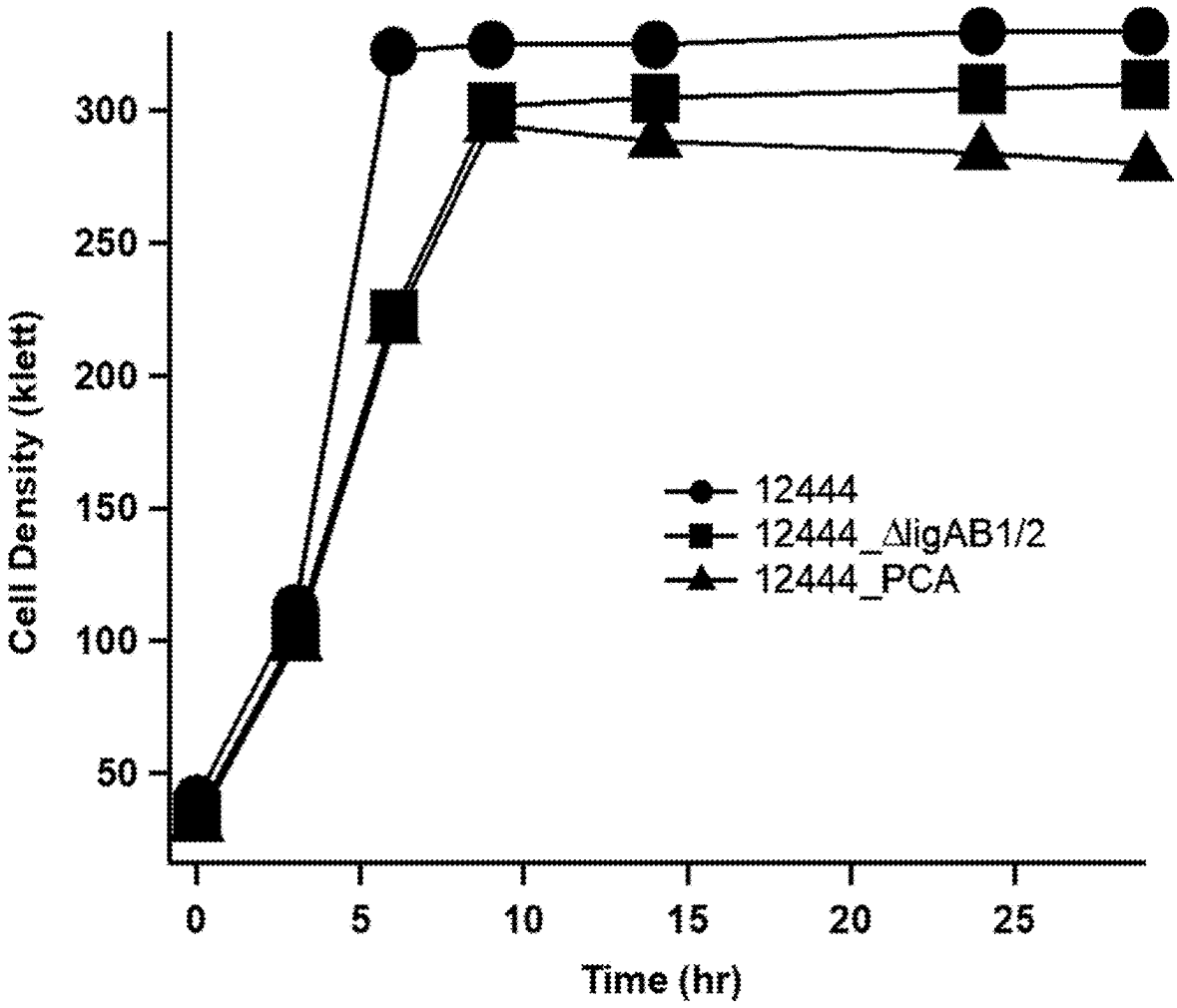

Cell Density (klett)
300
250
200
150
100
50
0
5
10
15
20
25
Time (hr)
12444
12444_ΔligAB1/2
12444_PCA

FIG. 4A

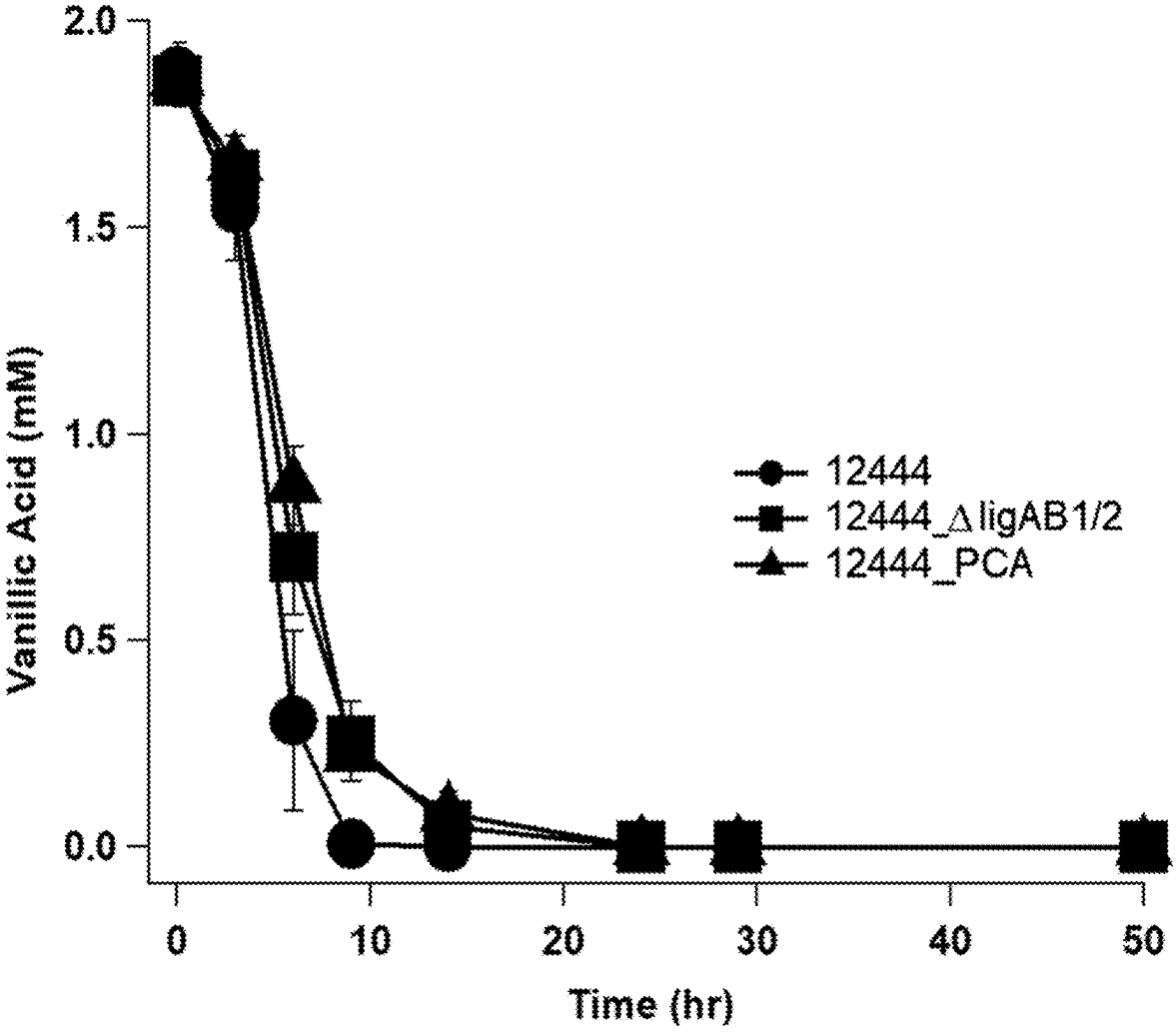

2.0
1.5
1.0
0.5
0.0
Vanillic Acid (mM)
0
10
20
30
40
50
Time (hr)
12444
12444_ΔligAB1/2
12444_PCA

FIG. 4B

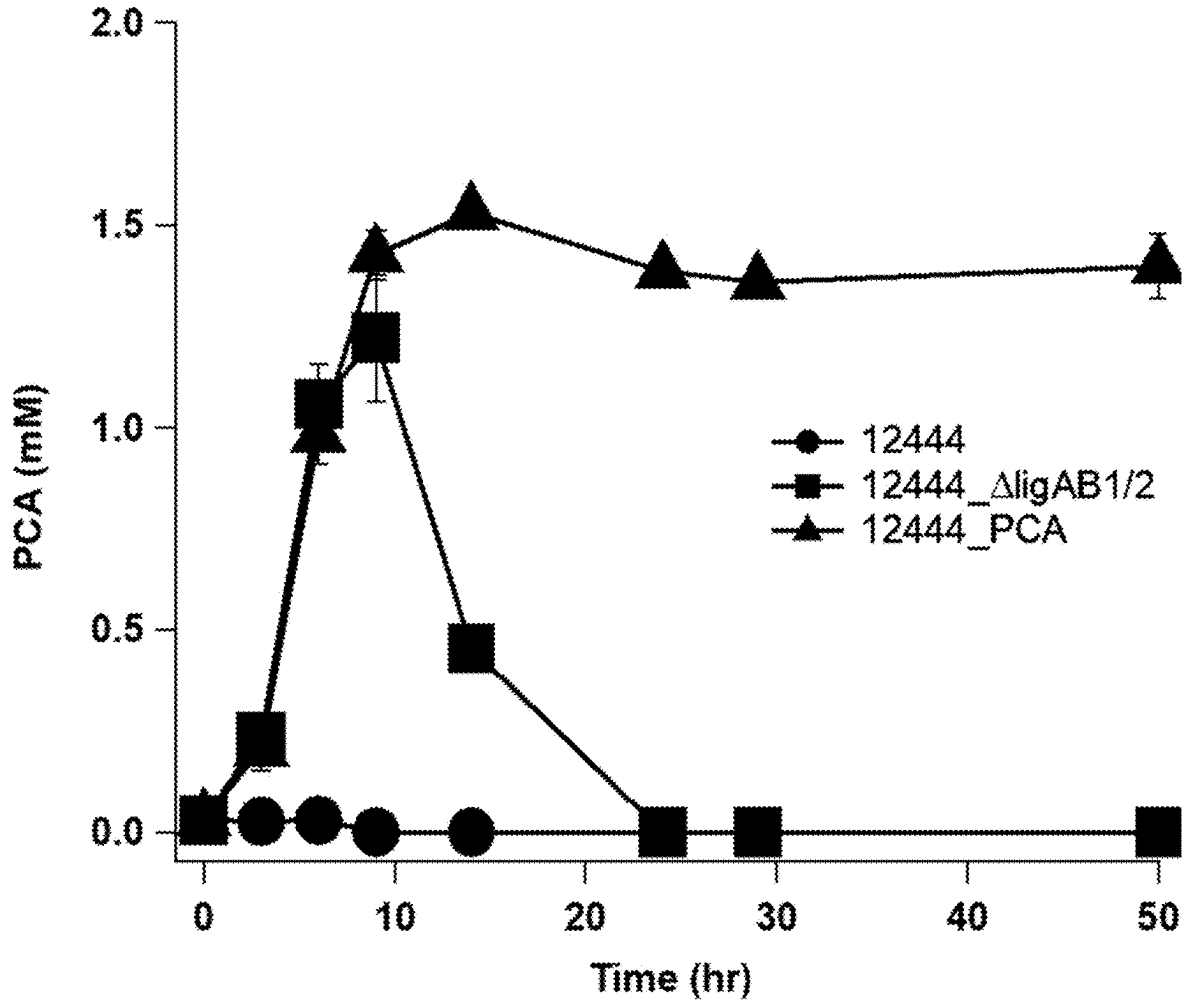

2.0
1.5
1.0
0.5
0.0
PCA (mM)
0
10
20
30
40
50
Time (hr)
12444
12444_ΔligAB1/2
12444_PCA

FIG. 4C

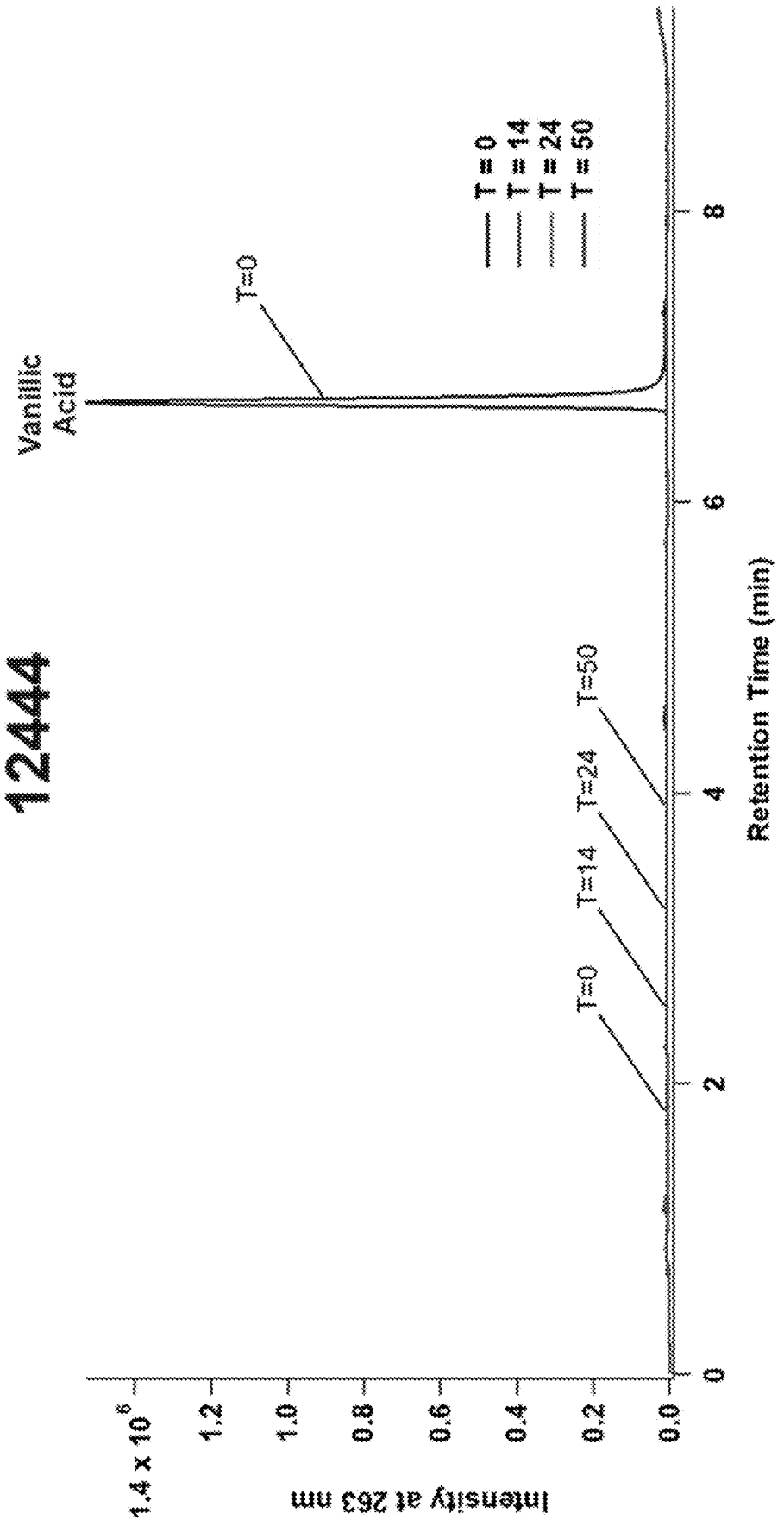

12444
Vanillic Acid
T=0
T = 0
T = 14
T = 24
T = 50
T=0
T=14
T=24
T=50
Intensity at 263 nm
1.4 x 10^6
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0
2
4
6
8
Retention Time (min)

FIG. 5A

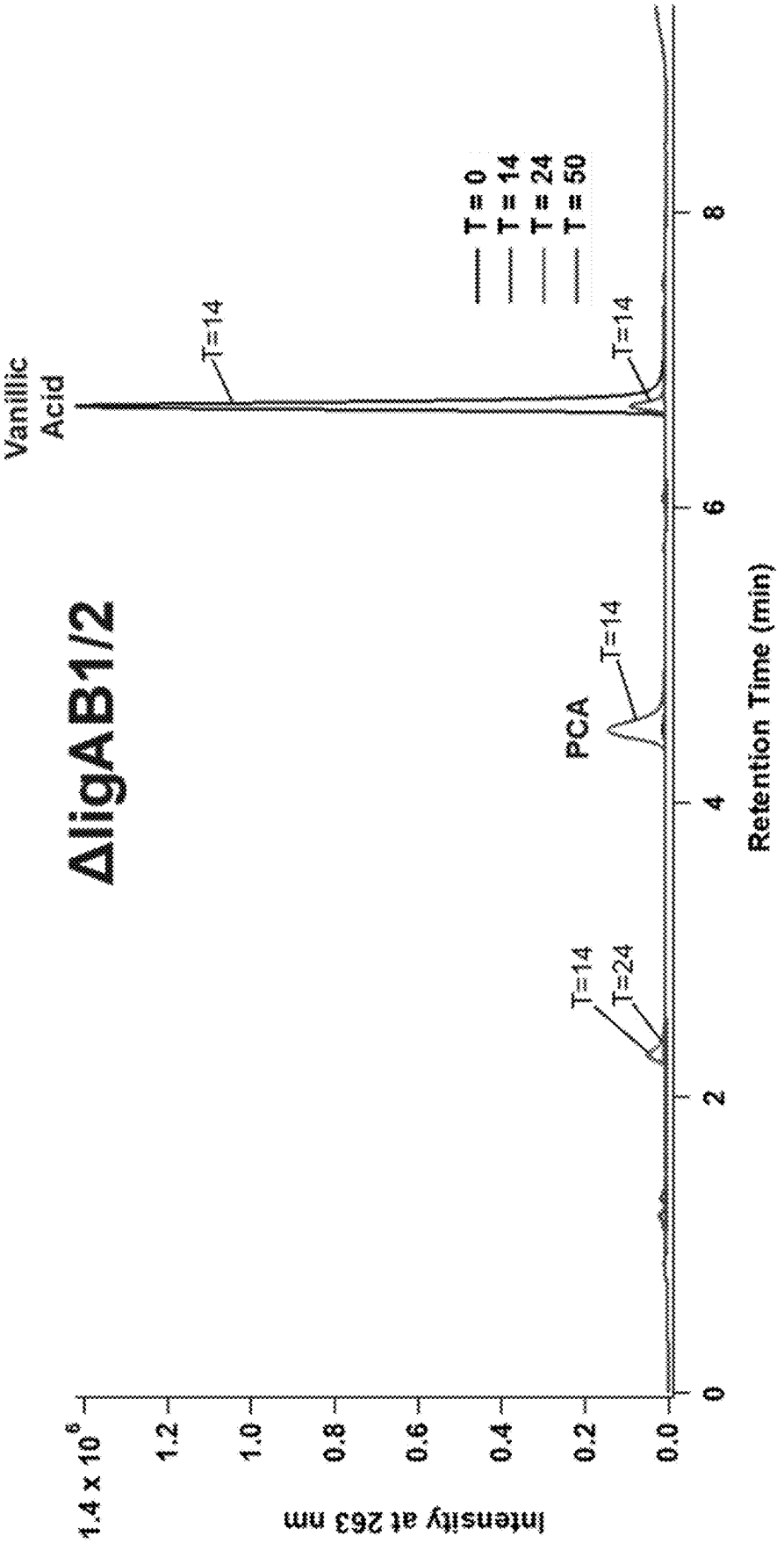

ΔligAB1/2
Vanillic Acid
PCA
T=14
T=24
T = 0
T = 14
T = 24
T = 50
Intensity at 263 nm
1.4 x 10^6
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
Retention Time (min)

FIG. 5B

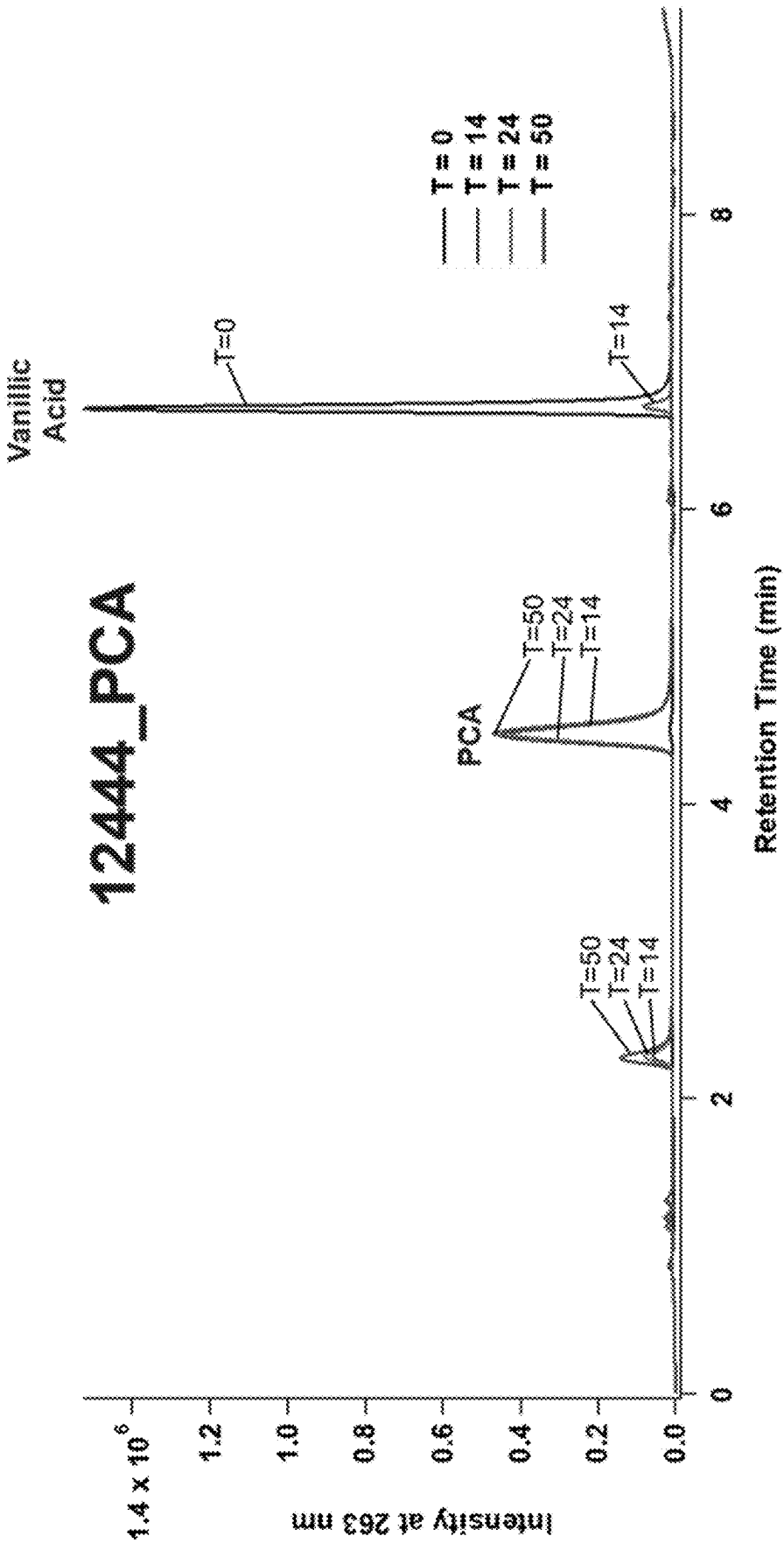

12444_PCA
Intensity at 263 nm
1.4 x 10^6
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
Retention Time (min)
T = 0
T = 14
T = 24
T = 50
Vanillic Acid
T=0
T=14
PCA
T=50
T=24
T=14
T=50
T=24
T=14

FIG. 5C

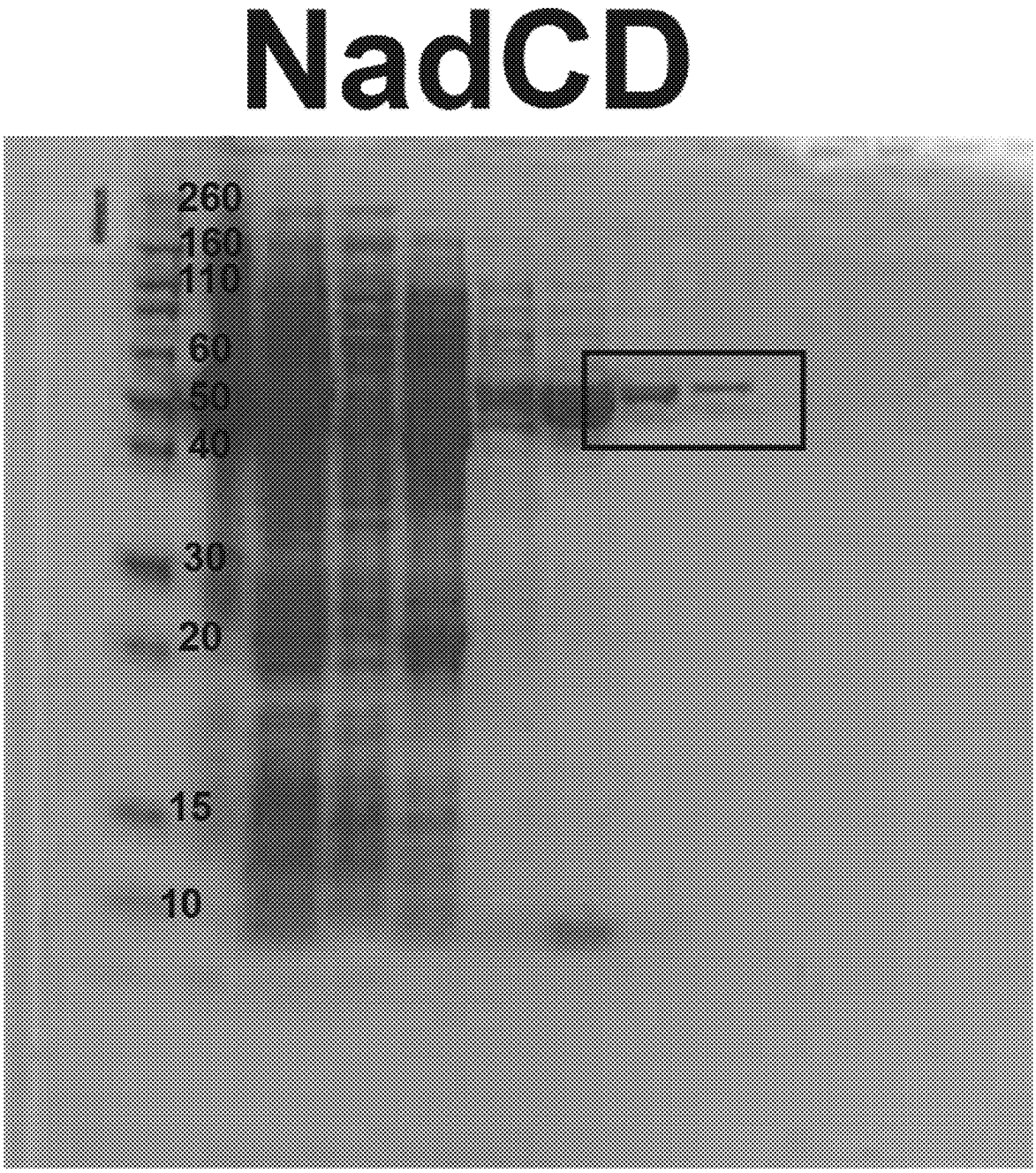


FIG. 6A

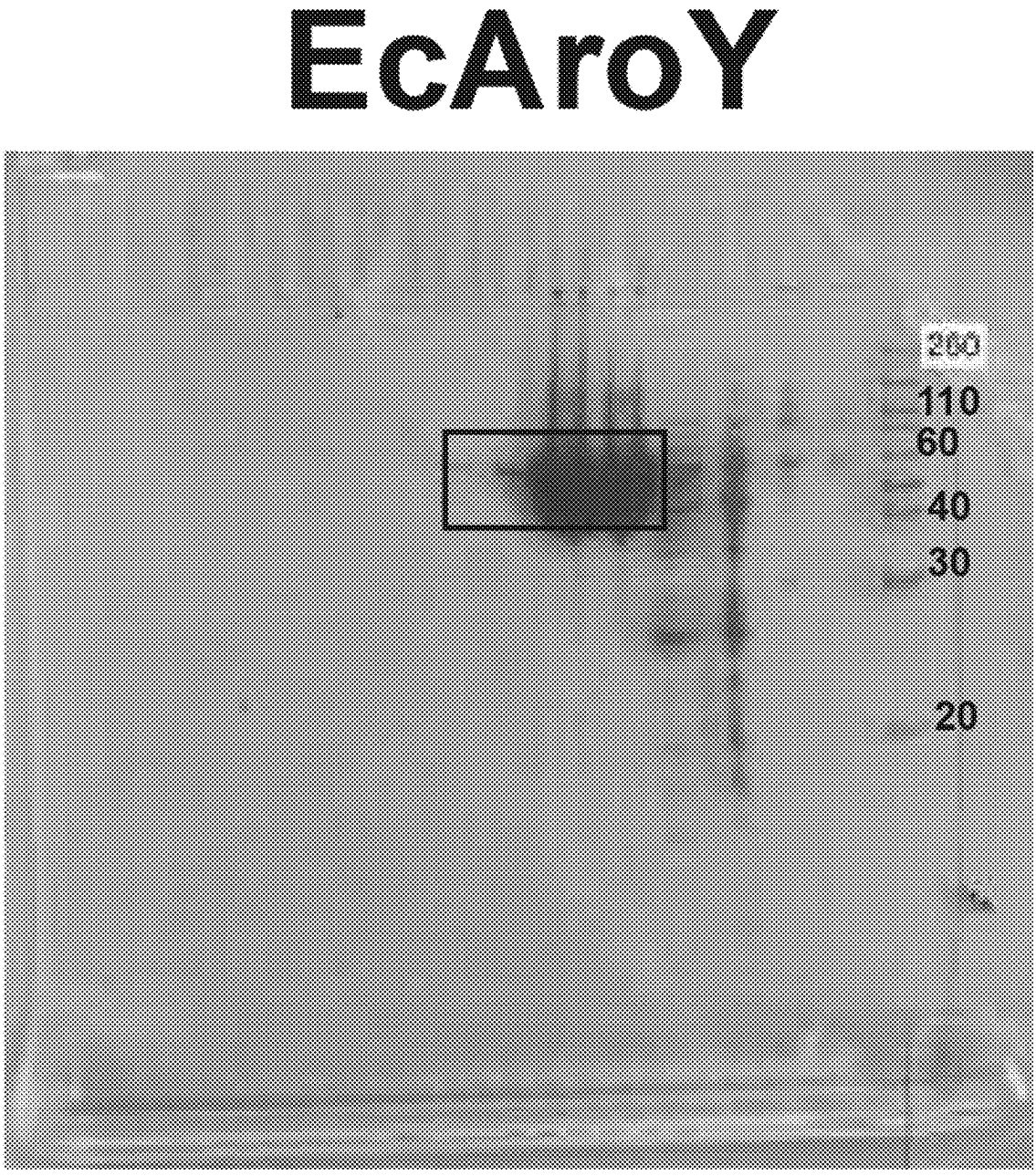


FIG. 6B

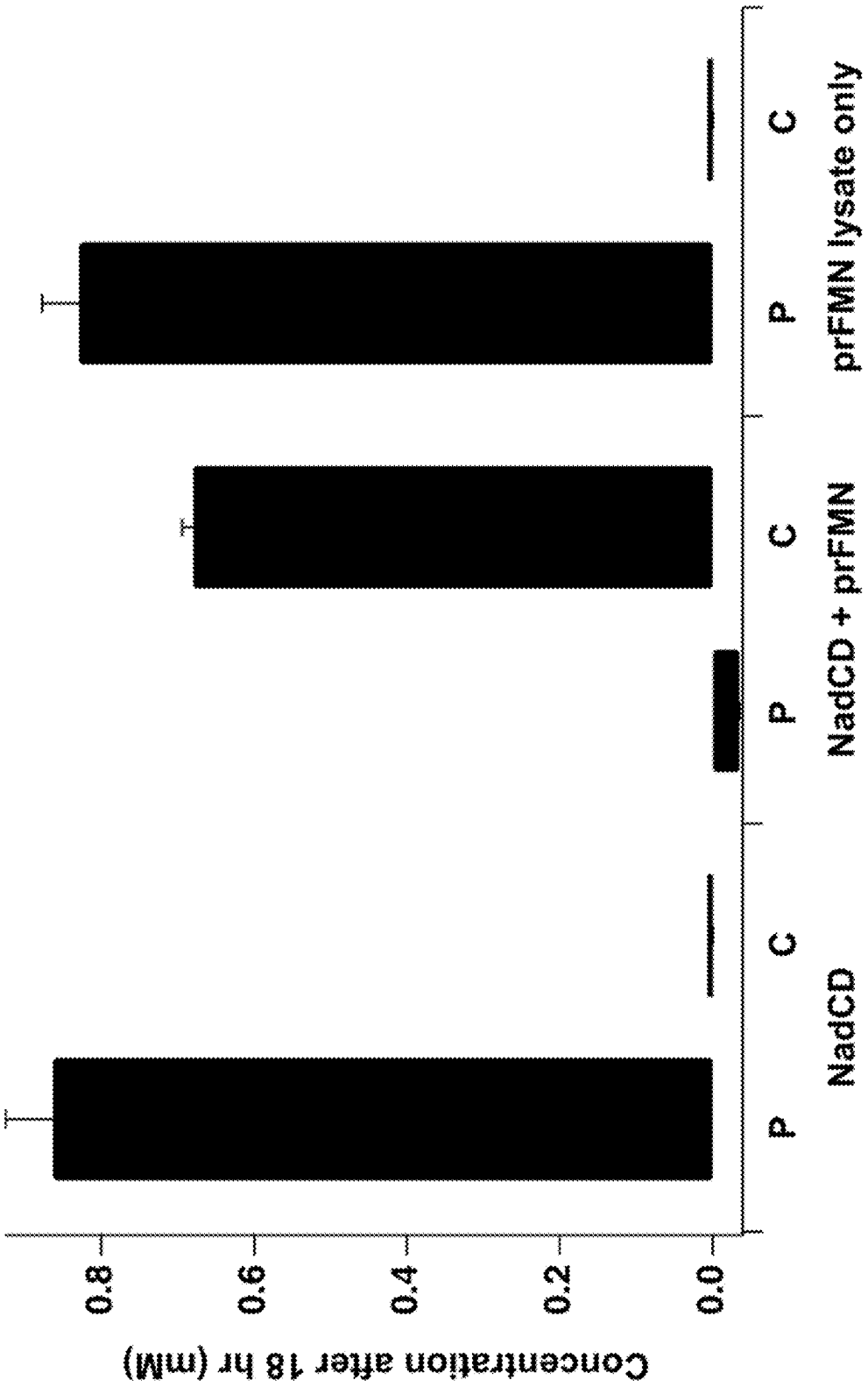

Concentration after 18 hr (mM)
0.0
0.2
0.4
0.6
0.8
P
C
NadCD
P
C
NadCD + prFMN
P
C
prFMN lysate only

FIG. 7

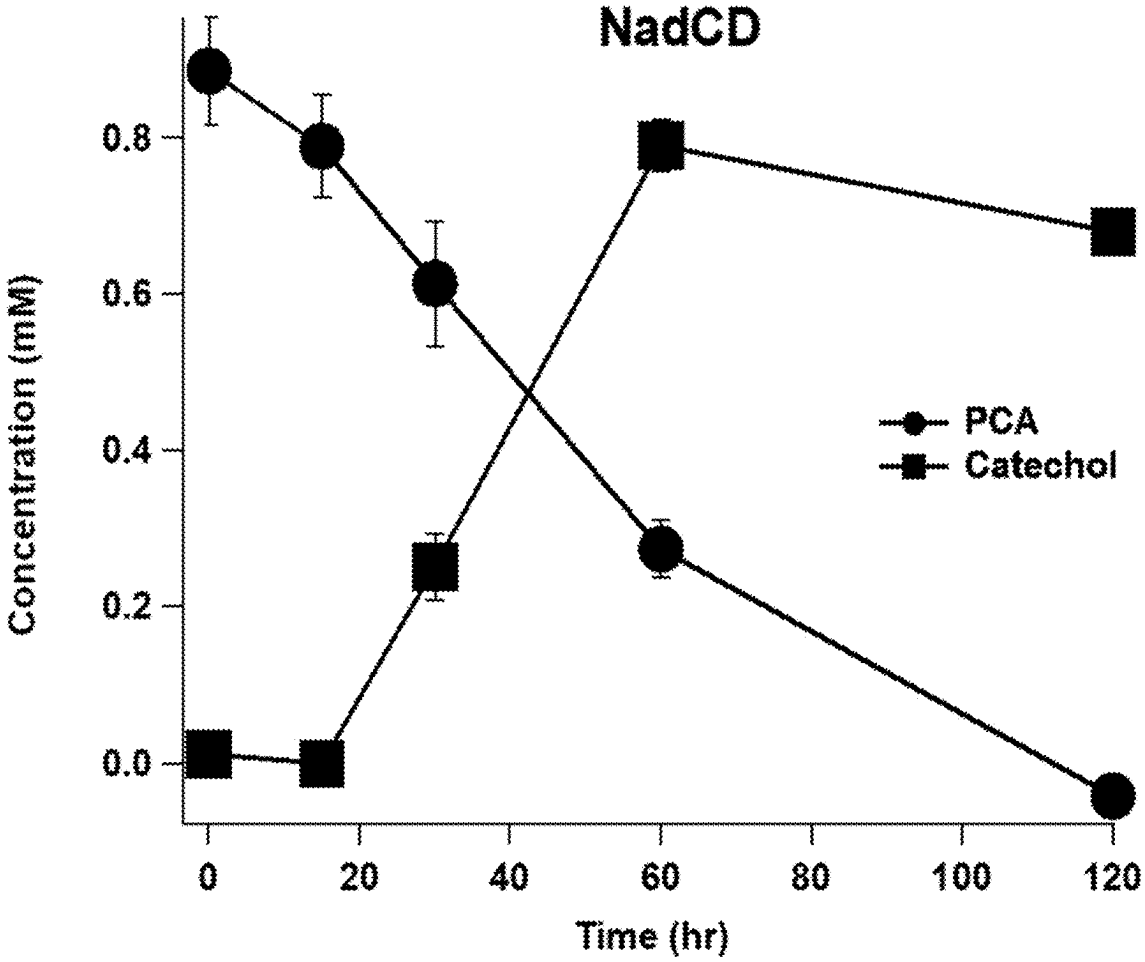

NadCD
Concentration (mM)
0.0
0.2
0.4
0.6
0.8
0
20
40
60
80
100
120
Time (hr)
PCA
Catechol

FIG. 8A

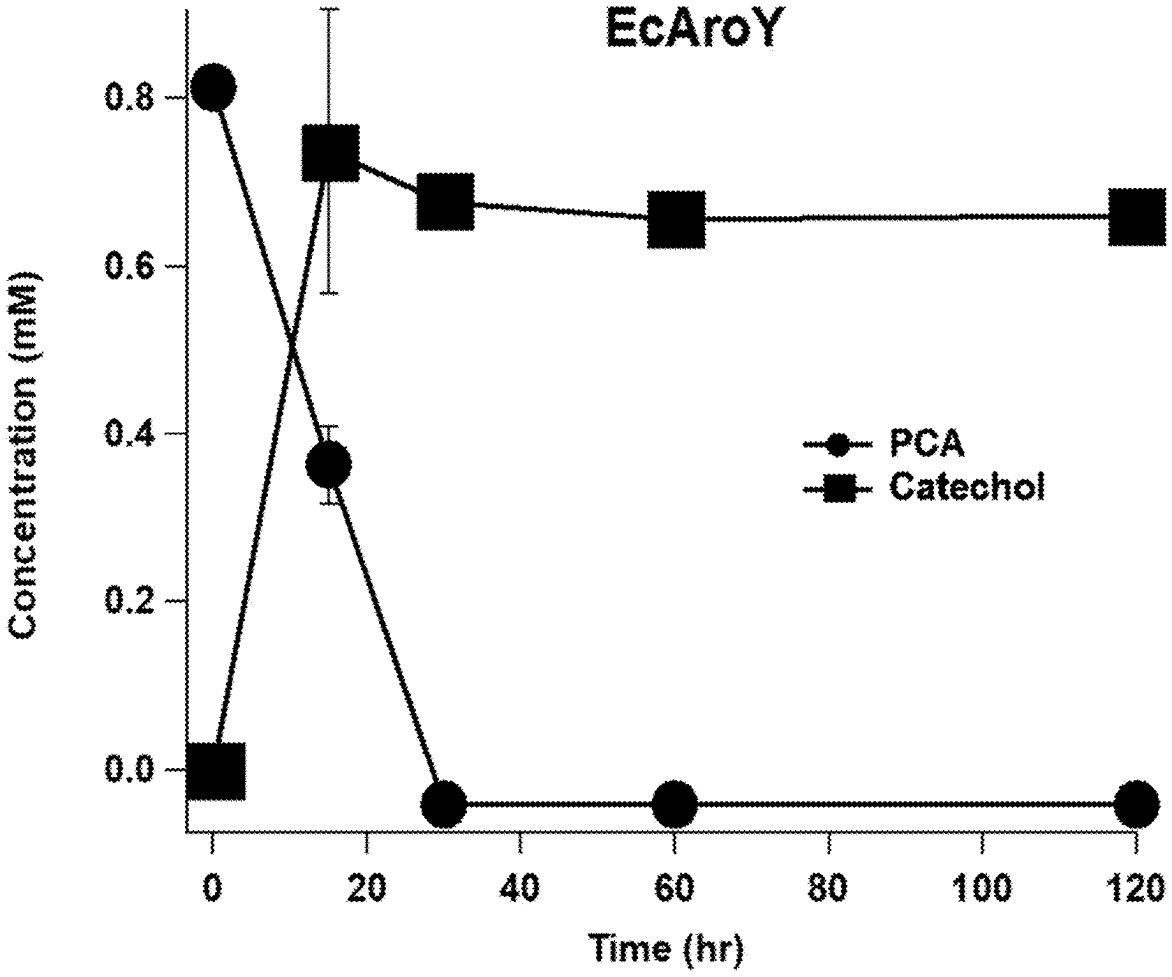

EcAroY
Concentration (mM)
0.0
0.2
0.4
0.6
0.8
0
20
40
60
80
100
120
Time (hr)
PCA
Catechol

FIG. 8B

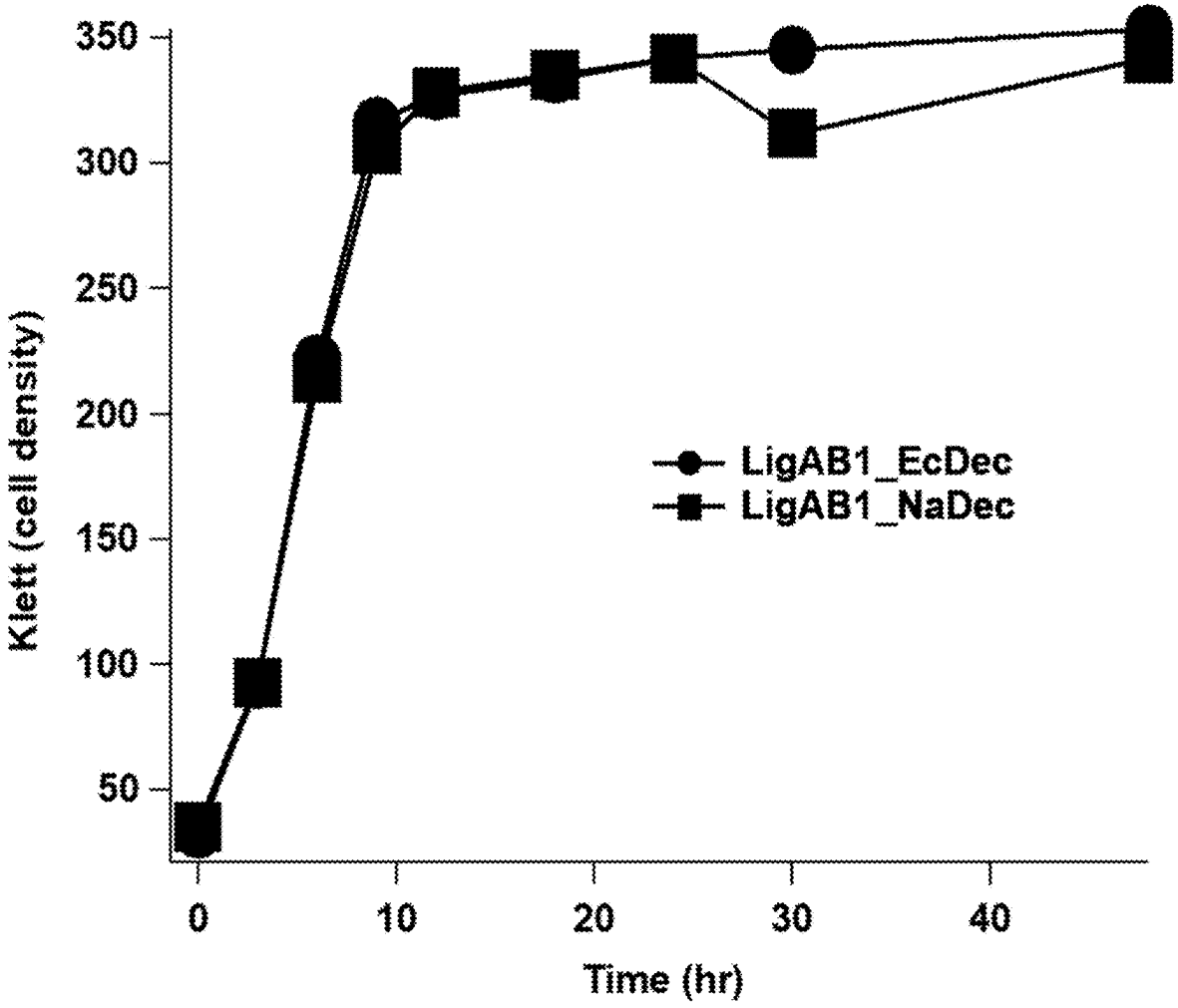

350
300
250
200
150
100
50
Klett (cell density)
0
10
20
30
40
Time (hr)
LigAB1_EcDec
LigAB1_NaDec

FIG. 9A

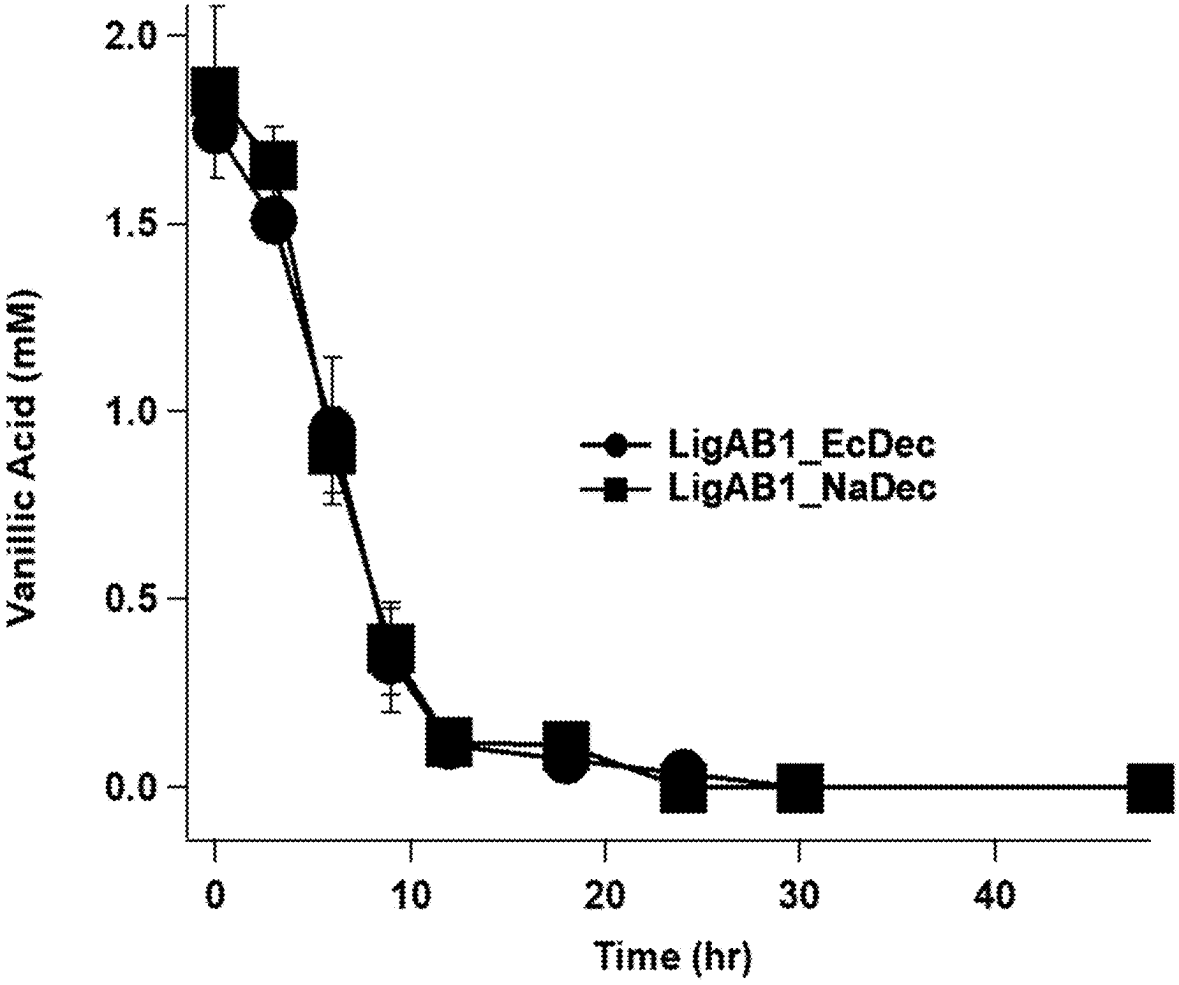

2.0
1.5
1.0
0.5
0.0
Vanillic Acid (mM)
0
10
20
30
40
Time (hr)
LigAB1_EcDec
LigAB1_NaDec

FIG. 9B

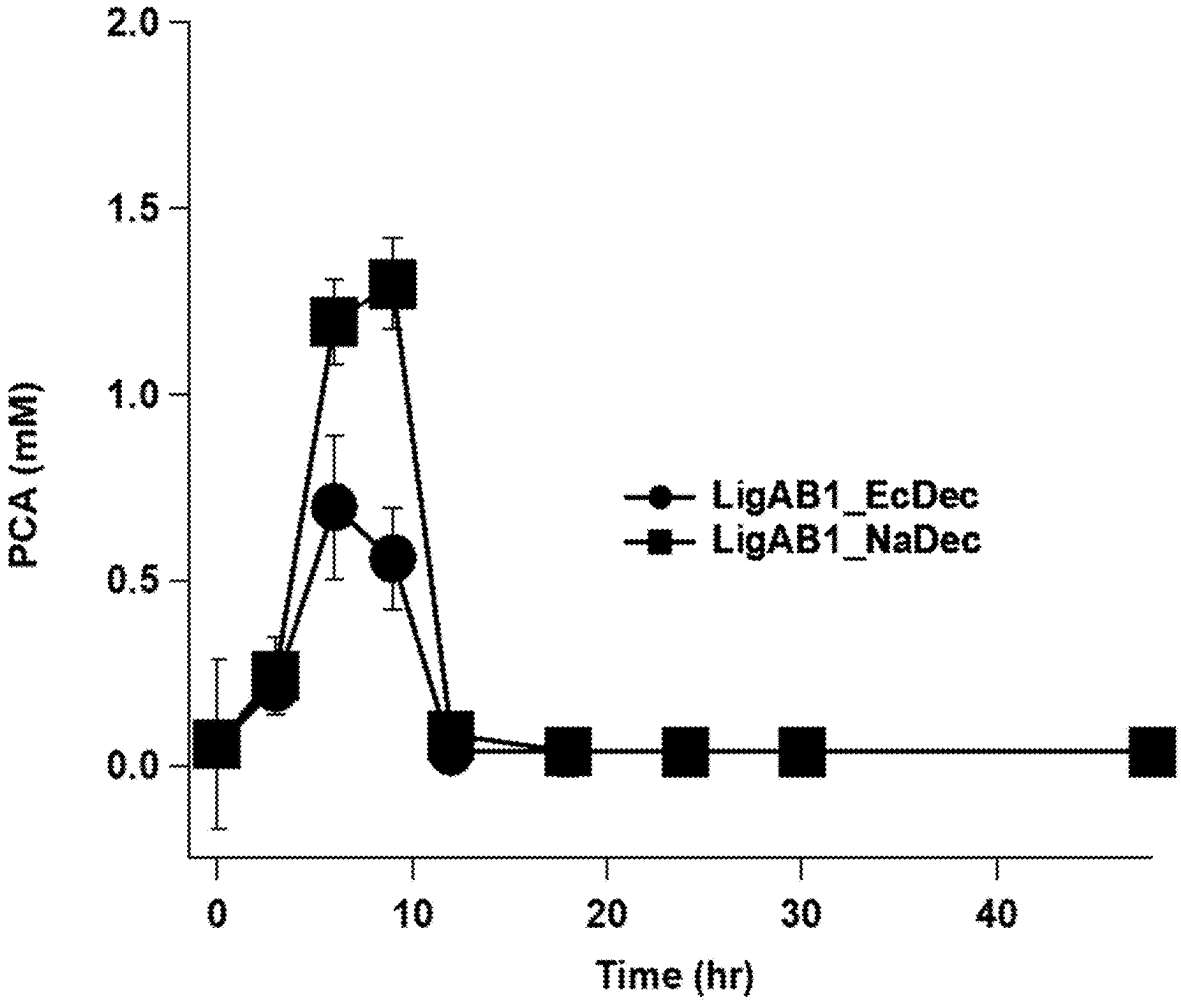

2.0
1.5
1.0
0.5
0.0
PCA (mM)
LigAB1_EcDec
LigAB1_NaDec
0
10
20
30
40
Time (hr)

FIG. 9C

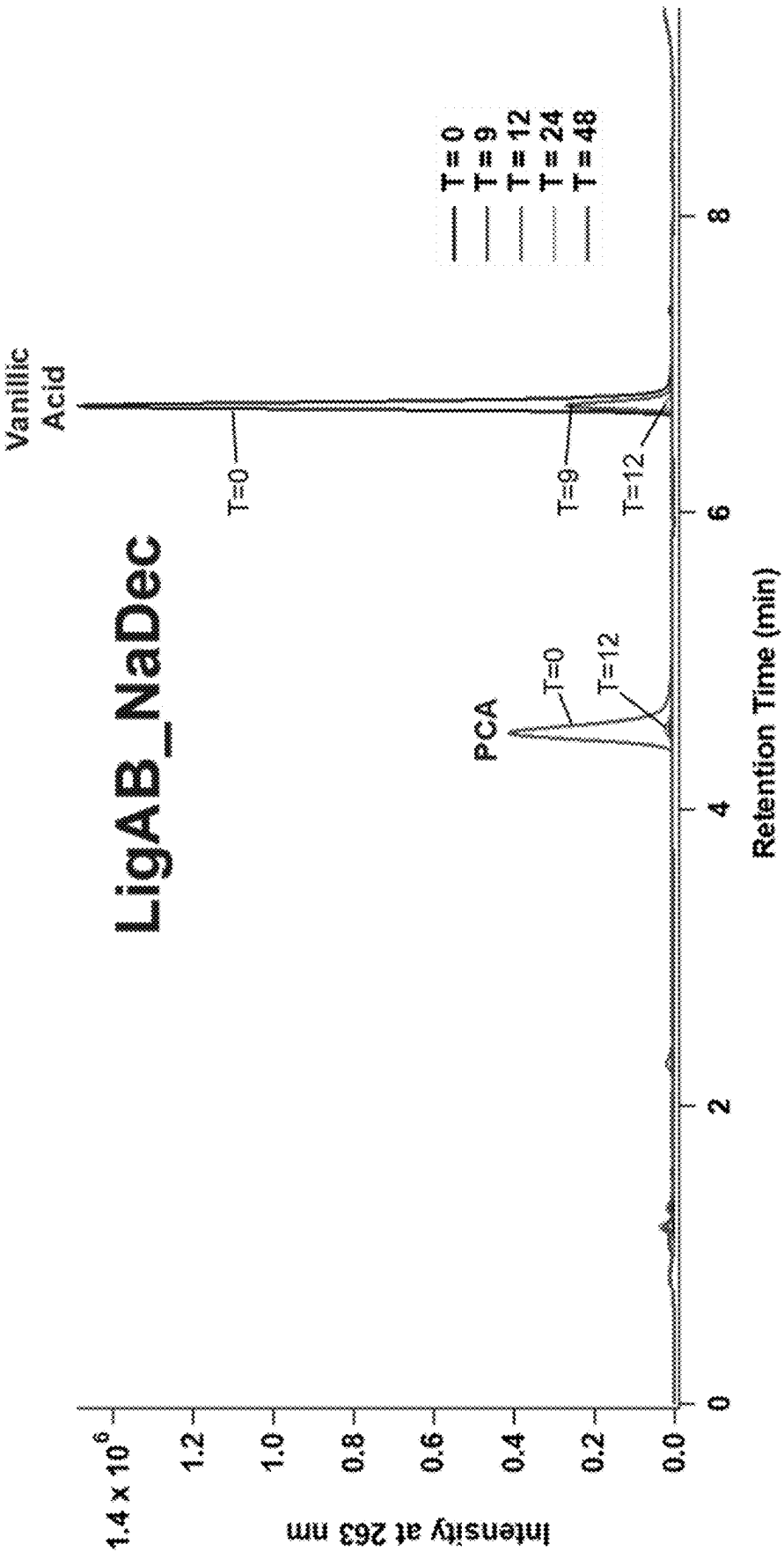

LigAB_NaDec
Vanillic Acid
PCA
T=0
T=9
T=12
T=0
T=12
T = 0
T = 9
T = 12
T = 24
T = 48
Intensity at 263 nm
1.4 x 10^6
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
Retention Time (min)

FIG. 10A

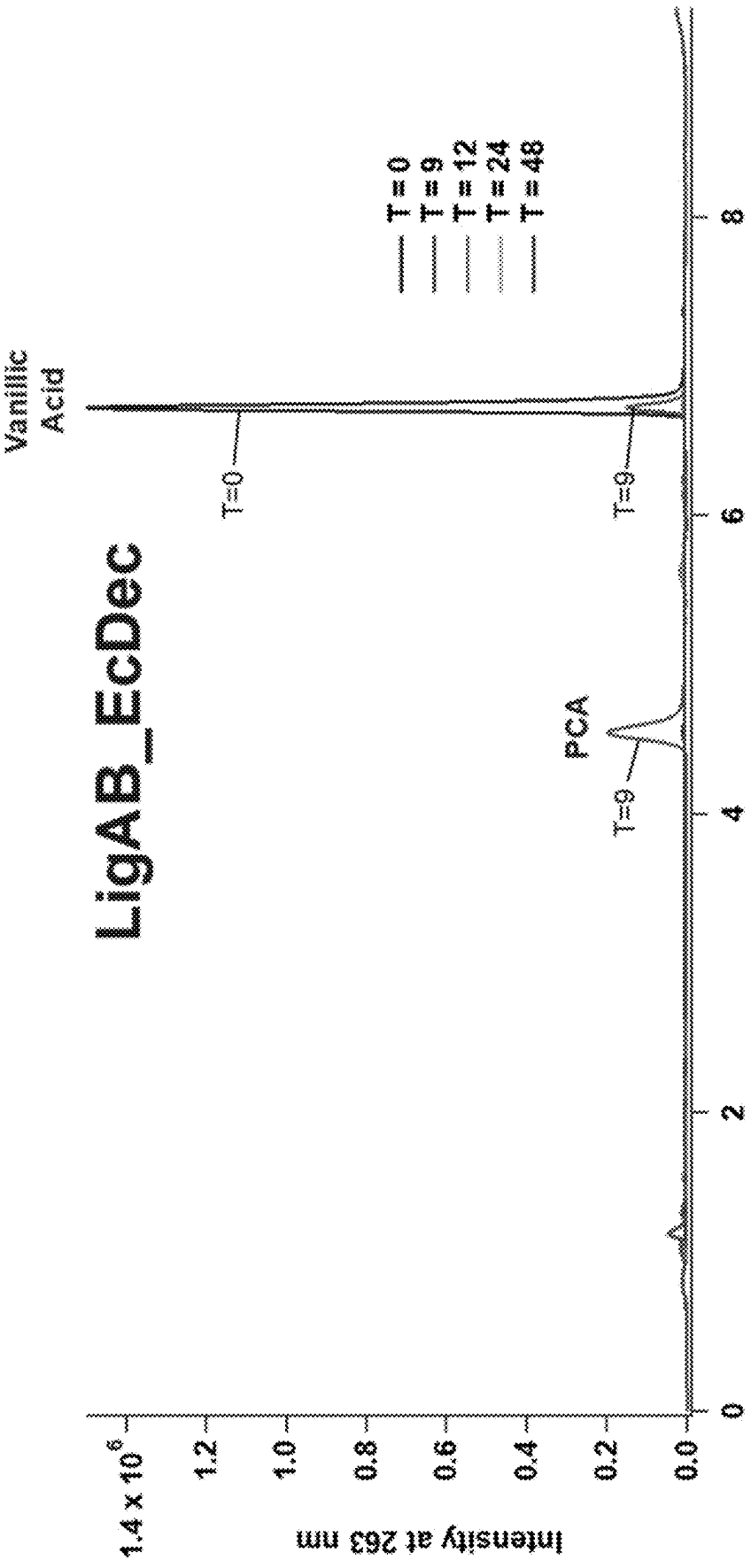

LigAB_EcDec
Intensity at 263 nm
1.4 x 10^6
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
Retention Time (min)
Vanillic Acid
PCA
T=0
T=9
T=9
T = 0
T = 9
T = 12
T = 24
T = 48

FIG. 10B

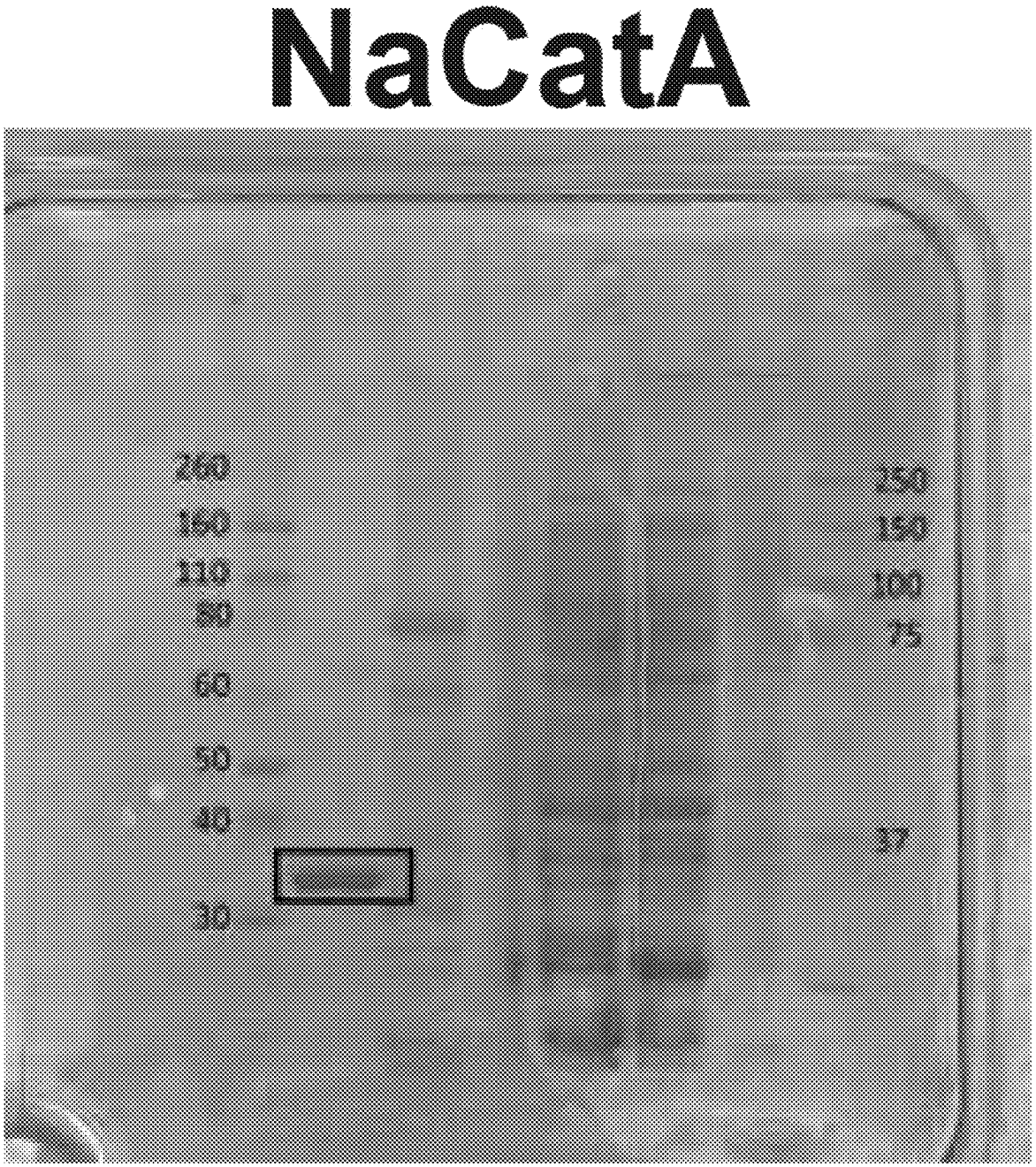


FIG. 11A

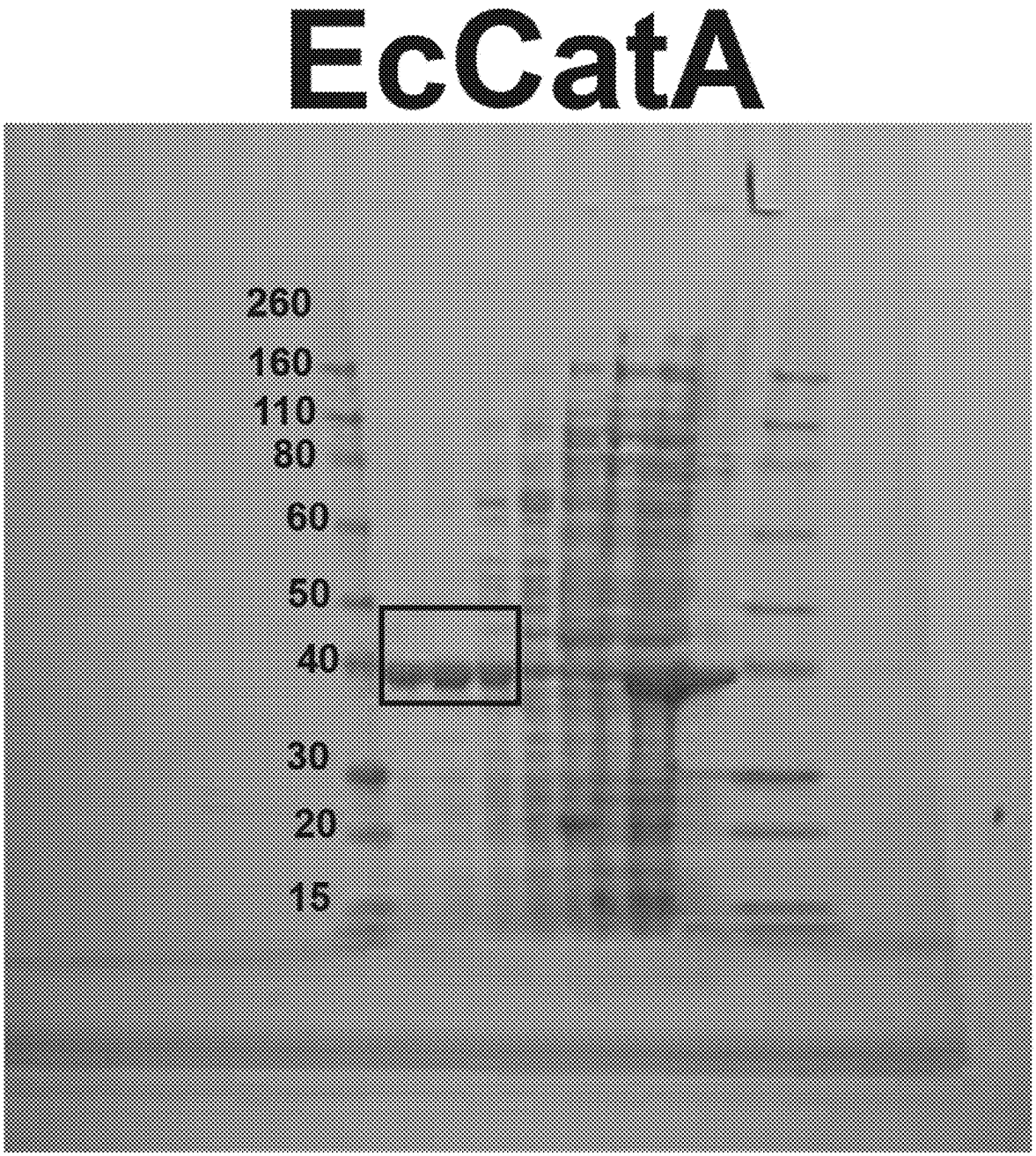


FIG. 11B

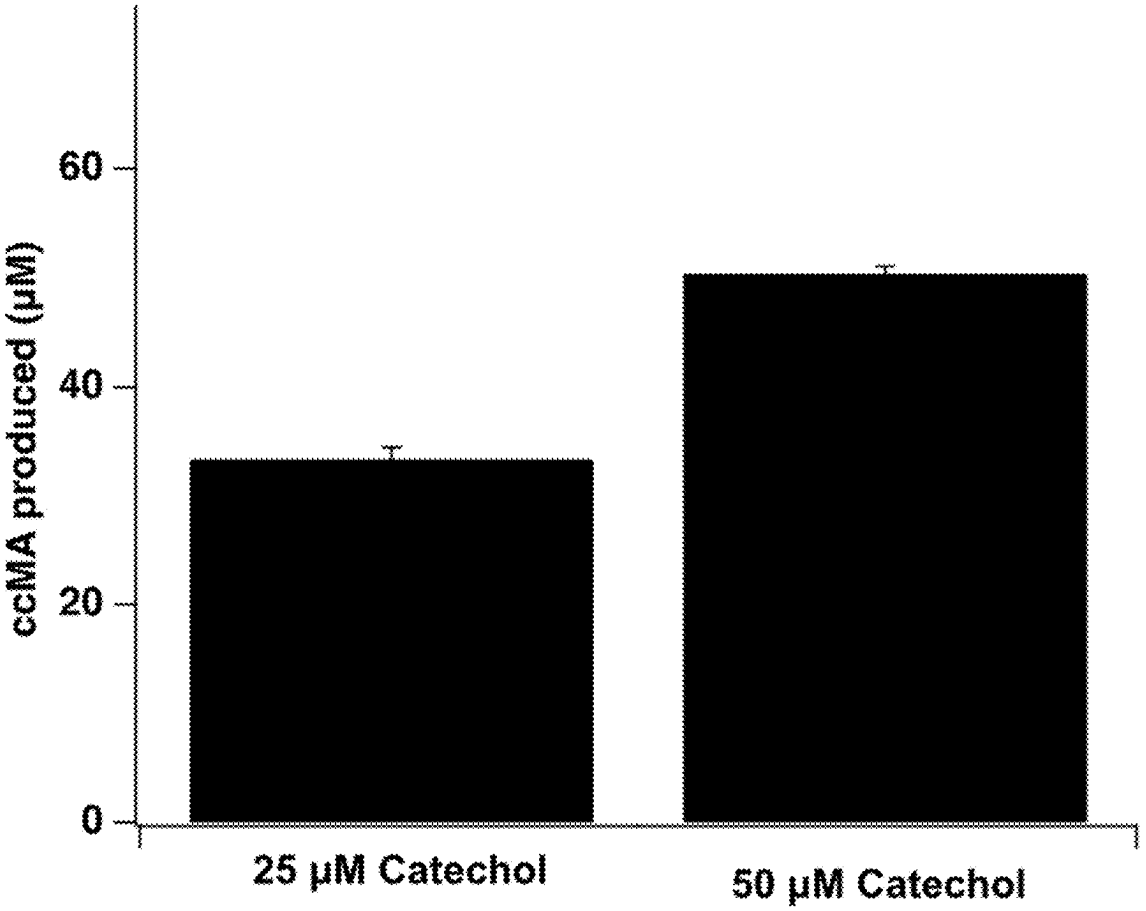


FIG. 12

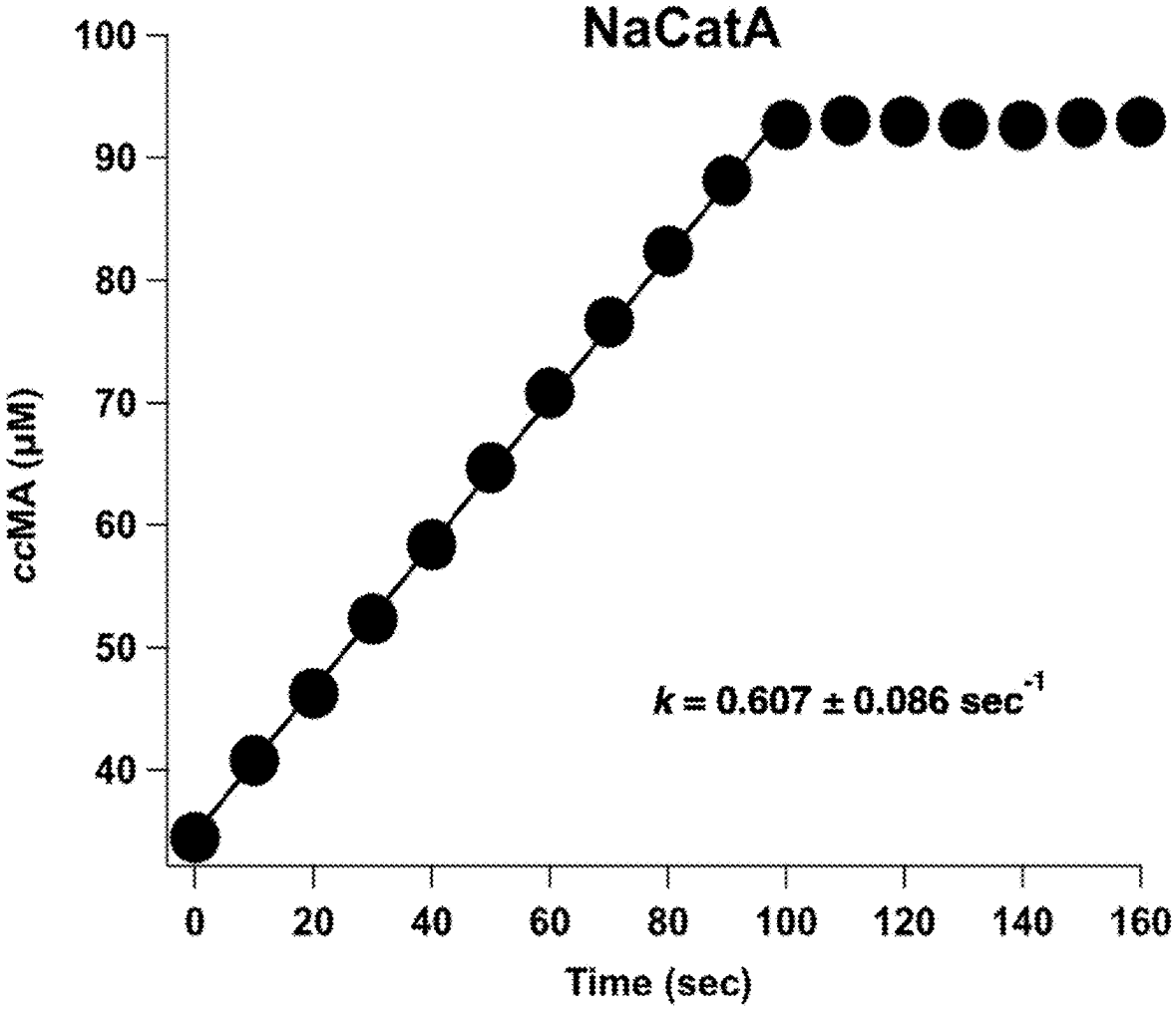

NaCatA
ccMA (μM)
100
90
80
70
60
50
40
0
20
40
60
80
100
120
140
160
Time (sec)
k = 0.607 ± 0.086 sec-1

FIG. 13A

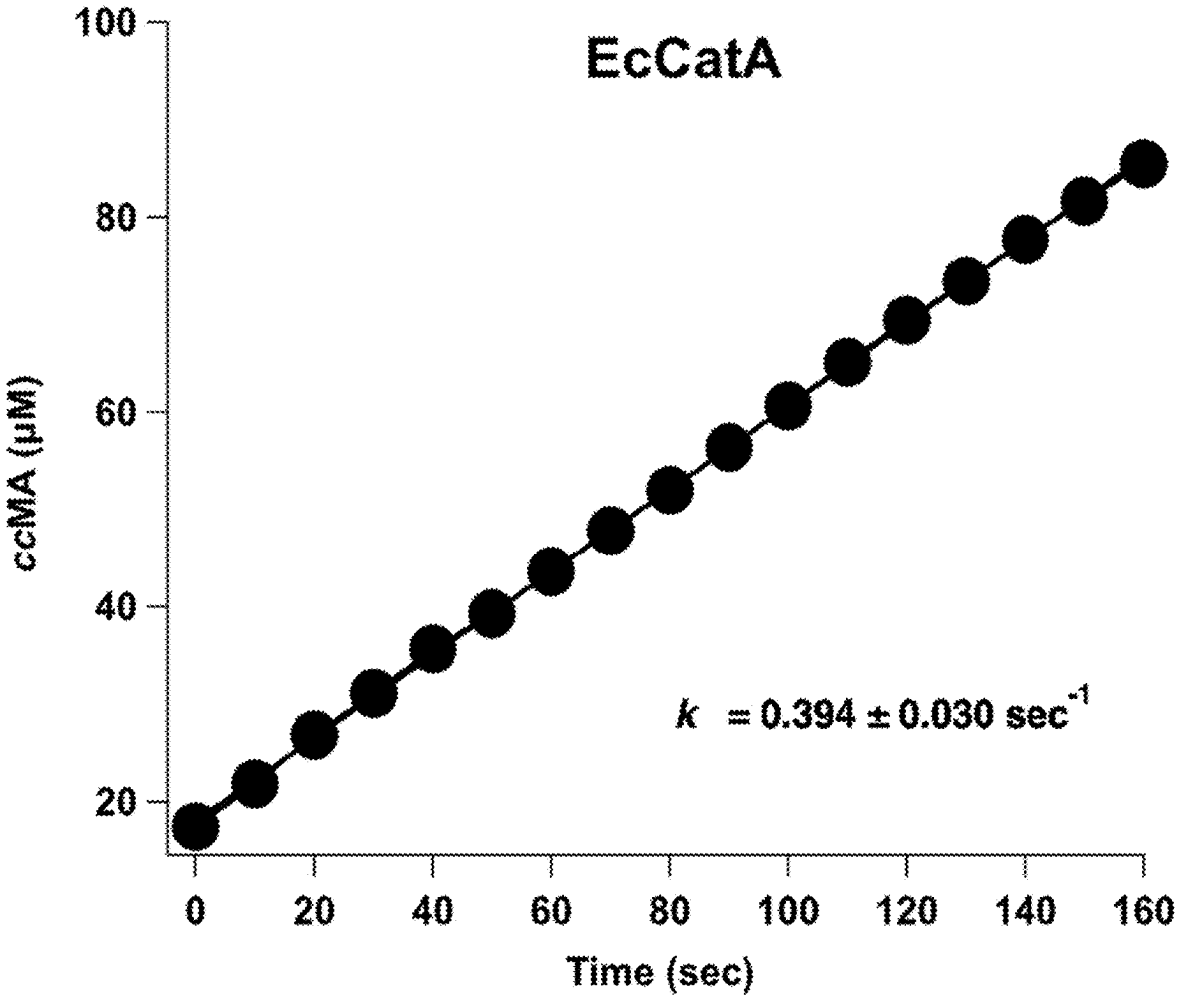

EcCatA
ccMA (μM)
100
80
60
40
20
0
20
40
60
80
100
120
140
160
Time (sec)
k = 0.394 ± 0.030 sec-1

FIG. 13B

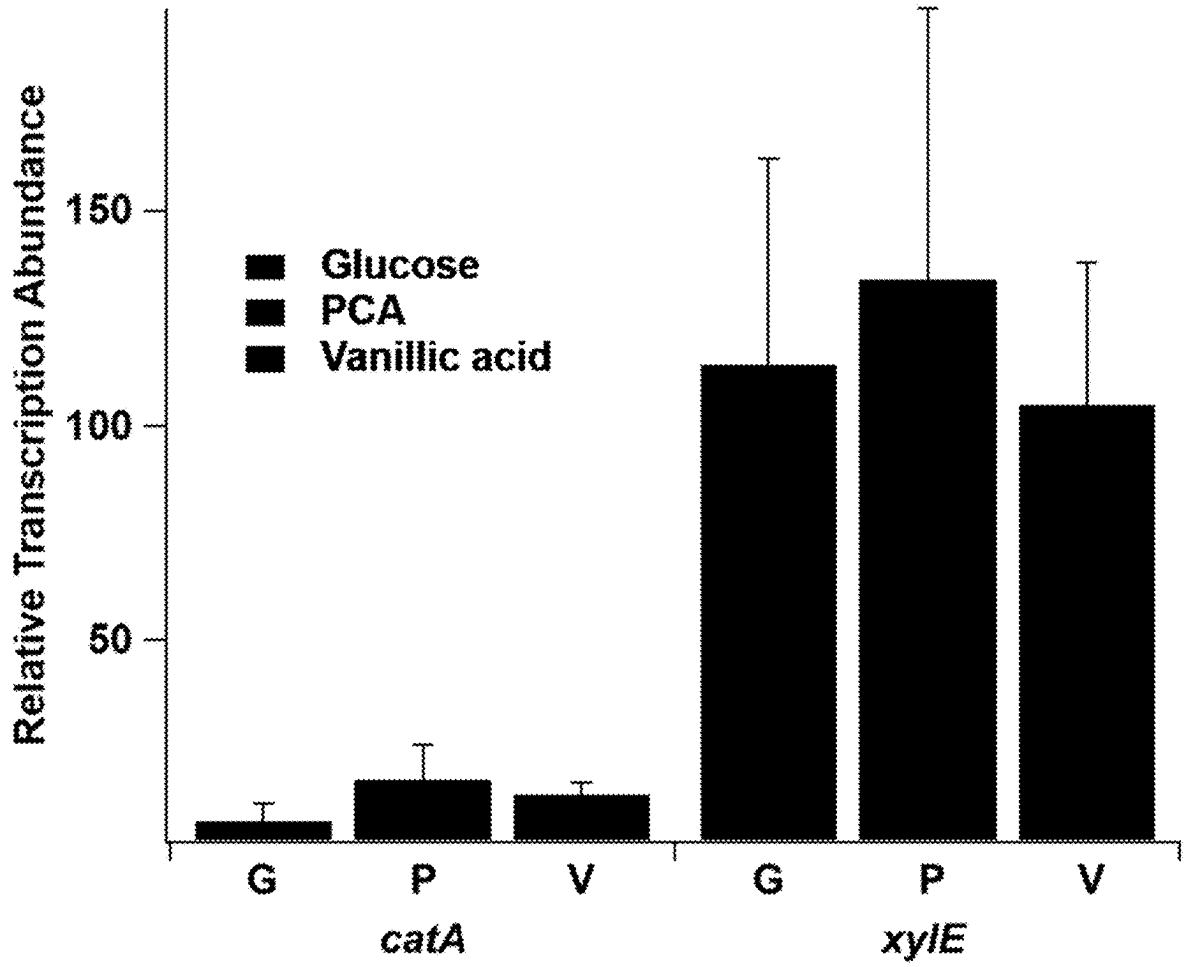

Relative Transcription Abundance
150
100
50
Glucose
PCA
Vanillic acid
G
P
V
G
P
V
catA
xylE

FIG. 14

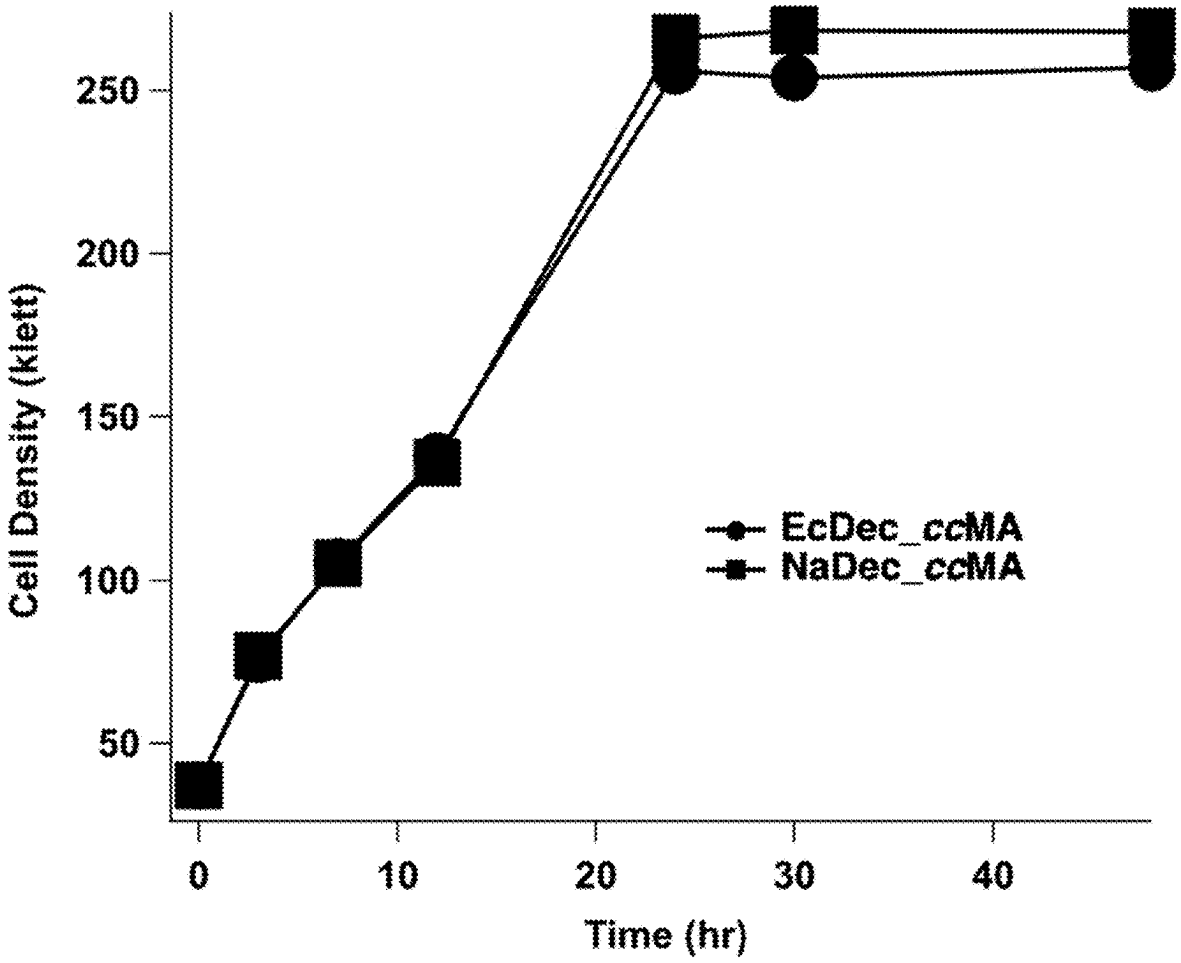

Cell Density (klett)
250
200
150
100
50
0
10
20
30
40
Time (hr)
EcDec_ccMA
NaDec_ccMA

FIG. 15A

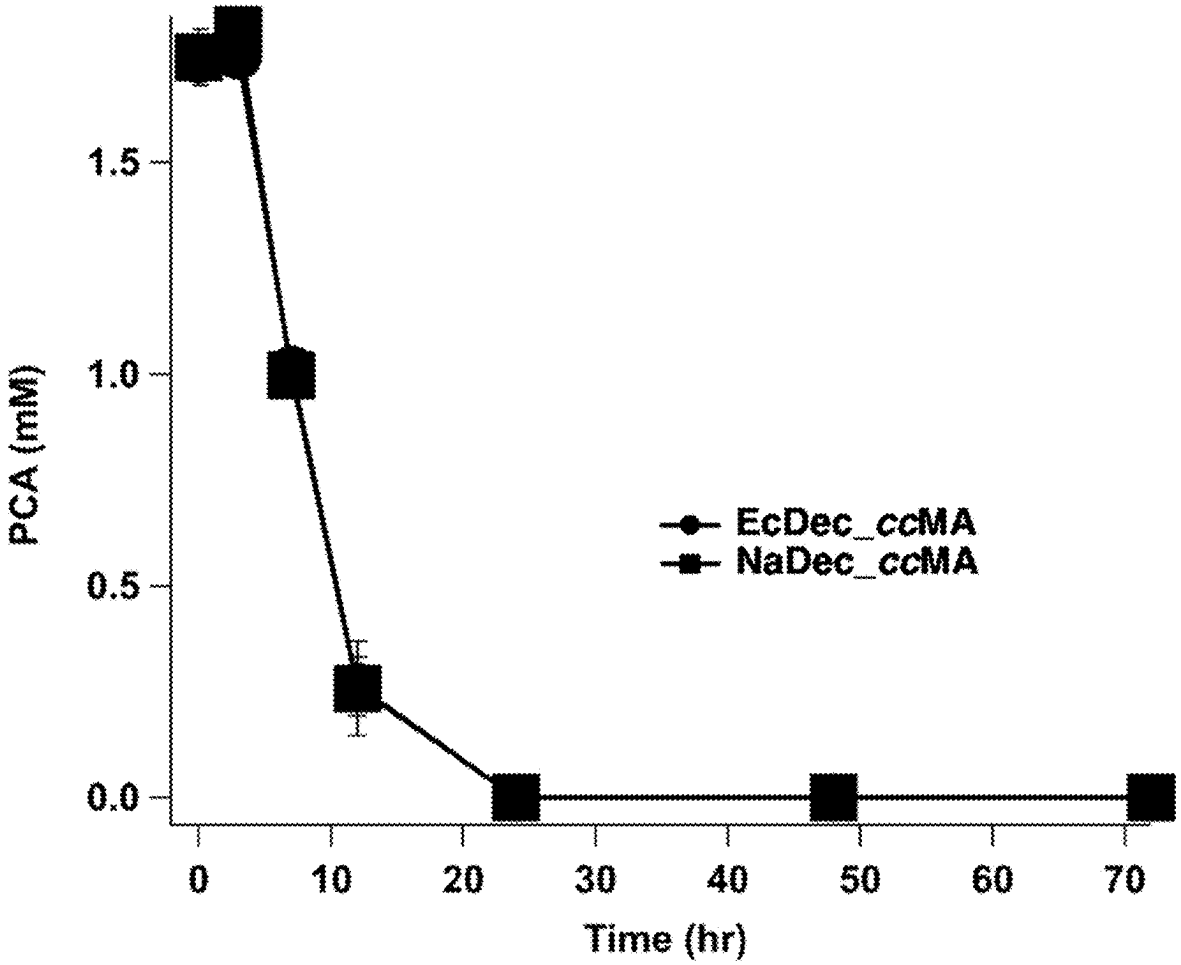

PCA (mM)
1.5
1.0
0.5
0.0
0
10
20
30
40
50
60
70
Time (hr)
EcDec_ccMA
NaDec_ccMA

FIG. 15B

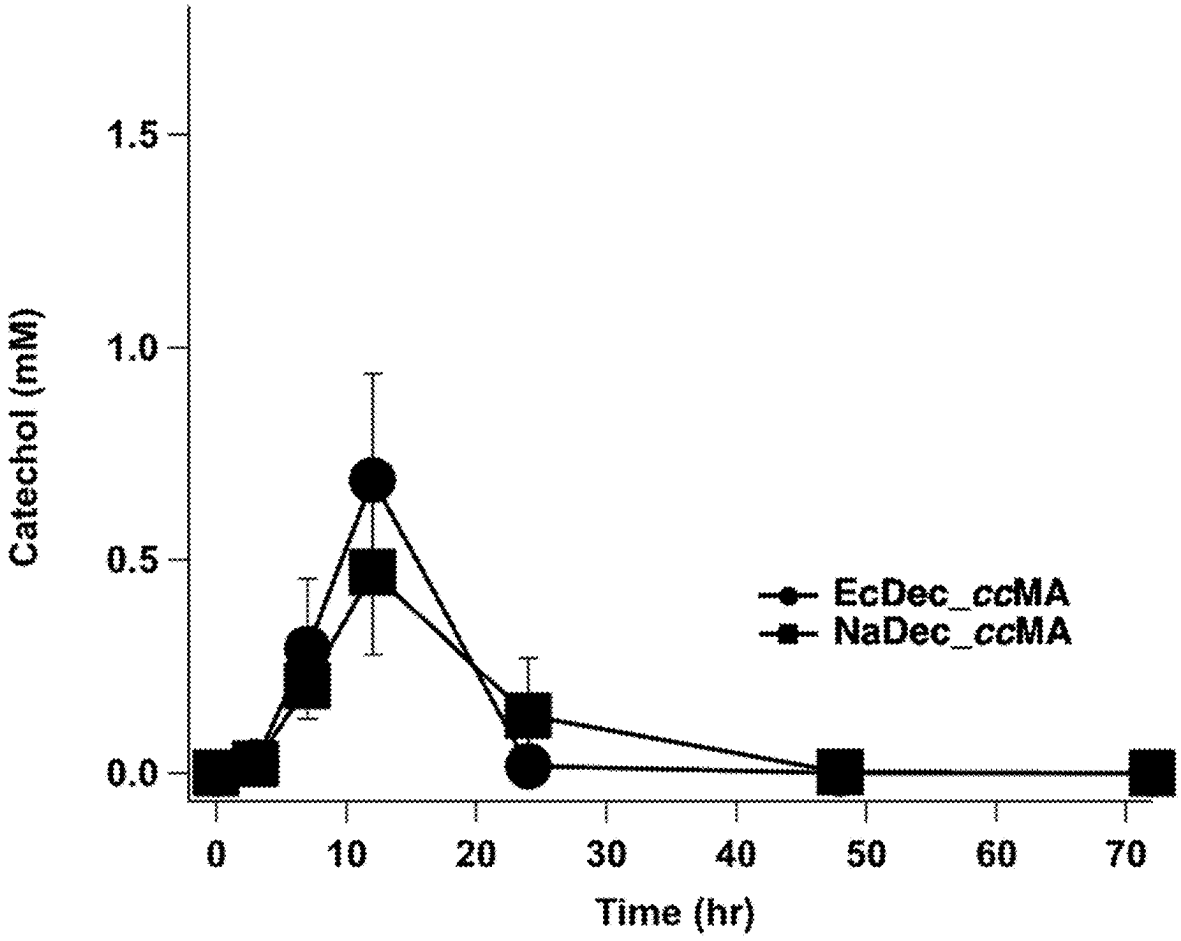

Catechol (mM)
1.5
1.0
0.5
0.0
0
10
20
30
40
50
60
70
Time (hr)
EcDec_ccMA
NaDec_ccMA

FIG. 15C

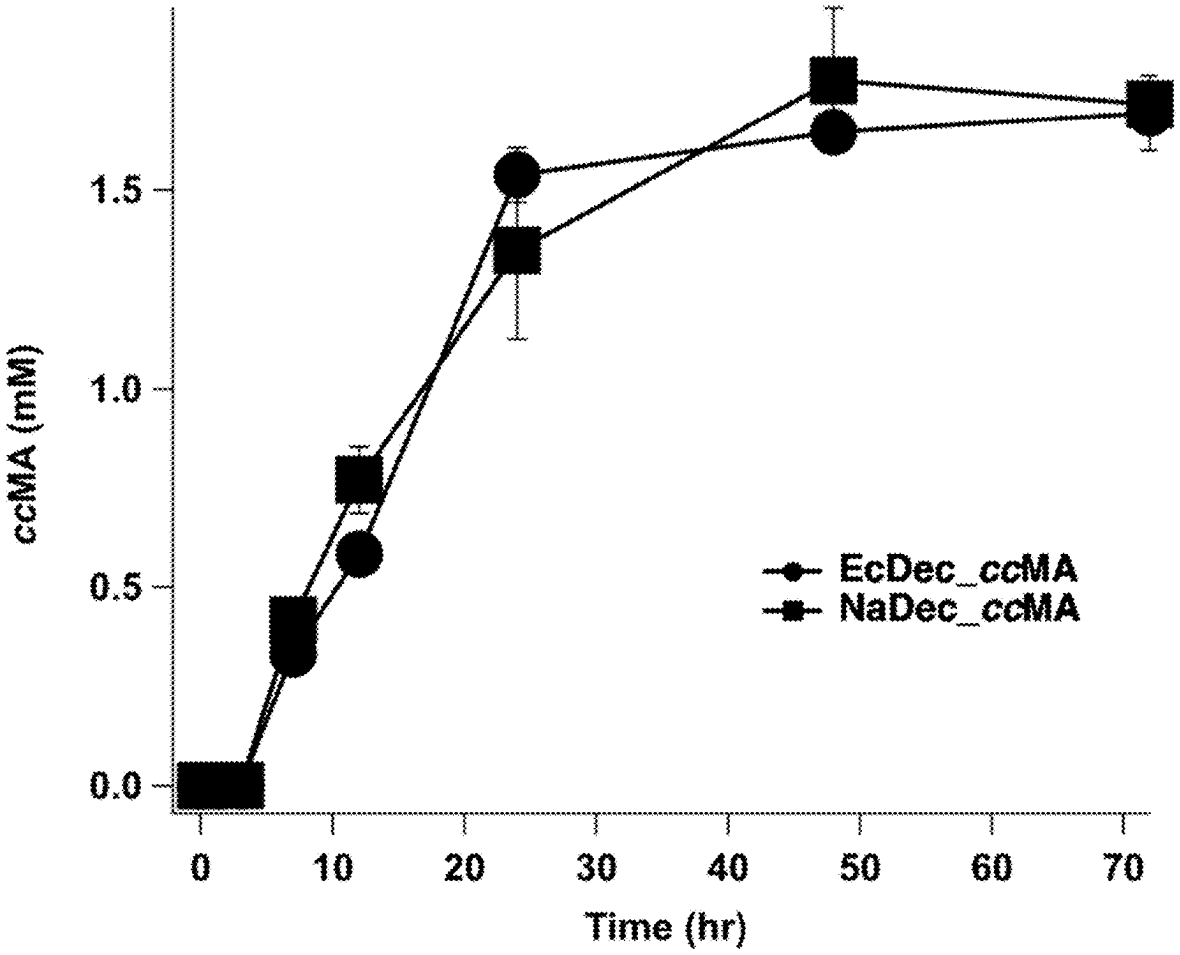

ccMA (mM)
1.5
1.0
0.5
0.0
0
10
20
30
40
50
60
70
Time (hr)
EcDec_ccMA
NaDec_ccMA

FIG. 15D

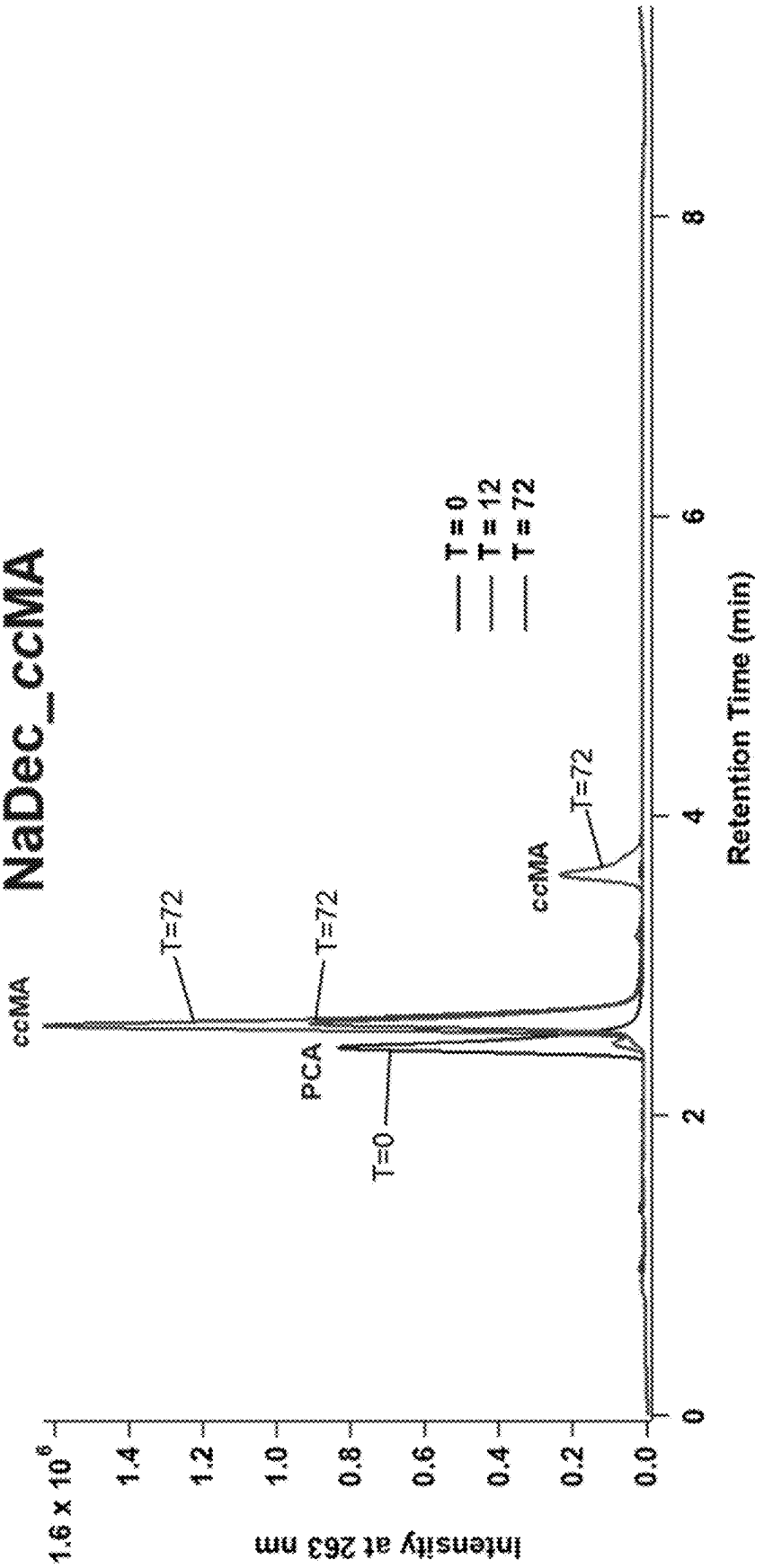

NaDec_ccMA
T = 0
T = 12
T = 72
ccMA
T=72
T=72
PCA
T=0
ccMA
T=72
Intensity at 263 nm
1.6 x 10^6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
Retention Time (min)

FIG. 16A

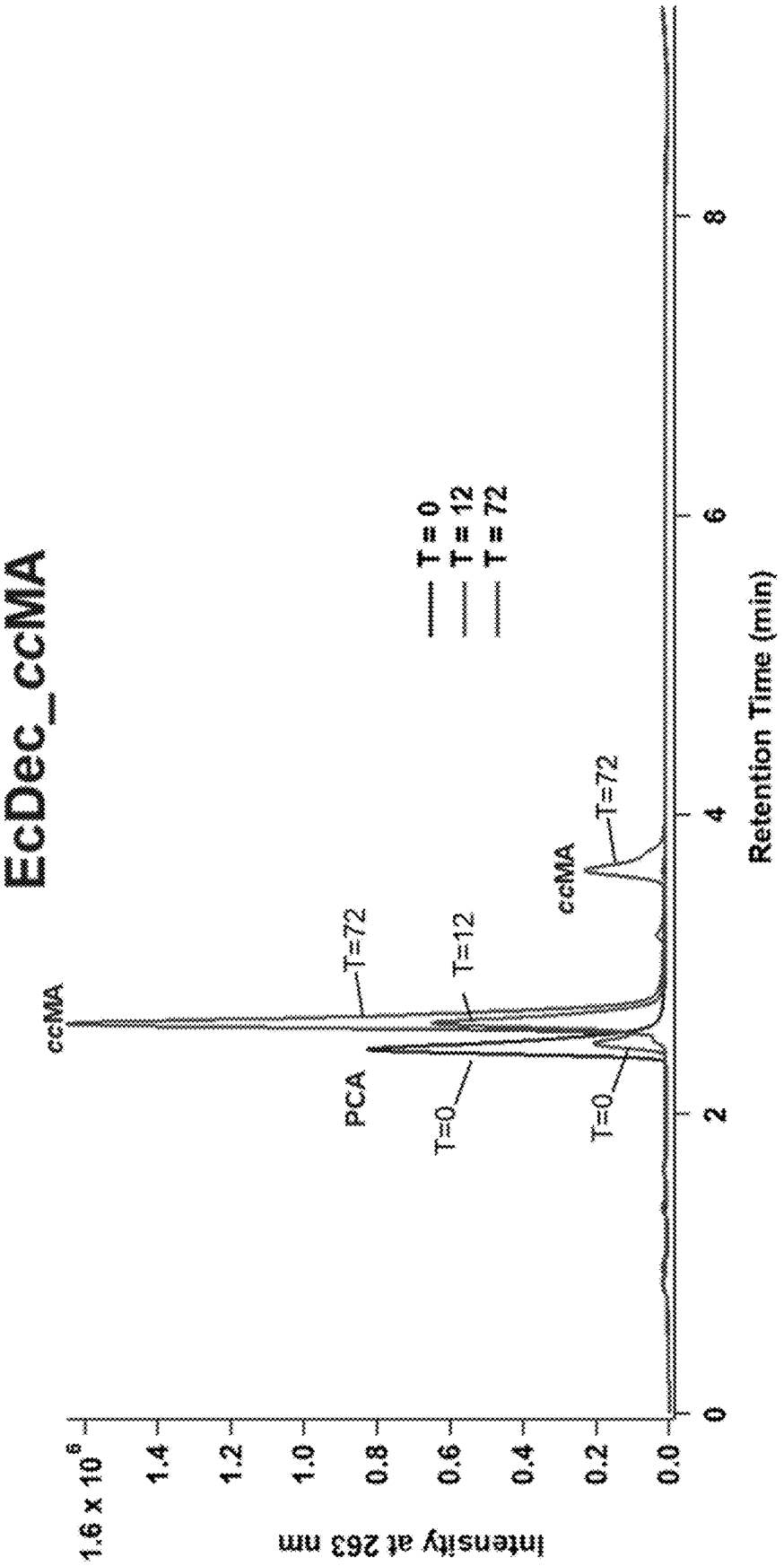

EcDec_ccMA
T = 0
T = 12
T = 72
ccMA
T=72
T=12
PCA
T=0
ccMA
T=72
T=0
Intensity at 263 nm
1.6 x 10^6
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
0
2
4
6
8
Retention Time (min)

FIG. 16B

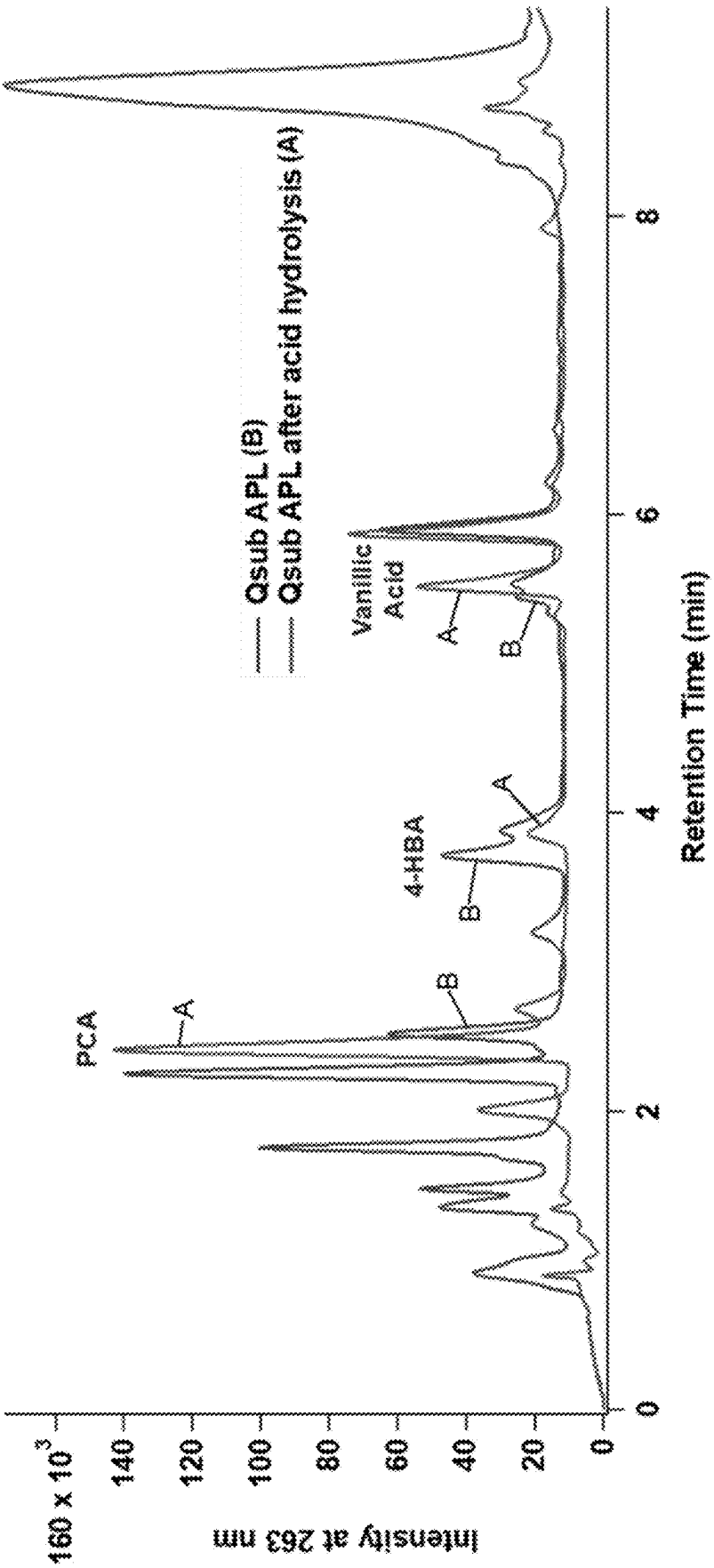

Qsub APL (B)
Qsub APL after acid hydrolysis (A)
PCA
4-HBA
Vanillic Acid
A
B
Intensity at 263 nm
160 x 10^3
140
120
100
80
60
40
20
0
0
2
4
6
8
Retention Time (min)

FIG. 17A

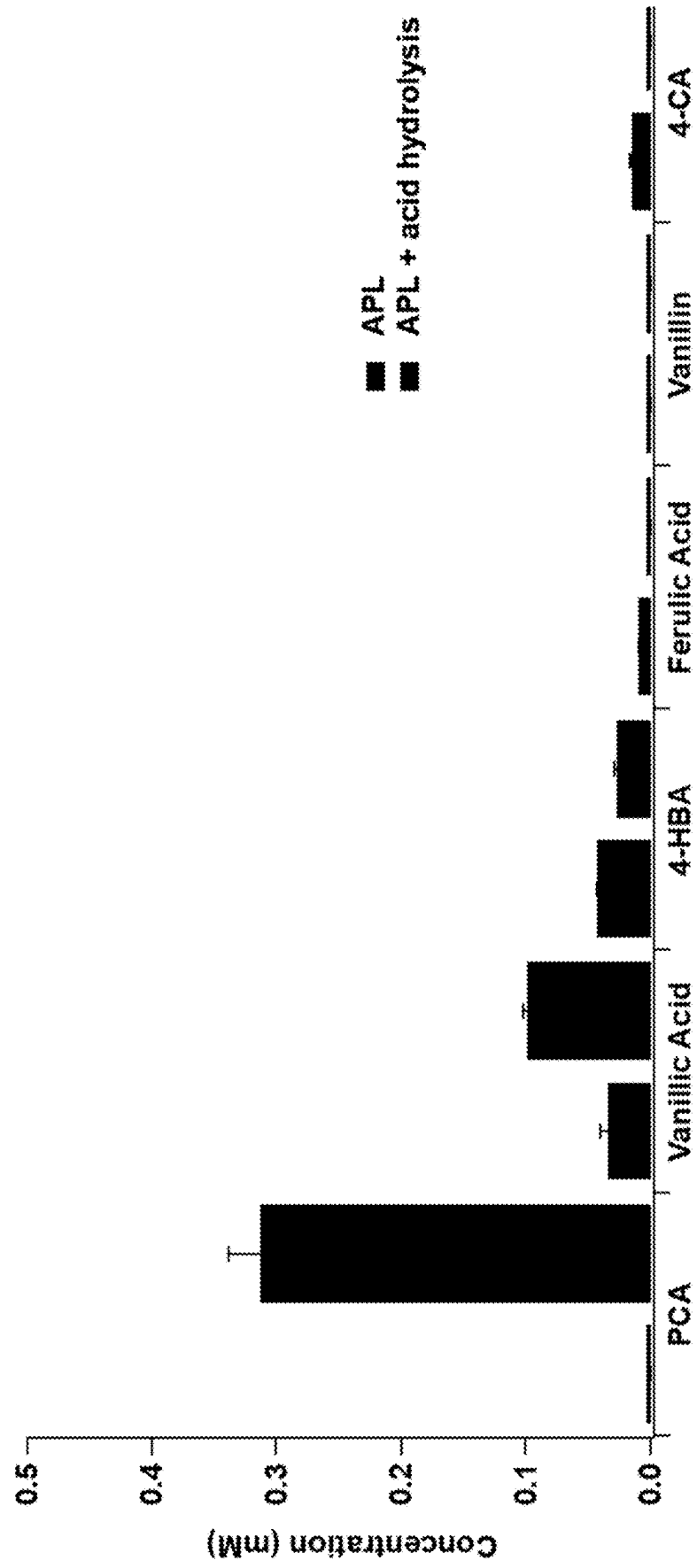

APL
APL + acid hydrolysis
Concentration (mM)
0.0
0.1
0.2
0.3
0.4
0.5
PCA
Vanillic Acid
4-HBA
Ferulic Acid
Vanillin
4-CA

FIG. 17B

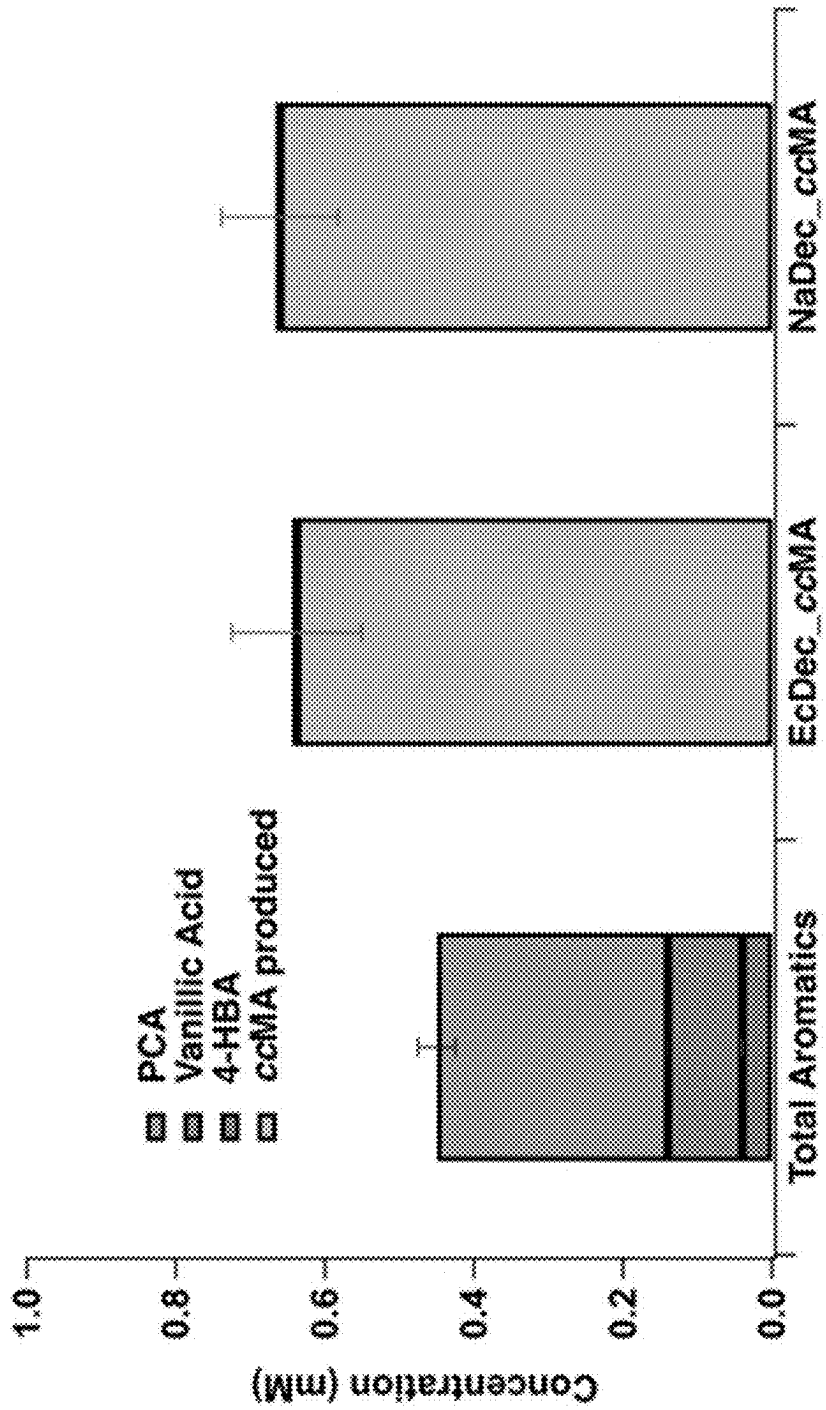

Concentration (mM)
1.0
0.8
0.6
0.4
0.2
0.0
PCA
Vanillic Acid
4-HBA
ccMA produced
Total Aromatics
EcDec_ccMA
NaDec_ccMA

FIG. 18

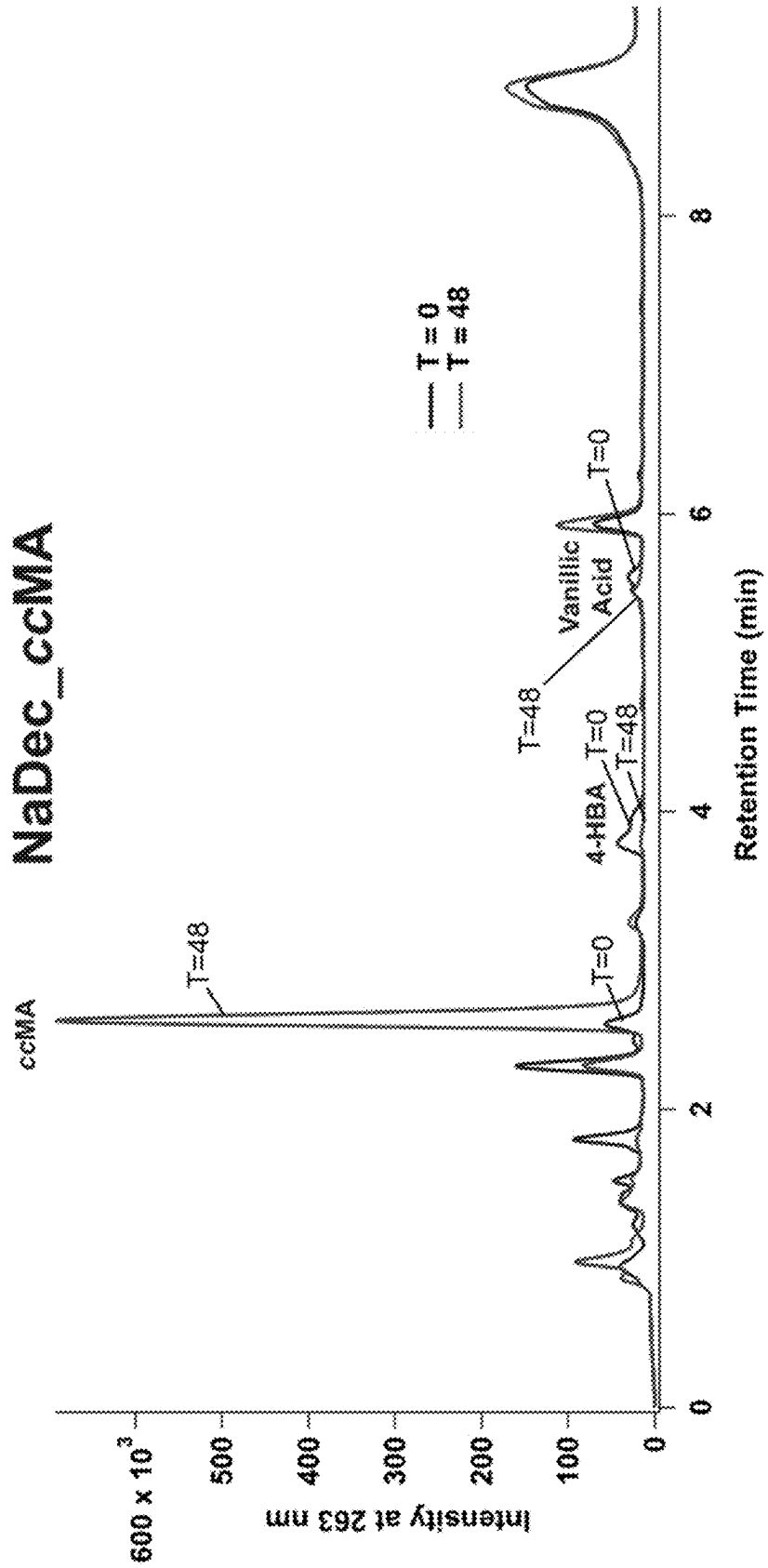

NaDec_ccMA
ccMA
T=48
T=0
4-HBA
T=0
T=48
T=48
Vanillic Acid
T=0
T = 0
T = 48
Intensity at 263 nm
600 x 10^3
500
400
300
200
100
0
0
2
4
6
8
Retention Time (min)

FIG. 19A

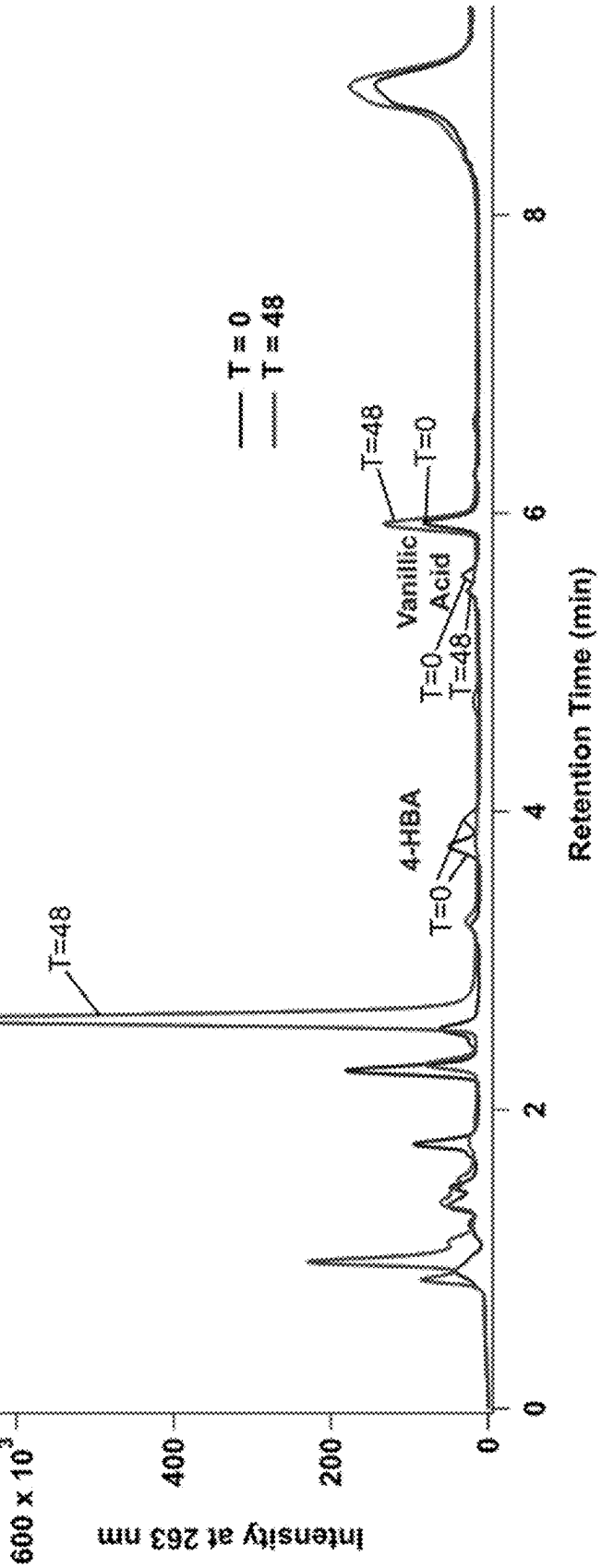

EcDec_ccMA
T = 0
T = 48
ccMA
T=48
4-HBA
T=0
Vanillic Acid
T=0
T=48
T=48
T=0
Intensity at 263 nm
600 x 10^3
400
200
0
0
2
4
6
8
Retention Time (min)

FIG. 19B

MICROORGANISMS AND METHODS FOR PRODUCING CIS,CIS-MUCONIC ACID

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0001] This invention was made with government support under DE-SC0018409 awarded by the US Department of Energy. The government has certain rights in the invention.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted in XML format and is hereby incorporated by reference in its entirety. The XML copy, created Sep. 10, 2024, is named USPTO-09824551-P240032US02-SEQ_LIST and is 40,403 bytes in size.

FIELD OF THE INVENTION

[0003] The invention is directed to recombinant microorganisms configured for enhanced production of cis, cis-muconic acid and methods of using the recombinant microorganisms for the production of same.

BACKGROUND

[0004] One strategy to increase the environmental and economic sustainability of chemical production relies on harnessing the native or engineered metabolic pathways of microbes to catalyze the conversion of renewable resources into valuable products. Advances in genomics have enabled metabolic engineering approaches to convert abundant renewable resources into a number of targets for bioproduct production. One of these bio-privileged molecules is cis,cis-muconic acid (ccMA), which can be used as a precursor for the production of polymers including nylon-6,6, polyurethane, and polyethylene terephthalate (1). This dicarboxylic acid is an intermediate in the β-ketoadipic acid pathway of many bacteria and thus its production is amenable to metabolic engineering approaches.

[0005] The biological production of ccMA has been reported from food-grade, non-renewable sugars like glucose (2). Recently, significant attention has been drawn towards the production of ccMA from biomass lignin (3-6). Lignin is the most abundant renewable source of aromatics on the planet (7, 8) and accounts for approximately 20-30% (w/w) of dry biomass (9-11). However, lignin remains an underutilized industrial carbon source due to the chemical heterogeneity of the lignocellulose polymers. Lignin is composed of phenolic monomers that contain either 2 methoxy groups (S), 1 methoxy group (G) or no methoxy group (H) on the aromatic ring (12). Furthermore, biomass deconstruction methods used to recover aromatics produce a diverse set of aromatic monomers, dimers and oligomers (13).

[0006] Microbes capable of converting diverse biomass aromatics into simple products such as ccMA are needed.

SUMMARY OF THE INVENTION

[0007] One aspect of the invention is directed to recombinant microorganisms comprising one or more modifications with respect to a corresponding microorganism not comprising the one or more modifications. In some versions, the one or more modifications comprise at least one of: a modification that increases flavin prenyltransferase activity with respect to the corresponding microorganism; a modification that increases protocatechuate decarboxylase activity with respect to the corresponding microorganism; a recombinant protocatechuate decarboxylase D gene encoding a protocatechuate decarboxylase D protein; a modification that increases catechol 1,2-dioxygenase activity with respect to the corresponding microorganism; a modification that decreases muconate lactonizing enzyme activity with respect to the corresponding microorganism; a modification that decreases muconolactone isomerase activity with respect to the corresponding microorganism; a modification that decreases catechol 2,3-dioxygenase activity with respect to the corresponding microorganism; and a modification that decreases protocatechuate 4,5-dioxygenase activity with respect to the corresponding microorganism.

[0008] In some versions, the one or more modifications comprise one or more genetic modifications in the recombinant microorganism with respect to the corresponding microorganism.

[0009] In some versions, the one or more modifications comprise the modification that increases flavin prenyltransferase activity with respect to the corresponding microorganism. In some versions, the modification that increases flavin prenyltransferase activity with respect to the corresponding microorganism, if present in the recombinant microorganism, comprises a recombinant gene encoding a flavin prenyltransferase. In some versions, the modification that increases flavin prenyltransferase activity with respect to the corresponding microorganism, if present in the recombinant microorganism, comprises a recombinant gene encoding a flavin prenyltransferase comprising a sequence having at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO:2.

[0010] In some versions, the one or more modifications comprise the modification that increases protocatechuate decarboxylase activity with respect to the corresponding microorganism. In some versions, the modification that increases protocatechuate decarboxylase activity with respect to the corresponding microorganism, if present in the recombinant microorganism, comprises a recombinant gene encoding a protocatechuate decarboxylase. In some versions, the modification that increases protocatechuate decarboxylase activity with respect to the corresponding microorganism, if present in the recombinant microorganism, comprises a recombinant gene encoding a protocatechuate decarboxylase comprising a sequence having at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO:4.

[0011] In some versions, the one or more modifications comprise the recombinant protocatechuate decarboxylase D gene. In some versions, the recombinant protocatechuate decarboxylase D gene, if present in the recombinant microorganism, encodes a protocatechuate decarboxylase D protein comprising a sequence having at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to any one of SEQ ID NOS:6, 24, and 26. In some versions, the recombinant protocatechuate decarboxylase D gene, if present in the recombinant microorganism, encodes a protocatechuate decarboxylase D protein comprising a sequence having at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO:6.

[0012] In some versions, the one or more modifications comprise the modification that increases catechol 1,2-dioxy-

genase activity with respect to the corresponding microorganism. In some versions, the modification that increases catechol 1,2-dioxygenase activity with respect to the corresponding microorganism, if present in the recombinant microorganism, comprises a recombinant gene encoding a catechol 1,2-dioxygenase. In some versions, the modification that increases catechol 1,2-dioxygenase activity with respect to the corresponding microorganism, if present in the recombinant microorganism, comprises a recombinant gene encoding a catechol 1,2-dioxygenase comprising a sequence having at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO:8.

[0013] In some versions, the one or more modifications comprise the modification that decreases muconate lactonizing enzyme activity with respect to the corresponding microorganism. In some versions, the modification that decreases muconate lactonizing enzyme activity with respect to the corresponding microorganism, if present in the recombinant microorganism, comprises a mutation to a gene in the corresponding microorganism encoding a muconate lactonizing enzyme. In some versions, the modification that decreases muconate lactonizing enzyme activity with respect to the corresponding microorganism, if present in the recombinant microorganism, comprises a mutation to a gene in the corresponding microorganism encoding a muconate lactonizing enzyme comprising a sequence having at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO:10.

[0014] In some versions, the one or more modifications comprise the modification that decreases muconolactone isomerase activity with respect to the corresponding microorganism. In some versions, the modification that decreases muconolactone isomerase activity with respect to the corresponding microorganism, if present in the recombinant microorganism, comprises a mutation to a gene in the corresponding microorganism encoding a muconolactone isomerase. In some versions, the modification that decreases muconolactone isomerase activity with respect to the corresponding microorganism, if present in the recombinant microorganism, comprises a mutation to a gene in the corresponding microorganism encoding a muconolactone isomerase comprising a sequence having at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO:12.

[0015] In some versions, the one or more modifications comprise the modification that decreases catechol 2,3-dioxygenase activity with respect to the corresponding microorganism. In some versions, the modification that decreases catechol 2,3-dioxygenase activity with respect to the corresponding microorganism, if present in the recombinant microorganism, comprises a mutation to a gene in the corresponding microorganism encoding a catechol 2,3-dioxygenase. In some versions, the modification that decreases catechol 2,3-dioxygenase activity with respect to the corresponding microorganism, if present in the recombinant microorganism, comprises a mutation to a gene in the corresponding microorganism encoding a catechol 2,3-dioxygenase comprising a sequence having at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO:14.

[0016] In some versions, the one or more modifications comprise the modification that decreases protocatechuate 4,5-dioxygenase activity with respect to the corresponding microorganism. In some versions, the modification that decreases protocatechuate 4,5-dioxygenase activity with respect to the corresponding microorganism, if present in the recombinant microorganism, comprises a mutation to a gene in the corresponding microorganism encoding a protocatechuate 4,5-dioxygenase subunit. In some versions, the modification that decreases protocatechuate 4,5-dioxygenase activity with respect to the corresponding microorganism, if present in the recombinant microorganism, comprises a mutation to any one, two, three, or each of: a gene in the corresponding microorganism encoding a protocatechuate 4,5-dioxygenase subunit comprising a sequence having at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO:16; a gene in the corresponding microorganism encoding a protocatechuate 4,5-dioxygenase subunit comprising a sequence having at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO:18; a gene in the corresponding microorganism encoding a protocatechuate 4,5-dioxygenase subunit comprising a sequence having at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO:20; and a gene in the corresponding microorganism encoding a protocatechuate 4,5-dioxygenase subunit comprising a sequence having at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO: 22.

[0017] In some versions, the recombinant microorganism exhibits enhanced production of cis, cis-muconic acid with respect to the corresponding microorganism.

[0018] In some versions, the recombinant microorganism is a bacterium. In some versions, the recombinant microorganism is a phenol-degrading microorganism. In some versions, the recombinant microorganism is from the genus *Novosphingobium*. In some versions, the recombinant microorganism is *Novosphingobium aromaticivorans*.

[0019] Another aspect of the invention is directed to methods for producing cis,cis-muconic acid. In some versions, the methods comprise culturing the recombinant microorganism of the invention in a medium. In some versions, the medium comprises a plant-derived phenolic. In some versions, the medium comprises a plant-derived phenolic selected from the group consisting of a syringyl phenolic, a guaiacyl phenolic, and a p-hydroxyphenyl phenolic. In some versions, the medium comprises depolymerized lignin. Some versions further comprise isolating the cis,cis-muconic acid from the medium and/or the recombinant microorganism.

[0020] The objects and advantages of the invention will appear more fully from the following detailed description of the preferred embodiment of the invention made in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021] FIG. **1**. Metabolic pathways for aromatic catabolism from H- and G-phenolic monomers (left) and other aromatics (benzoic acid, phenol and guaiacol, right). Two central intermediates, protocatechuic acid (PCA) and catechol, are shown.

[0022] FIG. **2**. Chromatogram of the indicated aromatics used in LC Method 1 (see Examples) to quantify the aromatics at 263 nm.

[0023] FIG. **3**. Chromatogram of the commercial compounds used as aromatics to quantify the aromatics present in Qsub poplar APL measured at 263 nm with chromato-

graphic Method 2. The aromatics measured with this method (1 mM final concentration) are shown and labeled above each peak with protocatechuic acid (PCA), cis,cis-muconic acid (ccMA), 4-hydroxybenzoic acid (4-HBA), vanillic acid, vanillin and 4-coumaric acid (4-CA) and ferulic acid.

[0024] FIGS. **4**A-**4**C. Cell density (FIG. **4**A) and extracellular concentrations of vanillic acid (FIG. **4**B) and protocatechuic acid (PCA) (FIG. **4**C) in cultures of the parent strain 12444, the 12444_ΔligAB1/2 strain, and the ΔligAB1/2 ΔnadBCD strain (12444_PCA). Cells were grown in batch cultures in minimal media containing 2 mM vanillic acid and 10 mM glucose. No other aromatics were detected in the media. All experiments were performed in triplicate. Error bars represent 1 standard deviation above and below the mean.

[0025] FIGS. **5**A-**5**C. Representative HPLC traces of extracellular aromatics using LC Method 1 from *N. aromaticivorans* strains 12444 (FIG. **5**A), ΔligAB1/2 (FIG. **5**B) or 12444_PCA (FIG. **5**C) grown with 2 mM vanillic acid and 10 mM glucose as carbon sources. The traces representing the sample taken at T=0 hr, T=14 hr, T=25 hr, and T=50 hr are labeled. All identified aromatics are labeled.

[0026] FIGS. **6**A and **6**B. SDS-PAGE gel of purified recombinant NadCD (FIG. **6**A), EcAroY/D (FIG. **6**B). The box highlights the purified protein.

[0027] FIG. **7**. The dependence of NadCD on a prFMN source for PCA decarboxylase activity. Experiments were performed with 100 nM NadCD with 1 mM PCA in either the presence or absence of a prFMN lysate. The bars represent the concentration of either PCA (P) or catechol (C) after 18 hr of incubation at room temperature. A control of lysate with 1 mM PCA was also performed to ensure no PCA to catechol conversion with lysate only. Both enzymes only produced catechol (C) from PCA (P) when in the presence of a prFMN source. Each bar is the average of 3 trials with error bars representing one standard deviation.

[0028] FIGS. **8**A and **8**B. Time-dependent conversion of PCA to catechol by either 100 nM NadCD (FIG. **8**A) or 100 nM EcAroY (FIG. **8**B) with 1 mM PCA in prFMN lysate in 50 mM HEPES, 150 mM NaCl pH 7.5. All experiments were performed in triplicate with each data point representing the average of 3 trials with error bars representing one standard deviation.

[0029] FIGS. **9**A-**9**C. Cell density (FIG. **9**A) and extracellular metabolite concentration of vanillic acid (FIG. **9**B) and protocatechuic acid (PCA) (FIG. **9**C) of LigAB1_NaDec and LigAB1_EcDec. Cultures were grown in triplicate with 2 mM vanillic acid and 10 mM glucose, and metabolite concentrations were analyzed by LCMS. The data points are the average of 3 trials with error bars representing one standard deviation.

[0030] FIGS. **10**A and **10**B. Representative HPLC traces of extracellular aromatics using LC Method 1 from *N. aromaticivorans* strains LigAB1_NaDec (FIG. **10**A) or LigAB1_EcDec (FIG. **10**B) grown with 2 mM vanillic acid and 10 mM glucose as carbon sources. The traces representing the sample taken at T=0 hr, T=9 hr, T=12 hr, T=24 hr, and T=48 hr are labeled. All identified aromatics are labeled.

[0031] FIGS. **11**A and **11**B. SDS-PAGE gel of purified recombinant NaCatA (FIG. **11**A) and EcCatA (FIG. **11**B). The box highlights the purified protein.

[0032] FIG. **12**. Catechol to ccMA production by NaCatA. The reaction was initiated with 0.5 μM enzyme with either 25 μM or 50 μM catechol in 50 mM HEPES 150 mM NaCl pH 7.5. The production of ccMA was measured by the increase in absorbance at 260 nm.

[0033] FIGS. **13**A and **13**B. Representative time courses of the formation of ccMA with either NaCatA (FIG. **13**A) or EcCatA (FIG. **13**B). Both reactions were performed in 50 mM HEPES 150 mM NaCl pH 7.5 with either 0.5 μM NaCatA (FIG. **13**A) or EcCatA (FIG. **13**B) and initiated with 100 μM catechol. The formation of ccMA was monitored by UV/vis absorption spectroscopy at A260-nm and the data points were best fit to a linear equation ([ccMA]=kt+[ccMA]0). The resulting fit is shown as the black line. The inset shows the average rate after 3 trials with error represented by 1 standard deviation above and below the mean.

[0034] FIG. **14**. Relative transcript abundance of catA (Saro_3830) and xylE (Saro_3857) when *N. aromaticivorans* strain 12444 is grown with either glucose (G), protocatechuic acid (P) or vanillic acid (V) as a carbon source.

[0035] FIGS. **15**A-**15**D. Cell density (FIG. **15**A) of NaDec_ccMA and EcDec_ccMA and extracellular metabolite concentration of protocatechuic acid (PCA) (FIG. **15**B), catechol (FIG. **15**C) and ccMA (FIG. **15**D). Cultures were grown in triplicate in a shake-flasks with 2 mM PCA and 10 mM glucose. Metabolite concentrations were analyzed by LCMS. Each data point represents the average of 3 trials with error bars representing one standard deviation.

[0036] FIGS. **16**A and **16**B. Representative HPLC traces of extracellular aromatics using LC Method 2 (see Examples) from *N. aromaticivorans* strains of NaDec_ccMA (FIG. **16**A) or EcDec_ccMA (FIG. **16**B) grown with 2 mM protocatechuic acid (PCA) and 10 mM glucose. The black trace represents the sample taken at T=0 and the blue trace represents the sample taken at T=72 hr. All identified aromatics are labeled.

[0037] FIGS. **17**A and **17**B. Aromatics identified in Qsub Poplar APL before (B) and after (A) acid hydrolysis. The HPLC trace (FIG. **17**A) is shown with the identified aromatics protocatechuic acid (PCA), vanillic acid, and 4-hydroxybenzoic acid (4-HBA). The corresponding calculated concentrations of aromatics identified before (left bar shown for each compound) and after (right bar shown for each compound) acid hydrolysis are shown in FIG. **17**B.

[0038] FIG. **18**. Conversion of Qsub APL into ccMA with either EcDec_ccMA or NaDec_ccMA *N. aromaticivorans* strains. The first bar represents the total concentration of the major free and glycosylated aromatics identified from Qsub APL with PCA (top portion), vanillic acid (middle portion) and 4-hydroxybenzoic acid (bottom portion). The second and third bars represent the concentration of ccMA produced from either the EcDec_ccMA or NaDec_ccMA strains after 48 h incubation with Qsub APL. Each bar is the average of 3 trials with error bars representing one standard deviation.

[0039] FIGS. **19**A and **19**B. Representative HPLC traces of extracellular aromatics using LC Method 2 from *N. aromaticivorans* strains NaDec_ccMA (FIG. **19**A) or EcDec_ccMA (FIG. **19**B) grown with Qsub APL popular as a carbon source. The respective traces representing the sample taken at T=0 hr and T=48 hr are labeled. All identified aromatics are labeled.

DETAILED DESCRIPTION OF THE INVENTION

[0040] One aspect of the invention is directed to recombinant microorganisms. The recombinant microorganisms of

the invention can be configured for enhanced production of cis,cis-muconic acid or other compounds. The recombinant microorganisms of the invention comprise one or more modifications that increase the activity of one or more genes or gene products, decrease the activity of one or more genes or gene products, or increase the activity of one or more genes or gene products and decrease the activity of one or more genes or gene products. The recombinant microorganisms with the modifications can exhibit enhanced production of cis,cis-muconic acid with respect to corresponding microorganisms not comprising the modifications.

[0041] "Modifications that increase the activity of one or more genes or gene products" refers to any modification to microorganism that increases expression of a gene in producing its gene product or increases the functioning of the gene product. "Increase" in this context encompasses increasing beyond a baseline activity. The baseline activity can be a positive baseline activity or null activity. Exemplary modifications that increase the activity of one or more genes or gene products include genetic modifications. The genetic modifications include genetic modifications to a gene in a manner that increases expression of the gene in producing the gene product. Such modifications include operationally connecting the coding sequence to a stronger promoter or enhancer, etc., and/or introducing additional copies of the gene (whether the native gene or a recombinant version). The genetic modifications also include mutations to a first gene (such as a transcription factor or an inhibitor of a transcription factor) that affects the expression of a second gene. The genetic modifications also include one or more copies of an exogenous or heterologous gene introduced into the microorganism. Other genetic modifications are described herein.

[0042] "Modifications that decrease the activity of one or more genes or gene products" refers to any modification to a microorganism that decreases expression of the gene and thus production of the gene product and/or decreases the functioning of the gene product per se. "Decrease" in this context encompasses reducing below a positive baseline level of expression and/or activity to a lower level of expression and/or activity. The lower level of expression and/or activity can be a lower positive level of expression and/or activity or null expression and/or activity. Decreasing the functioning of a gene product may comprise decreasing the specific activity of a gene product. Exemplary modifications that decrease the activity of one or more genes or gene products include genetic modifications. Exemplary genetic modifications include mutations to a gene that decrease expression of the gene in producing the gene product. Such mutations may include mutations to the coding sequence, the promoter, an enhancer, any other part of the gene, or the entire gene. Other exemplary genetic modifications include mutations to the coding sequence of a gene that decrease the functioning of a gene product expressed from the gene. Exemplary mutations include substitutions, insertions, and deletions, including partial and full deletions of a particular gene. Other exemplary genetic modifications include recombinant nucleotide sequences configured to express antisense RNAs or other molecules that decrease production of a gene product. Other exemplary genetic modifications include mutations to a first gene (such as a transcription factor or an inhibitor of a transcription factor) that affects the expression of a second gene. Other exemplary genetic modifications are described elsewhere herein. Other modifications include epigenetic modifications, such as methylation, etc.

[0043] "Corresponding microorganism" refers to a microorganism of the same species having the same or substantially same genetic and proteomic composition as a recombinant microorganism of the invention, with the exception of genetic and proteomic differences resulting from the modifications specified herein for the recombinant microorganisms of the invention in a given particular embodiment. In some versions, the corresponding microorganism is the native version of the recombinant microorganism of the invention, i.e., the unmodified microorganism as found in nature. The terms "microorganism" and "microbe" are used interchangeably herein.

[0044] In some versions, the recombinant microorganisms comprise one or more modifications with respect to a corresponding microorganism not comprising the one or more modifications. The one or more modifications can comprise, in any combination, a modification that increases flavin prenyltransferase activity with respect to the corresponding microorganism, a modification that increases protocatechuate decarboxylase activity with respect to the corresponding microorganism, a recombinant protocatechuate decarboxylase D gene encoding a protocatechuate decarboxylase D protein, a modification that increases catechol 1,2-dioxygenase activity with respect to the corresponding microorganism, a modification that decreases muconate lactonizing enzyme activity with respect to the corresponding microorganism, a modification that decreases muconolactone isomerase activity with respect to the corresponding microorganism, a modification that decreases catechol 2,3-dioxygenase activity with respect to the corresponding microorganism, and/or a modification that decreases protocatechuate 4,5-dioxygenase activity with respect to the corresponding microorganism.

[0045] Flavin prenyltransferase activity is characterized by EC 2.5.1.129 and comprises the ability to catalyze the addition of dimethylallyl-monophosphate (DMAP) (or dimethylallyl-pyrophosphate (DMAPP)) onto the N5 and C6 positions of flavin mononucleotide (FMN) to result in the formation of the prenylated FMN (prFMN) cofactor. Flavin prenyltransferase activity is performed by flavin prenyltransferases. An exemplary flavin prenyltransferase is nadB/NadB (Saro_3873) of *Novosphingobium aromaticivorans*, the nucleic acid coding sequence of which is SEQ ID NO:1 and the protein sequence of which is SEQ ID NO:2. Other exemplary flavin prenyltransferases include proteins with flavin prenyltransferase activity having a sequence at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% identical to SEQ ID NO:2. Other exemplary flavin prenyltransferases include the EcdB enzyme from *Enterobacter cloacae* and the KpdB enzyme from *Klebsiella pneumoniae* described in the following examples, as well as modified versions thereof. Other flavin prenyltransferases are known in the art. In some versions of the invention, the one or more modifications in the recombinant microorganisms can comprise a genetic modification that increases flavin prenyltransferase activity. A genetic modification that increases flavin prenyltransferase activity can comprise a recombinant flavin prenyltransferase gene. In some versions, the recombinant flavin prenyltransferase gene is an exogenous recombinant flavin prenyltransferase gene newly introduced to the microorganism. In some versions, the recombinant flavin prenyl-

transferase gene is a modified form of an endogenous flavin prenyltransferase gene already present in the microorganism.

[0046] Protocatechuate decarboxylase activity is characterized by EC 4.1.1.63 and comprises the ability to catalyze conversion of catalyzes the chemical reaction 3,4-dihydroxybenzoate to catechol CO_2. Protocatechuate decarboxylase activity is performed by protocatechuate decarboxylases. An exemplary protocatechuate decarboxylase is nadC/NadC (Saro_3877) of *Novosphingobium aromaticivorans*, the nucleic acid coding sequence of which is SEQ ID NO:3 and the protein sequence of which is SEQ ID NO:4. Other exemplary protocatechuate decarboxylases include proteins with protocatechuate decarboxylase activity having a sequence at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% identical to SEQ ID NO:4. Other exemplary protocatechuate decarboxylases include the EcdC and EcAroY enzymes from *Enterobacter cloacae* and the KpdC and KpAroY enzymes from *Klebsiella pneumoniae* described in the following examples, as well as modified versions thereof. Other protocatechuate decarboxylases are known in the art. In some versions of the invention, the one or more modifications in the recombinant microorganisms can comprise a genetic modification that increases protocatechuate decarboxylase activity. A genetic modification that increases protocatechuate decarboxylase activity can comprise a recombinant protocatechuate decarboxylase gene. In some versions, the recombinant protocatechuate decarboxylase gene is an exogenous protocatechuate decarboxylase gene newly introduced to the microorganism. In some versions, the recombinant protocatechuate decarboxylase gene is a modified form of an endogenous protocatechuate decarboxylase gene already present in the microorganism.

[0047] Protocatechuate decarboxylase D genes are genes encoding protocatechuate decarboxylase D proteins. Protocatechuate decarboxylase D genes are often found in aromatic-degrading microbes (e.g., *Novosphingobium aromaticivorans, Klebsiella pneumoniae, Enterobacter cloacae, Pseudomonas putida, Sedimentibacter hydroxybenzoicus, Streptomyces* sp. D7, *Bacillus subtilis, B. licheniformis, E. coli* O157:H7, *Shigella dysenteriae, Salmonella enterica, S. paratyphi, S. typhimurium, S. bongori*, and S. diarizonae, among others), are clustered in a single operon with flavin prenyltransferase and protocatechuate decarboxylase genes, have an open reading from overlapping with—or just downstream of—the protocatechuate decarboxylase gene, and encode a protein of about 70-80 amino acids (38). Such clusters are known in the art as hydroxyarylic acid decarboxylases (38). Exemplary protocatechuate decarboxylase D genes and proteins include nadD/NadD (Saro_3878) of *Novosphingobium aromaticivorans* (SEQ ID NOS:5 and 6), ecdD/EcdD of *Enterobacter cloacae* (SEQ ID NOS:23 and 24), and kpdD/KpdD of *Klebsiella pneumoniae* (SEQ ID NOS:25 and 26). Other protocatechuate decarboxylases are known in the art (Johnson C W, Salvachda D, Khanna P, Smith H, Peterson D J, Beckham G T. Enhancing muconic acid production from glucose and lignin-derived aromatic compounds via increased protocatechuate decarboxylase activity. Metab Eng Commun. 2016 Apr. 22; 3:111-119) (Payer S E, Marshall S A, Barland N, Sheng X, Reiter T, Dordic A, Steinkellner G, Wuensch C, Kaltwasser S, Fisher K, Rigby S E J, Macheroux P, Vonck J, Gruber K, Faber K, Himo F, Leys D, Pavkov-Keller T, Glueck S M. Regioselective para-Carboxylation of Catechols with a Prenylated Flavin Dependent Decarboxylase. Angew Chem Int Ed Engl. 2017 Oct. 23; 56(44):13893-13897) (Sonoki T, Morooka M, Sakamoto K, Otsuka Y, Nakamura M, Jellison J, Goodell B. Enhancement of protocatechuate decarboxylase activity for the effective production of muconate from lignin-related aromatic compounds. J Biotechnol. 2014 Dec. 20; 192 Pt A:71-7) (Lupa B, Lyon D, Gibbs M D, Reeves R A, Wiegel J. Distribution of genes encoding the microbial non-oxidative reversible hydroxyarylic acid decarboxylases/phenol carboxylases. Genomics. 2005 September; 86(3):342-51). In some versions of the invention, the one or more modifications in the recombinant microorganisms comprise a recombinant protocatechuate decarboxylase D gene. In some versions, the recombinant protocatechuate decarboxylase D gene encode a protocatechuate decarboxylase D protein having at least 80%, at least 85%, at least 90%, at least 95%, at least 99%, or 100% sequence identity to any one of SEQ ID NOS:6, 24, and 26. In some versions, the recombinant protocatechuate decarboxylase D gene is an exogenous protocatechuate decarboxylase D gene newly introduced to the microorganism. In some versions, the recombinant protocatechuate decarboxylase D gene is a modified form of an endogenous protocatechuate decarboxylase gene already present in the microorganism.

[0048] Catechol 1,2-dioxygenase activity is characterized by EC 1.13.11.1 and comprises the ability to catalyze the oxidative ring cleavage of catechol to form cis,cis-muconic acid. Catechol 1,2-dioxygenase activity is performed by catechol 1,2-dioxygenases. An exemplary catechol 1,2-dioxygenase is catA/CatA (Saro_3830) of *Novosphingobium aromaticivorans*, the nucleic acid coding sequence of which is SEQ ID NO:7 and the protein sequence of which is SEQ ID NO:8. Other exemplary catechol 1,2-dioxygenases include proteins with catechol 1,2-dioxygenase activity having a sequence at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% identical to SEQ ID NO:8. Other exemplary catechol 1,2-dioxygenases include the CatA enzymes from *Enterobacter cloacae* and *Pseudomonas putida* described in the following examples, as well as modified versions thereof. Other catechol 1,2-dioxygenases are known in the art, including those found in *Pseudomonas* sp. (Dorn E, Knackmuss H J. Chemical structure and biodegradability of halogenated aromatic compounds. Two catechol 1,2-dioxygenases from a 3-chlorobenzoate-grown pseudomonad. Biochem J. 1978 Jul. 15; 174(1):73-84), Pseudomonasfluorescens (Hayaishi O, Katagiri M, Rothberg S. Studies on oxygenases; pyrocatechase. J Biol Chem. 1957 December; 229(2):905-20.), *Aspergillus niger* (Ninnekar H, Vaidyanathan C. Catechol 1,2-dioxygenase from *Aspergillus niger*: Purification and properties. J. Indian Inst. Sci. 1981 63C:131-136), *Brevibacterium fuscum* (Nakagawa H, Inoue H, Takeda Y. Characteristics of Catechol Oxygenase from *Brevibacterium fuscum*. J Biochem. 1963 July; 54:65-74) (Hou C T, Patel R, Lillard M O. Extradiol cleavage of 3-methylcatechol by catechol 1,2-dioxygenase from various microorganisms. Appl Environ Microbiol. 1977 March; 33(3):725-7), *Acinetobacter calcoaceticus* (Patel R N, Hou C T, Felix A, Lillard M O. Catechol 1,2-dioxygenase from *Acinetobacter calcoaceticus*: purification and properties. J Bacteriol. 1976 July; 127(1):536-44), *Trichosporon cutaneum* (Itoh, M. Characteristics of a new catechol-1,2-oxygenase from *Trichosporon cutaneum* WY2-2. Agric. Biol. Chem. 1981 45(1):2787-2796), *Rhodococcus erythropolis*

(Murakami S, Kodama N, Shinke R, Aoki K. Classification of catechol 1,2-dioxygenase family: sequence analysis of a gene for the catechol 1,2-dioxygenase showing high specificity for methylcatechols from Gram+ aniline-assimilating *Rhodococcus erythropolis* AN-13. Gene. 1997 Jan. 31; 185(1):49-54), Frateuria sp. (Aoki K, Konohana T, Shinke R, Nishira H. Two catechol 1,2-dioxygenases from aniline-assimilating bacterium, *Frateuria* species ANA-18. Agric. Biol. Chem. 1984 48(1):2097-2104), *Rhizobium trifolii* (Chen Y, Glenn A, Dilworth M. Aromatic metabolism in *Rhizobium trifolii*-catechol 1,2-dioxygenase. Arch. Microbiol. 1985 141(1):225-228), *Pseudomonas putida* (Pascal RA Jr, Huang DS. Reactions of 3-ethylcatechol and 3-(methylthio)catechol with catechol dioxygenases. Arch Biochem Biophys. 1986 July; 248(1):130-7), *Candida tropicalis* (Krug M, Straube G. Degradation of phenolic compounds by the yeast *Candida tropicalis* HP 15. II. Some properties of the first two enzymes of the degradation pathway. J Basic Microbiol. 1986; 26(5):271-81), *Candida* maltose (Gomi K, Horiguchi. Purification and characterization of pyrocatechase from the catechol-assimilating yeast *Candida* maltose. Agric. Biol. Chem. 1988 52(2):585-587), *Rhizobium leguminosarum* (Chen Y P, Lovell C R. Purification and Properties of Catechol 1,2-Dioxygenase from *Rhizobium leguminosarum* biovar viceae USDA 2370. Appl Environ Microbiol. 1990 June; 56(6):1971-3), and *Nocardia* sp. (Smith M, Ratledge C, Crook S. Properties of cyanogen bromide-activated, Agarose-immobilized catechol 1,2-dioxygenase from freeze-dried extracts of *Nocardia* sp. NCIB 10503. Enzyme Microb. Technol. 1990 12(12):945-949). In some versions of the invention, the one or more modifications in the recombinant microorganisms can comprise a genetic modification that increases catechol 1,2-dioxygenase activity. A genetic modification that increases catechol 1,2-dioxygenase activity can comprise a recombinant catechol 1,2-dioxygenase gene. In some versions, the recombinant catechol 1,2-dioxygenase gene is an exogenous catechol 1,2-dioxygenase gene newly introduced to the microorganism. In some versions, the recombinant catechol 1,2-dioxygenase gene is a modified form of an endogenous catechol 1,2-dioxygenase gene already present in the microorganism.

[0049] Muconate lactonizing enzyme activity is characterized by EC 5.5.1.1 and comprises the ability to catalyze the conversion of cis,cis-muconate to (+)-muconolactone ((S)-5-oxo-2,5-dihydro-2-furylacetate, (S)-muconolactone, 2-[(2S)-5-oxo-2H-furan-2-yl]acetate, [(2S)-5-oxo-2,5-dihydrofuran-2-yl]acetate). Muconate lactonizing enzyme activity is performed by muconate lactonizing enzymes. An exemplary muconate lactonizing enzyme is catB/CatB (Saro_3828) of *Novosphingobium aromaticivorans*, the nucleic acid coding sequence of which is SEQ ID NO:9 and the protein sequence of which is SEQ ID NO: 10. Other exemplary muconate lactonizing enzymes include proteins with muconate lactonizing enzyme activity having a sequence at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% identical to SEQ ID NO:10. Other exemplary muconate lactonizing enzymes include the CatB enzymes from *Enterobacter cloacae* and *Pseudomonas putida* described in the following examples, as well as modified versions thereof. Other muconate lactonizing enzymes are known in the art. In some versions of the invention, the one or more modifications in the recombinant microorganisms can comprise a genetic modification that decreases muconate lactonizing enzyme activity. A genetic modification that decreases muconate lactonizing enzyme activity can comprise a genetic modification to a muconate lactonizing enzyme gene. A genetic modification to a muconate lactonizing enzyme gene can comprise a substitution or insertion in or a complete or partial deletion of the muconate lactonizing enzyme gene.

[0050] Muconolactone isomerase (muconolactone A-isomerase) activity is characterized by EC 5.3.3.4 and comprises the ability to catalyze the conversion of (+)-muconolactone to beta-ketoadipate-enol-lactone (5-oxo-4,5-dihydro-2-furylacetate, (5-oxo-4,5-dihydrofuran-2-yl)acetate, (4,5-dihydro-5-oxofuran-2-yl)-acetate, enol-lactone). Muconolactone isomerase activity is performed by muconate lactonizing enzymes. An exemplary muconolactone isomerase is catC/CatC (Saro_3829) of *Novosphingobium aromaticivorans*, the nucleic acid coding sequence of which is SEQ ID NO:11 and the protein sequence of which is SEQ ID NO:12. Other exemplary muconolactone isomerases include proteins with muconolactone isomerase activity having a sequence at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% identical to SEQ ID NO:12. Other exemplary muconolactone isomerases include the CatC enzymes from *Enterobacter cloacae* and *Pseudomonas putida* described in the following examples, as well as modified versions thereof. Other muconolactone isomerases are known in the art. In some versions of the invention, the one or more modifications in the recombinant microorganisms can comprise a genetic modification that decreases muconolactone isomerase activity. A genetic modification that decreases muconolactone isomerase activity can comprise a genetic modification to a muconolactone isomerase gene. A genetic modification to a muconolactone isomerase gene can comprise a substitution or insertion in or a complete or partial deletion of the muconolactone isomerase gene.

[0051] Catechol 2,3-dioxygenase activity is characterized by EC 1.13.11.2 and comprises the ability to catalyze the conversion of catechol and O_2 to 2-hydroxymuconate semialdehyde. Catechol 2,3-dioxygenase activity is performed by catechol 2,3-dioxygenases. An exemplary catechol 2,3-dioxygenase is xylE/XylE (Saro_3857) of *Novosphingobium aromaticivorans*, the nucleic acid coding sequence of which is SEQ ID NO:13 and the protein sequence of which is SEQ ID NO:14. Other exemplary catechol 2,3-dioxygenases include proteins with catechol 2,3-dioxygenase activity having a sequence at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% identical to SEQ ID NO:14. Other catechol 2,3-dioxygenases are known in the art. In some versions of the invention, the one or more modifications in the recombinant microorganisms can comprise a genetic modification that decreases catechol 2,3-dioxygenase activity. A genetic modification that decreases catechol 2,3-dioxygenase activity can comprise a genetic modification to a catechol 2,3-dioxygenase gene. A genetic modification to a catechol 2,3-dioxygenase gene can comprise a substitution or insertion in or a complete or partial deletion of the catechol 2,3-dioxygenase gene.

[0052] Protocatechuate 4,5-dioxygenase activity is characterized by EC 1.13.11.8 and comprises the ability to catalyze the conversion of protocatechuic acid and O_2 to 4-carboxy-2-hydroxy-cis,cis-muconate-6-semialdehyde (CHMS). Protocatechuate 4,5-dioxygenase activity is performed by protocatechuate 4,5-dioxygenases. Exemplary

protocatechuate 4,5-dioxygenases are ligAB1/LigAB1 (Saro_2813/2812) and ligAB2/LigAB2 (Saro_1233/1234) of *Novosphingobium aromaticivorans*. These enzymes are comprised of A subunits (LigA1 or LigA2) and B subunits (LigB1 or LigB2). The nucleic acid coding sequence of the LigA1 subunit is SEQ ID NO:15. The protein sequence of the LigA1 subunit is SEQ ID NO:16. The nucleic acid coding sequence of the LigB1 subunit is SEQ ID NO:17. The protein sequence of the LigB1 subunit is SEQ ID NO:18. The nucleic acid coding sequence of the LigA2 subunit is SEQ ID NO:19. The protein sequence of the LigA2 subunit is SEQ ID NO:20. The nucleic acid coding sequence of the LigB2 subunit is SEQ ID NO:21. The protein sequence of the LigB2 subunit is SEQ ID NO:22. Other exemplary protocatechuate 4,5-dioxygenases include proteins with A and B subunits that together exhibit protocatechuate 4,5-dioxygenase activity and have sequences at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% identical to SEQ ID NOS:16, 18, 20, and/or 22. Other protocatechuate 4,5-dioxygenases are known in the art. In some versions of the invention, the one or more modifications in the recombinant microorganisms can comprise a genetic modification that decreases protocatechuate 4,5-dioxygenase activity. A genetic modification that decreases protocatechuate 4,5-dioxygenase activity can comprise a genetic modification to a protocatechuate 4,5-dioxygenase subunit gene. A genetic modification to a protocatechuate 4,5-dioxygenase subunit gene can comprise a substitution or insertion in or a complete or partial deletion of the protocatechuate 4,5-dioxygenase gene.

[0053] "Gene" refers to a nucleic acid sequence capable of producing a gene product and may include such genetic elements as a coding sequence together with any other genetic elements required for transcription and/or translation of the coding sequence. Such genetic elements may include a promoter, an enhancer, and/or a ribosome binding site (RBS), among others. In some versions, multiple genes are configured in an operon, in which multiple coding sequences are operationally connected to a single promoter. Each coding sequence and promoter pair in such instances are considered herein to constitute separate genes, despite comprising the same promoter.

[0054] "Gene product" refers to products such as a polypeptide or an mRNA encoded and produced by a particular gene.

[0055] "Operationally connected" refers to a relationship between two genetic elements (e.g., a promoter and coding sequence), in which one of the genetic elements controls or affects the activity of the other genetic element.

[0056] "Endogenous" used in reference to a genetic element means that the genetic element is native to the microorganism in which it is disposed.

[0057] "Exogenous" used in reference to a genetic element means that the genetic element is not native to the microorganism in which it is disposed.

[0058] "Heterologous" used in reference to a genetic element means that the genetic element is derived from a different species than that in which it is disposed.

[0059] "Recombinant" as used herein with reference to nucleic acid molecules or polypeptides refers to nucleic acid molecules or polypeptides having a non-natural nucleic acid or polypeptide sequence, respectively. Recombinant" as used herein with reference to a gene refers to a gene having a non-natural nucleic acid sequence, is exogenous, is heterologous, or is endogenous to a given microbe but is disposed within the microbe (e.g., within the microbe's genome) at a locus different from the native form of the gene. "Recombinant" as used herein with reference to a cell or microorganism refers to a cell or microorganism that contains a recombinant nucleic acid molecule, polypeptide, or gene.

[0060] "Genetic modification" as used herein refers to any difference in the nucleic acid composition of a cell with respect to a corresponding cell, whether in the cell's native chromosome or in endogenous or exogenous non-chromosomal plasmids harbored within the cell.

[0061] "Overexpress" as used herein means that a particular gene product is produced at a higher level in one cell, such as a recombinant cell, than in a corresponding cell. For example, a microorganism that includes a recombinant nucleic acid configured to overexpress a gene product produces the gene product at a greater amount than a microorganism of the same species that does not include the recombinant nucleic acid.

[0062] A "homologous" gene or protein is a gene or protein inherited in two species from a common ancestor. While homologous genes or proteins can be similar in sequence, similar sequences are not necessarily homologous.

[0063] The terms "identical" or "percent identity", in the context of two or more nucleic acid or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same, when compared and aligned for maximum correspondence, as measured using one of the sequence comparison algorithms described herein (or other algorithms available to persons of skill) or by visual inspection. For sequence comparison and identity determination, one sequence typically acts as a reference sequence to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence based on the designated program parameters. A typical reference sequence of the invention is any nucleic acid or amino acid sequence described herein. Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, Adv. Appl. Math. 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, J. Mol. Biol. 48:443 (1970), by the search for similarity method of Pearson & Lipman, Proc. Nat'l. Acad. Sci. USA 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by visual inspection (see Current Protocols in Molecular Biology, F. M. Ausubel et al., eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (supplemented through 2008)). One example of an algorithm that is suitable for determining percent sequence identity and sequence similarity for purposes of defining homologs is the BLAST algorithm, which is described in Altschul et al., J. Mol. Biol. 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. This algorithm

involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al., supra). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always>0) and N (penalty score for mismatching residues; always<0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, a cutoff of 100, M=5, N=−4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff (1989) Proc. Natl. Acad. Sci. USA 89:10915). In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin & Altschul, Proc. Natl. Acad. Sci. USA 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001. The above-described techniques are useful in determining sequence identity of sequences described herein.

[0064] In addition to mechanisms described elsewhere herein, genetic modifications for increasing the activity of a gene or protein include but are not limited to placing the coding sequence under the control of a more active promoter, increasing the copy number of genes comprising the coding sequence, introducing a translational enhancer on a gene comprising the coding sequence (see, e.g., Olins et al. *Journal of Biological Chemistry,* 1989, 264(29):16973-16976), and/or modifying factors (e.g., transcription factors or genes therefor) that control expression of a gene comprising the coding sequence. Increasing the copy number of genes comprising a coding sequence can be performed by introducing one or more additional copies of the native gene to the microorganism, introducing one or more a heterologous homologs to the microorganism, introducing one or more copies of recombinant versions of the native gene or heterologous homolog to the microorganism, etc. Genes expressing a given coding sequence may be incorporated into the microbial genome or included on an extrachromosomal genetic construct such as a plasmid.

[0065] In addition to mechanisms described elsewhere herein, genetic modifications for decreasing the activity of a gene or protein include but are not limited to substitutions, partial or complete deletions, insertions, or other variations to a coding sequence or a sequence controlling the transcription or translation of a coding sequence, such as placing a coding sequence under the control of a less active promoter, etc. In some versions, the genetic modifications can include the introduction of constructs that express ribozymes or antisense sequences that target the mRNA of the gene of interest. Various other genetic modifications that decrease the activity of a gene or gene product are described elsewhere herein.

[0066] Various methods for introducing genetic modifications are well known in the art and include homologous recombination, among other mechanisms. See, e.g., Green et al., *Molecular Cloning: A laboratory manual,* 4th ed., Cold Spring Harbor Laboratory Press (2012) and Sambrook et al., *Molecular Cloning: A Laboratory Manual,* 3rd ed., Cold Spring Harbor Laboratory Press (2001).

[0067] The recombinant genes of the invention can be codon-optimized for the particular microorganism in which they are introduced. Codon optimization can be performed for any nucleic acid by a number of programs, including "GENEGPS"-brand expression optimization algorithm by DNA 2.0 (Menlo Park, CA), "GENEOPTIMIZER"-brand gene optimization software by Life Technologies (Grand Island, NY), and "OPTIMUMGENE"-brand gene design system by GenScript (Piscataway, NJ). Other codon optimization programs or services are well known and commercially available.

[0068] The recombinant microorganisms of the invention may comprise any type of microorganism. The microorganism may be prokaryotic or eukaryotic. Suitable prokaryotes include bacteria and archaea. Suitable types of bacteria include α- and γ-proteobacteria, gram-positive bacteria, gram-negative bacteria, ungrouped bacteria, phototrophs, lithotrophs, and organotrophs. Suitable eukaryotes include yeast and other fungi. The microorganism in some versions can be from an order selected from the group consisting of Sphingomonadales and Pseudomonadales. The microorganism in some versions can be from a family selected from the group consisting of Sphingomonadaceae, Pseudomonadaceae, and Enterobacteriaceae. The microorganism in some versions can be from a genus selected from the group consisting of *Sphingomonas, Sphingobium, Sphingosinicella, Sphingopyxis, Novosphingobium, Pseudomonas, Erythrobacter* (e.g., sp. SG61-1L), *Altererythrobacter, Enterobacter*, and *Klebsiella*, among others.

[0069] The microorganism in some versions can be a phenol-degrading microorganism, such as a phenol-degrading bacterium. Phenol-degrading microorganisms, including phenol-degrading bacteria, are well known in the art. See, e.g., Gu et al. 2016 (Gu Q, Wu Q, Zhang J, Guo W, Wu H, Sun M. Community Analysis and Recovery of Phenol-degrading Bacteria from Drinking Water Biofilters. Front Microbiol. 2016 Apr. 12; 7:495), Ramid-Pujol et al. 2013 (Ramid-Pujol S, Baneras L, Artigas J, Romani A M. Changes of the phenol-degrading bacterial community during the decomposition of submersed *Platanus acerifolia* leaves. FEMS Microbiol Lett. 2013 January; 338(2):184-91), Bastos et al. 2000 (Bastos A E, Moon D H, Rossi A, Trevors J T, Tsai S M. Salt-tolerant phenol-degrading microorganisms isolated from Amazonian soil samples. Arch

Microbiol. 2000 November; 174(5):346-52), van Schie et al. 1998 (van Schie P M, Young L Y. Isolation and characterization of phenol-degrading denitrifying bacteria. Appl Environ Microbiol. 1998 July; 64(7):2432-8), Paisio et al. 2012 (Paisio C E, Talano M A, Gonzilez P S, Busto V D, Talou J R, Agostini E. Isolation and characterization of a *Rhodococcus* strain with phenol-degrading ability and its potential use for tannery effluent biotreatment. Environ Sci Pollut Res Int. 2012 September; 19(8):3430-9), Kumari et al. 2013 (Kumari S, Chetty D, Ramdhani N, Bux F. Phenol degrading ability of *Rhodococcus* pyrinidivorans and *Pseudomonas aeruginosa* isolated from activated sludge plants in South Africa. J Environ Sci Health A Tox Hazard Subst Environ Eng. 2013; 48(8):947-53), among others. Examples of phenol-degrading microorganisms include *Pseudomonas putida* (Abu Hamed T., Bayraktar E., Mehmetoglu U., Mehmetoglu T. (2004). The biodegradation of benzene, toluene and phenol in a two-phase system. Biochem. Eng. J. 19 137-146), *Gliomastix indicus* (Singh R. K., Kumar S., Kumar S., Kumar A. (2008) Biodegradation kinetic studies for the removal of p-cresol from wastewater using *Gliomastix indicus* MTCC 3869. Biochem. Eng. J. 40 293-303), *Sphingomonas chlorophenolica* (Nair C. I., Jayachandran K., Shashidhar S. (2008). Biodegradation of phenol. Afr. J. Biotechnol. 7 4951-4958), *Bacillus brevis* (Arutchelvan V., Kanakasabai V., Elangovan R., Nagarajan S., Muralikrishnan V. (2006). Kinetics of high strength phenol degradation using *Bacillus brevis*. J. Hazardous Materials 129 216-222), and Cyanobacterium synechococcus (Song H., Liu Y., Xu W., Zeng G., Aibibu N., Xu L., et al. (2009). Simultaneous Cr (VI) reduction and phenol degradation in pure cultures of *Pseudomonas aeruginosa* CCTCC AB91095. Bioresour. Technol. 100 5079-5084), and *Acinetobacter* sp. (Gu Q, Wu Q, Zhang J, Guo W, Wu H, Sun M. Community Analysis and Recovery of Phenol-degrading Bacteria from Drinking Water Biofilters. Front Microbiol. 2016 Apr. 12; 7:495). Other examples of phenol-degrading microorganisms include *Achromobacter* sp., *Alcaligenes denitrificans, Arthrobacter* sp., *Arthrobacter sulphureus, Acidovorax delafieldii, Bacillus cereus, Brevibacterium* sp., *Burkholderia* sp., *Burkholderia cepacia, Burkholderia cocovenenans, Burkholderia xenovorans, Chryseobacterium* sp., *Cycloclasticus* sp., *Janibacter* sp., *Marinobacter, Mycobacterium* sp., *Mycobacterium flavescens, Mycobacterium vanbaalenii, Mycobacterium* sp., *Nocardioides aromaticivorans, Pasteurella* sp., *Polaromonas naphthalenivorans, Pseudomonas* sp., *Pseudomonas paucimobilis, Pseudomonas vesicularis, Pseudomonas putida, Pseudomonas fluorescens, Pseudomonas stutzeri, Pseudomonas saccharophilia, Ralstonia* sp., *Rhodococcus* sp., *Rhodococcus erythropolis, Staphylococcus* sp., *Stenotrophomonas maltophilia, Sphingomonas yanoikuyae, Sphingomonas* sp., *Sphingomonas paucimobilis, Sphingomonas wittichii, Terrabacter* sp., and *Xanthamonas* sp. (Seo J-S, Keum Y-S, Li Q X. Bacterial Degradation of Aromatic Compounds. International Journal of Environmental Research and Public Health. 2009; 6(1): 278-309.) Other examples of phenol-degrading microorganism include *Acinetobacter calcoaceticus, Rhodococcus aetherivorans, Rhodococcus ruber* SD3, *Aspergillus oryzae*, and *Aspergillus flavus* (Xu N, Qiu C, Yang Q, Zhang Y, Wang M, Ye C, Guo M. Analysis of Phenol Biodegradation in Antibiotic and Heavy Metal Resistant *Acinetobacter lwoffii* NL1. Front Microbiol. 2021 Sep. 10; 12:725755), among others.

[0070] An exemplary microorganism from the genus *Novosphingobium* is *Novosphingobium aromaticivorans. Novosphingobium aromaticivorans* DSM12444 can naturally catabolize multiple aromatic compounds containing H, G, and S units via protocatechuic acid.

[0071] The recombinant microorganisms are preferably configured to exhibit enhanced production of cis,cis-muconic acid with respect to a corresponding microorganism. "Production" in this context refers to the extracellular appearance of cis,cis-muconic acid in media in which the recombinant microorganism are cultured. The recombinant microorganisms in such versions may include any one or more of the modifications described herein, in any combination.

[0072] The recombinant microorganisms of the invention preferably exhibit enhanced cis,cis-muconic acid production with respect to the corresponding microorganism when the recombinant microorganism and the corresponding organism are grown under identical conditions. The cis,cis-muconic acid production may be enhanced by a factor of at least about 1.1, at least about 1.5, at least about 2, at least about 2.5, at least about 3, at least about 3.5, at least about 4, at least about 4.5, at least about 5, at least about 5.5, at least about 6, or at least about 6.5 and/or up to about 6.5, up to about 7, or more. Such increases may reflect an increase by mass.

[0073] The cis,cis-muconic can be produced by culturing a recombinant microorganism of the invention in a medium comprising a plant-derived phenolic. The plant-derived phenolic can comprise any of a number of phenolics obtained from processing plant lignocellulosic biomass. Exemplary plant-derived phenolics comprise syringyl phenolics, guaiacyl phenolics, and p-hydroxyphenyl phenolics. Exemplary syringyl phenolics include syringaldehyde, syringic acid, and S-diketone. Exemplary guaiacyl phenolics include vanillin, vanillic acid, and G-diketone. Exemplary hydroxyphenyl phenolics include p-coumaric acid, p-hydroxybenzaldehyde, and p-hydroxybenzoic acid. Other plant-derived phenolics include methyl guaiacol, propyl guaiacol, dihydroconiferyl alcohol, methyl syringol, p-hydroxy benzoic acid methyl ester, dihydrop-hydroxy cinnamic acid methyl ester, dihydrosyringol alcohol, and dihydroferulic acid methyl ester, among others.

[0074] The plant-derived phenolic can be derived and/or provided in the form of depolymerized lignin, such as chemically depolymerized lignin. Methods of depolymerizing lignin are well known in the art. See Pandey et al. 2010 (Pandey M P, Kim C S. Lignin Depolymerization and Conversion: A Review of Thermochemical Methods. *Chemical & Engineering Technology,* 2010, Vol. 34, Issue 1, pp. 3-145) and Wang et al. 2013 (Wang H, Tucker M, Ji Y. Recent Development in Chemical Depolymerization of Lignin: A Review. *Journal of Applied Chemistry,* 2013, Volume 2013, Article ID 838645).

[0075] The depolymerized lignin can be derived from pretreated lignocellulosic biomass. Methods of pretreating lignocellulosic biomass are well known in the art. See Kumar et al. 2017 (Kumar AK and Sharma S. Recent Updates on Different Methods of Pretreatment of Lignocellulosic Feedstocks: A Review. *Bioresour. Bioprocess.* (2017) 4:7); Kumar et al. 2009 (Kumar, P.; Barrett, D. M.; Delwiche, M. J.; Stroeve, P., Methods for Pretreatment of lignocellulosic Biomass for Efficient Hydrolysis and Biofuel Production. Industrial & Engineering Chemistry Research

2009, 48, (8), 3713-3729); Wang et al. 2013 (Wang H, Tucker M, Ji Y. Recent Development in Chemical Depolymerization of Lignin: A Review. (2013) *Journal of Applied Chemistry.* 2013:1-9), and Karlen et al. 2020 (Karlen S D, Fasahati P, Mazaheri M, Serate J, Smith R A, Sirobhushanam S, Chen M, Tymkhin V I, Cass C L, Liu S, Padmakshan D, Xie D, Zhang Y, McGee M A, Russell J D, Coon J J, Kaeppler H F, de Leon N, Maravelias C T, Runge T M, Kaeppler S M, Sedbrook J C, Ralph J. Assessing the viability of recovering hydroxycinnamic acids from lignocellulosic biorefinery alkaline pretreatment waste streams. *ChemSusChem.* 2020 Jan. 26). Examples include chipping, grinding, milling, steam pretreatment, ammonia fiber expansion (AFEX, also referred to as ammonia fiber explosion), ammonia recycle percolation (ARP), CO_2 explosion, steam explosion, ozonolysis, wet oxidation, acid hydrolysis, dilute-acid hydrolysis, alkaline hydrolysis, organosolv, ionic liquids, gamma-valerolactone, and pulsed electrical field treatment, among others.

[0076] The lignocellulosic biomass can be derived from any source, such as corn cobs, corn stover, cotton seed hairs, grasses, hardwood stems, leaves, newspaper, nut shells, paper, softwood stems, sorghum, switchgrass, waste papers from chemical pulps, wheat straw, wood, woody residues, mixed biomass species such as those produced by native prairie, and other sources.

[0077] The medium in some versions can additionally or alternatively comprise a fermentable sugar. Non-limiting examples of suitable fermentable sugars include adonitol, arabinose, arabitol, ascorbic acid, chitin, cellubiose, dulcitol, erythrulose, fructose, fucose, galactose, glucose, gluconate, inositol, lactose, lactulose, lyxose, maltitol, maltose, maltotriose, mannitol, mannose, melezitose, melibiose, palatinose, pentaerythritol, raffinose, rhamnose, ribose, sorbitol, sorbose, starch, sucrose, trehalose, xylitol, xylose, and hydrates thereof, among others.

[0078] In some versions, the fermentable sugar may be replaced by other organic compounds that support growth of the recombinant microorganism. This includes but is not limited to the other organic compounds that are present in the deconstructed biomass fractions from the crops or plant species mentioned above.

[0079] A recitation herein of a microorganism "comprising" a mutation in or to a particular gene refers to a gene that would be present were it not for the mutation, e.g., the gene present in a corresponding microorganism. Thus, the recitation of a microorganism "comprising" a mutation in or to a particular gene encompasses a mutated form of the gene present in the microorganism, a partially deleted remnant of the gene present in the microorganism, a complete absence of the gene (e.g., as resulting from a complete deletion of the gene) in the microorganism, or other configurations.

[0080] The methods can further comprise isolating cis,cis-muconic acid from the recombinant microorganism and/or the medium. Methods of isolating cis,cis-muconic acid from a medium are provided in the attached examples and otherwise known in the art.

[0081] The elements and method steps described herein can be used in any combination whether explicitly described or not.

[0082] All combinations of method steps as used herein can be performed in any order, unless otherwise specified or clearly implied to the contrary by the context in which the referenced combination is made.

[0083] As used herein, the singular forms "a," "an," and "the" include plural referents unless the content clearly dictates otherwise.

[0084] Numerical ranges as used herein are intended to include every number and subset of numbers contained within that range, whether specifically disclosed or not. Further, these numerical ranges should be construed as providing support for a claim directed to any number or subset of numbers in that range. For example, a disclosure of from 1 to 10 should be construed as supporting a range of from 2 to 8, from 3 to 7, from 5 to 6, from 1 to 9, from 3.6 to 4.6, from 3.5 to 9.9, and so forth.

[0085] All patents, patent publications, and peer-reviewed publications (i.e., "references") cited herein are expressly incorporated by reference to the same extent as if each individual reference were specifically and individually indicated as being incorporated by reference. In case of conflict between the present disclosure and the incorporated references, the present disclosure controls.

[0086] It is understood that the invention is not confined to the particular construction and arrangement of parts herein illustrated and described, but embraces such modified forms thereof as come within the scope of the claims.

Examples

Summary

[0087] The transition to production of commodity chemicals from renewable resources is an important goal towards increasing the environmental and economic sustainability of industrial processes. The aromatics in plant biomass are an underutilized and abundant renewable resource for the production of valuable chemicals. However, due to the chemical composition of plant biomass, many deconstruction methods generate a heterogenous mixture of aromatics, thus making it difficult to extract valuable chemicals using current methods. Therefore, recent efforts have focused on harnessing the native or engineered pathways of microorganisms to convert a diverse set of aromatics into a single product. *Novosphingobium aromaticivorans* DSM12444 has the native ability to metabolize a wide range of aromatics and thus is a potential chassis for conversion of these abundant compounds to commodity chemicals.

[0088] The platform chemical cis,cis-muconic acid (ccMA) provides facile access to a number of monomers used in the synthesis of commercial plastics. It is also a metabolic intermediate in the β-ketoadipic acid pathway of many bacteria and, therefore, a current target for microbial production from abundant renewable resources via metabolic engineering. This study investigates *Novosphingobium aromaticivorans* DSM12444 as a chassis for the production of ccMA from biomass aromatics. We show that the *N. aromaticivorans* genome encodes a previously uncharacterized PCA decarboxylase and a catechol 1,2-dioxygenase, which can be used for the conversion of aromatic metabolic intermediates to ccMA. This study confirmed the activity of these 2 enzymes in vitro. From these results, we generated one strain that is completely derived from native genes and another strain that contains heterologous genes. Both of these strains exhibited stoichiometric production of ccMA from PCA and produced greater than 100% yield of ccMA from the aromatic monomers that were identified in liquor derived from alkaline pretreated biomass. Our results show that a strain completely derived from native genes is com-

parable in producing ccMA from biomass aromatics to strains that are derived from heterologous genes. Overall, this work highlights the capacity of *N. aromaticivorans* as a chassis for ccMA production from biomass.

[0089] This study demonstrates that metabolic engineering of *N. aromaticivorans* can be used to produce the commodity chemical cis,cis-muconic acid (ccMA) from renewable and abundant biomass aromatics.

Introduction

[0090] Strategies for biological ccMA production from aromatics typically relies on the intradiol cleavage of catechol by a catechol 1,2-dioxygenase, CatA. Catechol is a known intermediate in the aromatic metabolism of benzoic acid, guaiacol and phenol (FIG. **1**) (17, 18). Pathways for production of ccMA from biomass-derived H- and G-type aromatics have been designed to divert the pathway intermediate protocatechuic acid (PCA) to catechol often through heterologous expression of a PCA decarboxylase that is native to either *Klebsiella pneumoniae* or *Enterobacter cloacae* (5, 6, 19). Thus far, *Pseudomonas putida* KT2440 is the best described bacterial host strain for ccMA production from either pure aromatics (p-coumaric or ferulic acids) or deconstructed biomass (20). Engineering of *P. putida* KT2440 for ccMA production required the use of exogenous promoters for expression of foreign genes and deletion of a gene that encodes a global catabolic transcriptional regulator (21, 22) to overcome metabolic bottlenecks that included accumulation of several pathway intermediates (PCA, vanillin and 4-hydroxybenzoic acid) (6, 23, 24). Therefore, we sought to expand the scope of bacterial hosts for producing ccMA from biomass aromatics by leveraging the native metabolic pathways of the aromatic metabolizing bacterium *Novosphingobium aromaticivorans* DSM12444.

[0091] *N. aromaticivorans* is one of several sphingomonads that are being studied as a potential chassis for production of chemicals from biomass aromatics (4, 25). This α-protobacterium, isolated from a polyaromatic hydrocarbon-contaminated site, can utilize many aromatics as a sole carbon source (26-28). *N. aromaticivorans* and other sphingomonads have the native ability to cleave major inter-subunit linkages of lignin aromatic oligomers making these microbes ideal for converting renewable sources of mixed aromatics into products (11, 29-32). The genetic tractability of *N. aromaticivorans* has enabled the engineering of mutant strains that can stoichiometrically produce the commodity chemical 2-pyrone-4,6-dicarboxylic acid (PDC) from native G-, H-, and S-aromatics or aromatic diketones that are generated during lignin deconstruction (31-33). These characteristics of *N. aromaticivorans* make it attractive for microbial funneling of the heterogeneous mixture of aromatics in deconstructed biomass. However, knowledge gaps still remain on the number and diversity of enzymes that compose this bacterium's aromatic metabolic pathways. This work sought to address some of these knowledge gaps by investigating the ability of *N. aromaticivorans* to serve as a host for ccMA production from biomass aromatics.

[0092] Here, we evaluated the potential of engineering *N. aromaticivorans* as a chassis for ccMA production from lignin biomass. We determined possible PCA decarboxylase and CatA enzymes in the genome of *N. aromaticivorans*. These enzymes are both important for the diversion of PCA to catechol and subsequent production of ccMA (FIG. **1**). We used in vitro assays to confirm the activity of these putative enzymes and applied this information to generate defined mutants that test the predicted pathway for conversion of PCA to ccMA by *N. aromaticivorans*. Through the combination of these in vitro assays and the analysis of defined mutants, we were able to identify a previously unreported PCA catabolic pathway in *N. aromaticivorans*. This new information makes *N. aromaticivorans* unique in comparison to other reported ccMA production hosts which do not possess a native decarboxylase for conversion of PCA to catechol (20). We then explored whether the expression of these native genes could compete with expression of heterologous genes used previously for ccMA production by placing individual genes under control of native aromatic-inducible promoters in the genome. Overall, this study revealed the ability to engineer *N. aromaticivorans* to stoichiometrically convert PCA to ccMA and to produce greater than 100% yields of ccMA from the measured aromatics in biomass alkaline pretreatment liquor (APL). It also highlights that an engineered *N. aromaticivorans* strain completely derived from native genes produces ccMA at a similar rate and production yield as seen with a *N. aromaticivorans* strain that heterologously expresses the enzymes from *E. cloacae* (4-6, 34). This study illustrates the tractability and scope of *N. aromaticivorans* aromatic metabolism and the ability to use this host for production of ccMA as a valuable product from biomass-derived aromatics.

Materials and Methods

Genes and Sequences

[0093] Genes and the sequences of the genes used in this study are provided in Tables 1 and 2.

TABLE 1

N. aromaticivorans genes used in this study with associated locus tags.

Gene	Organism	Locus tag
ligAB1	*Novosphingobium aromativicorans* DSM 12444	Saro_2812-13
ligAB2	*Novosphingobium aromativicorans* DSM 12444	Saro_1233-34
nadB	*Novosphingobium aromativicorans* DSM 12444	Saro_3873
nadC	*Novosphingobium aromativicorans* DSM 12444	Saro_3877
nadD	*Novosphingobium aromativicorans* DSM 12444	Saro_3878
ecdB	*Enterobacter cloacae*	ECL_04083
ecaroY	*Enterobacter cloacae*	ECL_01944
ecdD	*Enterobacter cloacae*	ECL_04081
nacatA	*Novosphingobium aromativicorans* DSM 12444	Saro_3830
eccatA	*Enterobacter cloacae*	HWQ17_22740
naxylE	*Novosphingobium aromativicorans* DSM 12444	Saro_3857

TABLE 2

DNA sequences of genes inserted into the genome of *N. aromaticivorans*. Start and stop codons are shown in bold. Coding sequences are indicated by upper case and relevant ribosomal binding sites are shown in lower case. In the EcDec or NaDec decarboxylase gene clusters, sequences in underline denote the prenyltransferase coding region, those in underlined italics denote the PCA decarboxylase coding region and those in italics represent the coding sequence for a gene of unknown function.

Gene	Sequence
NaCatA	**ATG**CCTGCCACCTTCGCCAGTTCCGATTCCGTGCAGAAGCTCTTCGATC GCGCCTGCGGTCTTGATTGCGCAGGCGGCAATCCCCGCCTCAAGGCGAT CATGCGCGACCTTCTCCAGGCAACGGCCGACATCATCGTCAAGCATGAC GTGTCCGAAAGCGAGTTCTGGCAGGCGACCCGCTATCTTGCCGATGGCG CCGGCGAGATCGGCCTGATCGTCCCCGGCATCGGCCTCGAACACTTCCT CGATCTCTACATGGACGCCAAGGACGCCGAAGCCGGCCTCACCGGCGG AACCCCGCGCACGATCGAAGGCCCGCTCTACGTCGCTGGTGCACCGCTG GTGGATGGCAGTGACGAAGTGGACCTGACTTCCGACCCCGACGATACC GACACGCTGCACATGACCGGCACGATCACCGGCCCCGATGGCGAGCCG GTCAAGGACGCGATCCTCCACGTCTGGCACGCGAACAGCAAGGGCTGG TATTCGCACTTCGATCCCACGAGCGAGCAGACCCCGTTCAACAACCGCC GCCGCATCCGCGTCCCCGCCGACGGTCGCTACGCCTTCCGCTCCAAGAT GCCGCATGGCTATTCCGTGCCGCCGGGTGGCGCCACCGACGTGCTGATG CAGGCGCTCGGCCGCCACGGCAATCGCCCAGCGCACGTCCACTTCTTCG TCGAGGCGCCGGGCTACCGCACGCTGACCACGCAGATCAACTTCGGCG ACGACCCCTTCGCGGCCGACGATTTCGCCTTCGGCACGCGAGAGGGCTT GCTGCCGGTGCCGAGCCGCCAGGGCGATACCGCCCACATCGCGTTCGA CTTCCAGCTCCAGCGCGCCCGCTCGGAGGACGAGCAGCGGTTCTCGGA ACGCACCCGCGCCCAGGCCTGA (SEQ ID NO: 7)
EcCatA	**ATG**TCGAAGAACCCGTCGCAGCAGTCGGAACTCGAAACCCTCCTCGCC ATCTCGTCGGGCCTCAACACCGACGGCGGCAACGAACGCGTCAAGCGC GTCATCCACCAGCTCCTCAACGACCTCTGCCACACCATCAAGACCTTCG ACATCTCGGACGAAGAATTCTGGATCGCCGTCAACTACCTCAACGAACT CGGCGAACGCAAGGAAGCCGCCCTCCTCGCCGCCGGCCTCGGCCTCGA ACACTACCTCGACATGCGCGCCGACGAAAAGGAAGCCGCCTCGGTCTC GCGCGCCGGCACCCCGCGCACCATCGAAGGCCCGCTCTACGTCGCCAA CGCCCCGCTCTCGGACCACTTCGCCCGCATGGACGACGGCTCGGAACGC GCCGAAGCCATGTGGCTCCACGGCACCGTCACCGACATCGACGGCAAG CCGGTCGCCGGCGCCATCGTCGACATCTGGCACGCCAACACCCACGGC GGCTACTCGTTCTTCGACCCGTCGCAGTCGGAATACAACCTCCGCCGCC GCGTCAAGACCGCCGCCGACGGCTCGTACGCCGTCCGCTCGATCGTCCC GTGCGGCTACGGCTGCCCGCCGGACGGCCCGACCCAGAAGCTCCTCAA CGAACTCGGCCGCCACGGCAACCGCCCGGCCCACATCCACTTCTTCGTC TCGGCCCCGGGCTTCAAGCACCTCACCACCCAGATCAACCTCAACGGCG ACCGCTACCTCTGGGACGACTTCGCCTTCGCCACCCGCGAAGAACTCAT CGCCGACCCGGTCAAGGTCACCGACTCGACCCTCGCCCGCGAACGCGA CATCCACGAACCGCACACCGAAGTCTCGTTCTCGTTCACCCTCGTCAAG GCCGCCGGCGCCGAAGAAGAAGCCCGCGGCAAGCGCGCCCGCGTCAAG GAATGA (SEQ ID NO: 27)
NaDec	**GTG**AAGCGCATGGTCGTGGGGATTACCGGCGCAACCGGCTCGGTCTAT GGTCTTCGCCTGCTTGAGCTGCTGCGCGAGACGGGCGGTTGGGAAACCC ATCTGGTAATGTCTCCGGCTGCGCTGCTCAACATTCGCGAGGAACTGCC CGAAGGCAAAGCCCGGCTCGAAGCGCTGGCCGATGTGGTGCACAACGT CCGCAACGTCGGCGCCTCGATCGCCAGCGGTTCGTTCGTATGCGAAGGC ATGGCGATTGCGCCCTGTTCGATGCGCACGCTGGGCGCGGTGGCGCACG CCCTGTCCGACAACCTTATCACCCGCGCGGCCGACGTGATGCTGAAGGA ACGGCGCCGCCTGGTGATGATCACCCGCGAAGCGCCGCTCAACCTGGC GCACCTGCGCAACATGACGGCCTGCACCGAAATGGGGGCGGTGATCTT CCCCCCGGTGCCGGCCTTCTATGCGCGGCCGACCTCGCTGGCCGACGTG GTCGATCACACCTGCATGCGGGTACTGGATCTGTTCGGGCTTCATGCGA AGTCGGAGAAACGCTGGCAAGGCCTTAGCAAAGAGGCGGCAAGCCTTG TTCCGGGTGCTGGGCAAATGGAAGGGAAT**TGA**ga***ATG***A*CCATGAACGATC* *TCCCTAACCGCGCCCGCTCGATCTCGTCGCTGCGCGACTTCCTCGAACTGC* *TCGAGGATGCCGGCCAGGCGATCACCTGGAGCGATGCGGTGATGCCCGAA* *CCCGGCGTGCGCAACATAGCCGTCGCCGCATCGCGCGATGCCAACGGCGC* *GCCGGCGATCGTATTCGACAATATCACCGGTTACCCCGGCAAGCGCTTGGC* *GGTGGGCGTCCATGGTTCGTGGGACAACATCGCCCTGCTGCTGGGCCGAC* *CTAAAGGCACGACCATCCGCGAGCTTTTCTTCGAGATCGCCGGCCGCTGGG* *GCGATCAGGAAGCGCAAATCAGCTTTGTCCCAGAAGCCCAGGCCCCGGTGC* *ACGAATGCCGGATCGAACAGGACATCAACCTTTACGATGTCCTGCCGGTCTA* *TCGGATCAACGAATACGATGGCGGGTTCTACATCGGCAAGGCCTCGGTCGC* *CTCGCGCGATCCGCTCGATCCAGACAATTTCGGCAAGCAGAATGTCGGCAT* *CTATCGCCTGCAGATCCAGGGGCCGGACACCTTCACCCTGATGACGATCCC* *CTCCCACGACATGGGACGTCAGATCATGGCGGCCGAACGGGAAGGCGTTCC* *GCTAAAGATTGCGGTCATGCTGGGTAATCATCCCGGCCTTGCGGTGTTTGCT*

TABLE 2-continued

DNA sequences of genes inserted into the genome of *N. aromaticivorans*. Start and stop codons are shown in bold. Coding sequences are indicated by upper case and relevant ribosomal binding sites are shown in lower case. In the EcDec or NaDec decarboxylase gene clusters, sequences in underline denote the prenyltransferase coding region, those in underlined italics denote the PCA decarboxylase coding region and those in italics represent the coding sequence for a gene of unknown function.

Gene	Sequence
	GCCACCCCGATCGGCTACGAGGAATCGGAATATTCCTATGCCTCGGCGATG
	ATGGGCGCGCCAATCCGGCTGACCAAATCGGGCAACGGGATCGACATCCTG
	GCCGACAGCGAAATCGTGATAGAGGCCGAACTGCAACCGGGTGGACGCGA
	GCTGGAAGGGCCGTTCGGCGAATTCCCCGGTTCCTACAGCGGCGTGCGCAA
	GGCGCCGATCTTCAAGGTCACGGCGGTGTCGCACCGGCGCGATCCGATCTT
	CGAGAACATTTACATCGGGCGCGGCTGGACCGAGCACGATACGCTGATCGG
	CCTGCACACCTCCGCCCCGATCTATGCCCAGCTGCGCCAGAGCTTCCCCGA
	AGTCACCGCGGTCAACGCGCTTTACCAGCACGGACTGACCGGGATCATCTC
	GGTCAAAAACCGCATGGCCGGCTTTGCCAAGACGGTCGCGCTGCGCGCGCT
	GAGCACGCCGCACGGCGTGATGTACCTCAAGAACCTGATTATGGTCGATGC
	CGATGTCGATCCGTTCGATCTCAACCAAGTGATGTGGGCGCTTTCGACCCGC
	ACCCGTGCGGACGATATCATCGTGCTGCCCAACATGCCTGCCGTGCCGATC
	GATCCTTCGGCAGTGGTCCCGGGCAAGGGGCACCGCCTGATCATCGACGC
	GACCAGCTATCTCCCGCCCGATCCGGTGGGTGAAGCGCACCTTGTCACCCC
	GCCGACCGGGGACGAGATCGACGCCCTGAGCAAGCGGATCCGCGAAATGC
	*AGCTGGGAGCCCTGTC**ATGA**CCACCACCGTCTGCGGGCGCTGCAAATCGA*
	GCGGCGCTGTCACCGATCATCAGGGCAGGCAGGACGGCGCGGTCGTGTGG
	ACGATCCTGCGCTGCCCGACCTGCAACTTTTCCTGGCGCGACAGCGAACCG
	GCCCGCGCTATCGACCCGGCTGTGCGCTCGGCCGATTTCGCCGTCGATGTC
	*GGCGATCTCCAGCGTTATCCCAAGATTCTCCAGCAA**TAA** (SEQ ID NO: 28)
EcDec	**ATG**AGGCTCATCGTGGGCATGACGGGAGCCACGGGCGCTCCGCTTGGC
	GTGGCCCTCCTGCAGGCGCTCCGCGACATGCCCGAGGTTGAAACCCATC
	TGGTGATGTCGAAGTGGGCGAAGACCACCATCGAGCTGGAAACGCCGT
	ATACCGCGCAGGACGTCGCTGCCCTGGCCGACGTCGTCCACAGCCCTGC
	CGATCAGGCAGCCACCATCTCGTCGGGCTCGTTCCGCACCGATGGCATG
	ATCGTCATTCCCTGCAGCATGAAGACGCTTGCAGGCATTCGCGCGGGCT
	ATGCCGAAGGGCTTGTCGGTCGTGCGGCAGATGTTGTGCTGAAAGAAG
	GTCGCAAGCTGGTGCTGGTCCCGCGCGAAACGCCGCTCAGCACCATCCA
	TCTGGAGAACATGCTCGCGCTTTCCCGCATGGGGGTGGCGATGGTGCCG
	CCCATGCCCGCGTACTACAACCATCCGCAAACCGCCGACGACATCACCC
	AGCACATCGTGACCCGCGTCCTCGACCAGTTCGGTCTGGAGCACAAGA
	AGGCACGTCGCTGGAATGGCCTGCAGGCGGCGAAGCACTTCAGCCAGG
	AGAACAACGACGGCATC**TGA**tgctgggcaaatggaagggaattgaga***ATG***CAGAACCC
	CATCAACGACCTCCGCTCTGCCATCGCGCTGCTGCAACGCCATCCCGGTCA
	CTATATCGAAACCGACCACCCGGTCGATCCCAATGCTGAACTGGCGGGCGT
	CTATCGCCATATCGGCGCGGGCGGTACCGTCAAACGCCCCACCCGCACGG
	GCCCGGCCATGATGTTCAACAGCGTGAAGGGCTACCCTGGCTCCCGCATCC
	TGGTCGGTATGCATGCCAGCCGGGAACGCGCGGCGCTTCTGCTGGGCTGT
	GTCCCCTCGAAGCTGGCACAGCACGTCGGTCAGGCGGTGAAGAACCCGGTT
	GCACCGGTGGTGGTTCCAGCCTCGCAGGCACCGTGCCAGGAGCAGGTCTT
	CTATGCCGACGATCCGGACTTCGACCTGCGTAAGCTGCTTCCGGCCCCGAC
	CAACACGCCGATTGATGCAGGCCCGTTCTTCTGCCTGGGGCTGGTCCTGGC
	AAGCGATCCGGAAGACACCTCGCTGACCGATGTGACCATTCACCGTCTCTGC
	GTGCAGGAGCGAGACGAACTCTCGATGTTCCTTGCCGCCGGCCGCCATATC
	GAAGTCTTTCGCAAGAAGGCCGAAGCGGCGGGCAAACCGCTGCCGGTCAC
	CATCAACATGGGACTTGACCCGGCTATCTACATAGGGGCCTGCTTCGAAGCG
	CCCACCACGCCCTTCGGTTACAACGAGCTTGGCGTTGCCGGGGCACTCCGC
	CAGCAACCGGTGGAGCTGGTCCAGGGCGTAGCGGTCAAGGAGAAAGCGAT
	CGCGCGGGCGGAAATCATCATCGAGGGCGAACTGCTTCCCGGCGTGCGCG
	TCCGCGAAGATCAGCACACCAACACCGGCCACGCCATGCCGGAGTTCCCGG
	GCTACTGCGGCGAGGCGAATCCGTCGCTGCCGGTGATCAAGGTGAAAGCC
	GTGACGATGCGAAACCATGCGATCCTGCAGACGCTGGTGGGCCCTGGCGAA
	GAGCACACCACGCTTGCCGGTCTGCCGACCGAGGCCAGCATTCGCAACGC
	GGTCGAAGAGGCCATTCCCGGCTTTCTGCAGAACGTCTACGCCCACACCGC
	CGGAGGCGGTAAGTTCCTCGGCATCCTACAGGTGAAGAAGCGCCAGCCGTC
	GGACGAAGGACGTCAGGGCCAGGCGGCACTCATCGCCCTGGCCACCTATTC
	CGAGCTGAAGAACATCATCCTCGTGGACGAAGACGTGGACATCTTCGACAG
	CGACGACATCCTGTGGGCAATGACCACCCGCATGCAGGGCGATGTGAGCAT
	CACCACGCTTCCGGGGATCCGCGGCCACCAGCTGGATCCGTCGCAGTCGC
	CGGACTACAGCACCTCGATCCGTGGAAACGGCATCTCCTGCAAGACTATCTT
	CGACTGCACGGTGCCGTGGGCGCTGAAGGCGCGGTTCGAACGGGCGCCGT
	TCATGGAGGTCGACCCCACACCGTGGGCGCCGGAGCTGTTCAGCGACAAGA
	*AG**TGA**cagctgggagccctgtc**ATG**ATCTGCCCGCGCTGCGCCGACGAGCAGATCG*
	AGGTCATGGCCACCAGCCCGGTGAAGGGCATCTGGACCGTCTACCAGTGCC
	AGCACTGCCTGTACACCTGGCGGGACACCGAACCGCTTCGTCGCACCTCGC
	GCGAGCACTATCCCGAAGCGTTCCGCATGACGCAGAAGGACATCGATGAAG

TABLE 2-continued

DNA sequences of genes inserted into the genome of *N. aromaticivorans*. Start and stop codons are shown in bold. Coding sequences are indicated by upper case and relevant ribosomal binding sites are shown in lower case. In the EcDec or NaDec decarboxylase gene clusters, sequences in underline denote the prenyltransferase coding region, those in underlined italics denote the PCA decarboxylase coding region and those in italics represent the coding sequence for a gene of unknown function.

Gene	Sequence
	*CGCCGCAGGTGCCCACGATTCCTCCGCTCCTG**TGA**gctgaccagacaggagtagtaccc* (SEQ ID NO: 29)

Bacterial Strains, Growth Media, and Culturing Conditions

[0094] *N. aromaticivorans* 12444A1879 (31) (called 12444 in Table 3) is a derivative of wild-type strain DSM 12444 in which a putative sacB gene (Saro_1879) was deleted to create a strain amenable to genomic modifications using a variant of the pK18mobsacB plasmid (59) that contains both kanamycin resistance and sacB. Plasmids for cloning were constructed with the NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs; Ipswich, MA), as described in the supplementary material SI. A complete list of the mutant strains and a list of the primers used to generate these mutant strains is shown in Table 3 and Table 4.

TABLE 3

N. aromaticivorans mutant strains used in this study.

Strain name	Genotype	Source
12444	12444Δ1879	Perez et al. 2019 (31)
12444_ΔligAB1/2	12444Δ1879ΔligABΔligAB2	Perez et al. 2021 (32)
12444_PCA	ΔligAB1/2 ΔSaro3873-3878	This study
LigAB1_EcDec	12444_PCA ΔligAB1:EcdB/EcaroY/EcdD	This study
LigAB1_NaDec	12444_PCA ΔligAB1:NadBCD	This study
EcDec_cat	LigAB1_EcDec ΔxylE ΔcatBC	This study
NaDec_cat	LigABI_NaDec ΔxylE ΔcatBC	This study
EcDec_ccMA	EcDec_cat ΔxylE: EccatA	This study
NaDec_ccMA	EcDec_cat ΔxylE: NacatA	This study

TABLE 4

Primers used for deletion and gene insertions in *N. aromaticivorans*. All Primers are shown in the 5' to 3' orientation.

Genomic Modification	PCR Reaction	Primers	Notes
Deletion of Saro_3877-8	Amplify upstream of Saro_3877	Saro_3877-8 Ampl F pk18 HiFi **CGATTCATTAATGCAGCTGGCACG ACAG**CGATCTGCTCGCTAAATTGTG GAAGGAG (SEQ ID NO: 30)	Bold and bold underline sequences are complementary to the end regions of linearized pK18mobsacB. Regions in italics or underlined italics are complementary to each other.
		Saro_3877-8 HiFi Del R *GCTGGAGAATCTTGGGATAACCGTTCC CGCCTGTCAG*TGTC (SEQ ID NO: 31)	
	Amplify downstream of Saro_3878	Saro_3877-8 HiFi Del F *CTGACAGGCGGGAACGGTTATCCCAA GATTCTCCAGC*AATAAGGCTC (SEQ ID NO: 32)	
		Saro_3877-8 Ampl R pk18 HiFi **GTTTCTGCGGACTGGCTTTCTAGA TGTTC**CGTAAAGGTTCCAGTAGCCT AGTCCG (SEQ ID NO: 33)	
Deletion of Saro_3873-8	Amplify upstream of Saro_3873	Saro3873-8 pk18 HiFi Ampl-F **CGATTCATTAATGCAGCTGGCACG ACAG**GATGTTACGAAGTTCACCTTC ACCGGTTC (SEQ ID NO: 34)	Bold and bold underline sequences are complementary to the end regions of linearized pK18mobsacB. Regions in italics
		Saro3873-8 HiFi Del R *CTGGAGAATCTTGGGATAACCAGGAGA CTTTCCTGCGCGTTTTGG* (SEQ ID NO: 35)	

TABLE 4-continued

Primers used for deletion and gene insertions in *N. aromaticivorans*. All Primers are shown in the 5' to 3' orientation.

Genomic Modification	PCR Reaction	Primers	Notes
	Amplify downstream of Saro_3878	Saro3873-8 HiFi Del F *CCCAAAACGCGCAGGAAAGTCTCCTG* *GTTATCCCAAGATTCTCCAGC*AATAAG GCTC (SEQ ID NO: 36) Saro_3877-8 Ampl R pk18 HiFi **GTTTCTGCGGACTGGCTTTCTAGA TGTTC**CGTAAAGGTTCCAGTAGCCT AGTCCG (SEQ ID NO: 37)	or underlined italics are complementary to each other.
Inserting Saro_3877-8 into the Saro_2812-3 genomic locus	Linearize pK18msB-ΔSaro2812/3	Saro_2812-3 Del R **CTGACCAGACAGGAGTAGTACCC ATG** (SEQ ID NO: 38) Saro_2812-3 Del F *CAtAGGCCCCTCTCCTTCAGCTTG* (SEQ ID NO: 39)	Regions in bold or italics are complementary to each other. Underlined regions are start or stop codons.
	Amplify Saro_3877-8	Saro3877-8-LigAB R CG**CATGGGTACTACTCCTGTCTGG TCAG**CTTATTGCTGGAGAATCTTGG GATAACGCTG (SEQ ID NO: 40) Saro3877-8-LigAB F *CCAAGCTGAAGGAGAGGGGCCTATGA* CCATGAACGATCTCCCTAACC (SEQ ID NO: 41)	The lowercase letter is non-complementary to its initial target sequence.
Inserting Saro_3873 and Saro_3877-8 into the Saro_2812-3 genomic locus	Linearize pK18msB-3877-8ΔligAB	Saro_2812-3 Del F2 **CACAGGCCCCTCTCCTTCAGCTTG** (SEQ ID NO: 42) Saro3877-8_HiFi 3873 F *GGCAAATGGAAGGGAATTGAGAATGA* CCATGAACGATCTCCCTAACC (SEQ ID NO: 43)	Regions in bold or italics are complementary to each other. Underlined regions are start or stop codons.
	Amplify Saro_3873	Saro3873 pVP HiFi Ampl F **CAAGCTGAAGGAGAGGGGCCTGT GA**AGCGCATGGTCGTGGGGATTAC (SEQ ID NO: 44) Saro3873 expr Ampl R *CATTCTCAATTCCCTTCCATTTGCCCA* GCACCCGGAAC (SEQ ID NO: 45)	
Deletion of Saro_3857 (xylE)	Upstream of xylE	Saro3857_pK18_Amp_F **GTTTCTGCGGACTGGCTTTCTACG TGTTCCGTTC**ATTACTTCACCCAGC AGGGC (SEQ ID NO: 46) Saro3857_pK18_Hifi_del_R *GGTCTCAAAGGCTGAAC*ggaaaggGCAA *GGCGATCTTCTACTACGAAAAGG* (SEQ ID NO: 47)	Bold and bold underlined sequences are complementary to the end regions of linearized pK18mobsacB.
	Downstream of xylE	Saro3857_pK18_Hifi_del_F *CCTTTTCGTAGTAGAAGATCGCCTTGC* *cctttccgtTCAGCCTTTGAGACC* (SEQ ID NO: 48) Saro3857_pK18_Amp_R **CGATTCATTAATGCAGCTGGCACG ACAG**CGAAGGTCTCATCTGATCGAA GAGCG (SEQ ID NO: 49)	Regions in italics or underlined italics are complementary to each other.
Deletion of Saro_3828-3829	Upstream Fragment	pK18_amp_delSaro3829_F **GTTTCTGCGGACTGGCTTTC**TACG TGTTCTCGCCGCAAGCTAGGACCGC (SEQ ID NO: 50) Del_Saro3828-3829_R *TCGTTGCTTGCCACATCGAAGATCGAC* *GGCCACAGGACTAAGCGTTGC* (SEQ ID NO: 51)	Bold and bold underlined sequences are complementary to the end regions of linearized pK18mobsacB.
	Downstream Fragment	Del_Saro3828-3829_F *GCAACGCTTAGTCCTGTGGCCGTCGAT* *CTTCGATGTGGCAAGCAACGA* (SEQ ID NO: 52) pK18_amp_delSaro3829_R **CGATTCATTAATGCAGCTGGCACG ACAG**CGTTATGTGGTGATTTCAGCG ATCGTCG (SEQ ID NO: 53)	Regions in italics or underlined italics are complementary to each other.

TABLE 4-continued

Primers used for deletion and gene insertions in *N. aromaticivorans*. All Primers are shown in the 5' to 3' orientation.

Genomic Modification	PCR Reaction	Primers	Notes
Insertion of catA into ΔxylE	Linearizing ΔxylE pK18 plasmid	XylE_catA_ATW_F **CGGACTACTCCCGTTATGTGGTGA** TCAGCCTTTGAGACCATTCCTAAAG AA (SEQ ID NO: 54) XylE_catA_ATW_R **CAAGGCGATCTTCTACTACGAAA**A GGCGCTGAACGACCGCTTCATGACG G (SEQ ID NO: 55)	Regions in bold and bold underline are complementary to each other. Italicized region is a ribosomal binding site from the native catA operon.
	Insertion of Saro3830 (NacatA) into ΔxylE	XylE_Saro3830_hifi_F **TTTCGTAGTAGAAGATCGCCTTG**C TCAGGCCTGGGCGCGGGTGCGTTCC (SEQ ID NO:56) XylE_Saro3830_hifi_R ***TCACCACATAACGGGAGTAGTCCG***AT GCCTGCCACCTTCGCCAGTTCCGAT (SEQ ID NO: 57)	
	Insertion of EcCatA into ΔxylE	XylE_EcCatA_hifi_F **TTTCGTAGTAGAAGATCGCCTTG**T CATTCCTTGACGCGGGCGCGCTTGC CG (SEQ ID NO: 58) XylE_EcCatA_hifi_R ***TCACCACATAACGGGAGTAGTCCG***AT GTCGAAGAACCCGTCGCAGCAGTCG (SEQ ID NO: 59)	

[0095] *E. coli* DH5α cells were used in all plasmid preparations and either *E. coli* S17 or *E. coli* WM6026 were used as a conjugal donor for mobilization of DNA into *N. aromaticivorans*. Procedures for conjugation and modifying the *N. aromaticivorans* genome via homologous recombination are found below. All *E. coli* strains were grown in Lysogeny Broth (LB) media containing 50 mg/L kanamycin or 0.3 mM diaminopimelic acid (DAP) when necessary. All *N. aromaticivorans* strains were grown in SMB minimal media (60) and supplemented with 10 mM glucose and an additional aromatic when specified. For genomic modifications of *N. aromaticivorans* the media was either supplemented with 50 mg/L of kanamycin or with 10% sucrose (100 g/L).

N. aromaticivorans Growth Experiments

[0096] Starter cultures of the *N. aromaticivorans* strains were grown aerobically (~18 h) in 5 mL of SMB media supplemented with 10 mM glucose, in 18×150 mm culture tubes at 30° C. The cells were then diluted by 1:1 and regrown to mid-exponential growth phase. The SMB vanillic acid and SMB PCA solutions were prepared fresh by dissolving either 34 mg of vanillic acid or 30 mg of PCA into 100 mL of SMB, which was then passed through a sterile 0.22 m filter. A 1:10 dilution was performed by adding 1.2 mL of starter culture into a 125 mL Erlenmeyer flask equipped with a side arm that contained 12 mL of SMB media with the specified carbon source. The cell density was measured at various time points using a Klett-Summerson photoelectric colorimeter with a red filter (31). Aliquots of culture samples (0.2 mL) were removed at indicated time points and filtered (31) prior to storage at 4° C. Liquid chromatography mass spectroscopy (LCMS) analysis was performed on the day of the last time point.

Protein Expression and Purification

[0097] Genes Saro_3877-78 and Saro_3830 were amplified via PCR from the *N. aromaticivorans* genome and independently cloned into the pVP302K plasmid, which contains an 8× His tag (32). EcaroY/D was PCR amplified from the ΔligAB1:EcDec_pK18mobsacB plasmid, and eccatA was PCR amplified out of a pUC57 plasmid synthesized by Genscript. Both genes were independently cloned into the pVP32K plasmid. The list of primers used to generate these protein expression plasmids and corresponding plasmids are shown in Table 5. Purified plasmid was then transformed into *E. coli* B834 containing the pRARE2 plasmid (32). Identical methods for heterologous protein expression and purification were performed and detailed below.

TABLE 5

Primers used for protein expression. The regions in bold are complementary to the linearized regions of the pVP302K plasmid. All primers are listed in the 5' to 3' orientation.

Primers	Sequence	Plasmid
EcdB_pVP_HiFi_F	**GTATTTTCAGAGCGCGATC GCAGGA**ATGAGGCTCATCG TGGGCATGACG (SEQ ID NO: 60)	EcdB_pVP302K

TABLE 5-continued

Primers used for protein expression. The regions in bold are complementary to the linearized regions of the pVP302K plasmid. All primers are listed in the 5' to 3' orientation.

Primers	Sequence	Plasmid
EcdB_pVP_HiFi_R	**CTAACTTTGTTATTTTCGG CTTTCTG**TCAGATGCCGTCG TTGTTCTCCTGG (SEQ ID NO: 61)	EcdB_pVP302K
EcAroY_EcdD_pVP_HiFi_F	**GTATTTTCAGAGCGCGATC GCAGGA**ATGCAGAACCCCA TCAACGACCTC (SEQ ID NO: 62)	EcAroY_D_pVP302K
EcAroY_EcdD_pVP_HiFi_R	**CTAACTTTGTTATTTTCGG CTTTCTG**GCTCACAGGAGC GGAGGAATCGTG (SEQ ID NO: 63)	EcAroY_D_pVP302K
Saro3830_pVP_HIFI_F2	**GTATTTTCAGAGCGCGATC GCA**ATGCCTGCCACCTTCGC CAG (SEQ ID NO: 64)	NaCatA_pVP302K
Saro3830_pVP_HIFI_R2	**CTAACTTTGTTATTTTCGG CTTTCTG**TCAGGCCTGGGCG CGGGTG (SEQ ID NO: 65)	NaCatA_pVP302K
Saro_3877_8_pVP_F	**GTATTTTCAGAGCGCGATC GCA**ATGACCATGAACGATCT CCCTAACC (SEQ ID NO: 66)	NadCD_pVP302K
Saro_3877_8_pVP_R	**CTAACTTTGTTATTTTCGG CTTTCTG**TTATTGCTGGAGA ATCTTGGGATAAC (SEQ ID NO: 67)	NadCD_pVP302K
EcCatA_PVP_HIFI_F	**GTATTTTCAGAGCGCGATC GCAGGA**ATGTCGAAGAACC CGTCGCAGCA (SEQ ID NO: 68)	EcCatA_pVP302K
EcCatA_pVP_HIFI_R	**CTAACTTTGTTATTTTCGG CTTTCTG**TTCCTTGACGCGG GCGCGCTTG (SEQ ID NO: 69)	EcCatA_pVP302K

[0098] For protein expression, a single colony was used to inoculate a 20 mL starter culture of cells grown in LB media containing kanamycin (50 mg/L) and chloramphenicol (20 mg/L) in a 125 mL Erlenmeyer flask that was shaken at 200 rpm overnight (~18 h) at 37° C. Next, the entire 20 mL starter culture was used to inoculate a 2 L Erlenmeyer flask containing 500 mL of Terrific Broth (TB) (61) media containing kanamycin (50 mg/L) and chloramphenicol (20 mg/L). The 500-mL culture was shaken at 200 rpm at 37° C. for 4 to 5 h until reaching an optical density (GD) OD_{600} of ~0.7. Once the cells reached an OD_{600} of 0.7, protein expression was induced with the addition of Isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.3 mM final concentration). For expression of the 1,2-catechol dioxygenase proteins, the media also included $Fe(II)SO_4$ at a final concentration of 0.160 mM at the time of induction. Induction was allowed to proceed overnight (~18 h) at 20° C. for both the PCA decarboxylase and the 1,2 catechol dioxygenase cultures. After induction, cells were harvested by centrifugation and suspended in the resuspension buffer which contains 50 mM HEPES (2-[14-(2-hydroxyethyl)piperazin-1-yl]ethane-1-sulfonic acid), 150 mM NaCl and 0.1% Triton X-100 at pH 7.5. The cells were then lysed by sonication and clarified by centrifugation at 20,000 rpm for 30 min. The soluble fraction was applied directly to a Ni-NTA column and washed with 50 mM HEPES, 150 mM NaCl and 30 mM Imidazole at pH 7.5. The proteins were eluted by applying a high imidazole elution buffer (50 mM HEPES, 150 mM NaCl and 300 mM Imidazole at pH 7.5). Fractions were collected and protein purity was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (FIGS. **6**A, **6**B, **11**A, and **11**B). The protein concentration was determined by the Bradford protein assay measuring the absorbance at 595 nm (Fisher Scientific).

Generation of a prFMN-Containing Crude Cell Lysate

[0099] A source of prFMN was generated as previously described (62) with slight modification. The gene for the EcdB prenyl transferase was PCR amplified from the dLigAB1:EcDec pK18 plasmid, and cloned into pVP302K. The resulting expression vector was then transformed into B834 *E. coli* containing the pRARE2 plasmid. A single colony of the EcdB BP86 *E. coli* was used to inoculate 10 mL of LB media containing kanamycin (50 mg/L) and chloramphenicol (20 mg/L). The culture was incubated for ~18 h at 37°

C. with shaking (200 rpm). Next, the 10-mL culture was used to inoculate 1 L of TB kanamycin and chloramphenicol media and incubated with shaking for 5 to 6 h until an OD of 0.7 was reached. The culture was then transferred to 1 L screw top bottle with a magnetic stir bar for anaerobic growth and was amended to include a final concentration of 1% dimethylsulfoxide (DMSO), 1 mM prenol, 0.1 mM riboflavin and 0.4 mM IPTG as previously described (62). After incubation overnight (~18 h) the cells were then lysed via sonication and clarified by centrifugation. The resulting crude cell lysate (prFMN lysate) was used as a source of prFMN for the PCA decarboxylase activity assays.

PCA Decarboxylase In Vitro Activity Assays

[0100] All PCA decarboxylase activity assays were performed in triplicate in the reaction buffer (50 mM HEPES, 150 mM NaCl pH 7.5) using purified enzymes. A stock solution of 25 mM PCA was generated by dissolving 39 mg into 10 mL of reaction buffer. To test the dependence of NadCD activity on prFMN, reactions were initiated by adding 0.1 μM (final concentration) NadCD to a 2 mL (final volume) reaction mixture that contained 1 mM PCA. For reactions that included the prFMN crude cell lysate, the assay mixture also contained 1 mL of prFMN lysate (50% lysate) and 1 mM PCA at a final volume of 2 mL. The enzyme assay was quenched by the addition of 40 μL of 1M HCl to a 0.2 mL aliquot of the reaction mixture at T=0 and T=18 h. For temporal analysis of PCA decarboxylase activity by NadCD or EcAroY, those reaction were initiated by the addition of enzyme to final concentration of 0.1 μM and added to the reaction mixture that included the prFMN crude cell lysate detailed above. Aliquots of 0.2 mL were removed and the reaction terminated as above at various time points. A control reaction of the prFMN lysate reaction mixture without the addition of enzyme was also performed. All reaction products were filtered through 0.22 μm nylon syringe tip filter (Fisher Scientific) and analyzed by LCMS to test for PCA to catechol conversion.

1,2 Catechol Dioxygenase In Vitro Activity Assays

[0101] Catechol dioxygenase activity was tested in triplicate with either NaCatA or EcCatA in a reaction buffer of 50 mM HEPES, 150 mM NaCl pH 7.5. A stock solution of 0.1 M catechol was freshly prepared by dissolving 22 mg into 2 mL of the reaction buffer. A series of dilutions were performed on the stock solution to obtain a 1 mM catechol working solution. The reactions were performed in a 96-well plate in a total volume of 200 μL with orbital shaking at 28° C. using a Tecan infinite M1000 Pro to ensure O_2 dissolution. The reaction mixture contained 0.5 μM of purified enzyme in reaction buffer and the assay was initiated by the addition of catechol. The formation of ccMA was monitored at 260 nm (50) and the resultant data were best fit to a linear equation ($[ccMA]=kt+[ccMA]_0$) to yield zeroth order rate constants (k) for each enzyme.

N. aromaticivorans Extracellular Metabolite Analysis by HPLC-MS Extracellular metabolite analysis was carried out on a Shimadzu triple-quadrupole liquid chromatography-mass spectrometer (LC-MS, Nextera XR HPLC-8045 MS/MS). The mobile phase used a binary gradient with solvent A (0.2% formic acid in water) and solvent B (acetonitrile) using the protocols listed as Method 1 in Table 6 and Method 2 in Table 7.

TABLE 6

Liquid chromatography (LC) program for elution of phenolics using Method 1.

Time (min)	% B conc
0.05	0
1.9	0
3	0
3.25	2
3.5	4
3.75	6
4	8
4.25	10
4.5	12
4.75	14
5	16
6	20
7.5	50
8	95
9	95
9.25	5
10	0
11	End of Run

TABLE 7

LC program for elution of phenolics in Method 2.

Time (min)	% B conc
0.05	5
3.5	8
4	11
5.5	12
6.5	14
7	30
7.5	50
8	75
8.5	95
9.25	95
9.5	5
10	5
10.5	1
11	End of run

The stationary phase used was a Kinetex C18 column (Kinetex 2.6 μm pore size, 100 Å 150 length×2.1 mm ID, P/N: 00F-4462-AN). Quantification of the metabolites was performed by preparing standard solutions of compounds (Sigma-Aldrich). A series of dilutions were performed to obtain a set of 5 concentrations for each compound that was within the range of the predicted amount of analyte (FIGS. **2** and **3**). The compounds were analyzed by both UV/vis absorption and multiple-reaction-monitoring (MRM) (Tables 8 and 9). Each of the compounds were quantified using the maximum absorbance wavelength in the UV/visible absorption spectrum and a standard curve was applied using the area under the curve at that maximum absorbance (Tables 8 and 9).

TABLE 8

Multiple Reaction Monitoring (MRM) transitions and maximum UV/vis absorption data used to quantify phenolics in Method 1 (see Materials and Methods).

Compound	Retention Time	Maximum UV/vis absorption	MRM m/z
Vanillic acid	6.692	263 nm	167.00 > 152.10
PCA	4.473	263 nm	153.00 > 109.10
Catechol	4.701	275 nm	109.20 > 91.10

TABLE 8-continued

Multiple Reaction Monitoring (MRM) transitions and maximum UV/vis absorption data used to quantify phenolics in Method 1 (see Materials and Methods).

Compound	Retention Time	Maximum UV/vis absorption	MRM m/z
ccMA 1	5.570	263 nm	141.15 > 97.10
ccMA 2	6.217	263 nm	141.15 > 97.10

TABLE 9

Multiple Reaction Monitoring (MRM) transitions and maximum UV/vis absorption data used to quantify extracellular aromatics in Qsub poplar APL Method 2.

Compound	Retention Time	Maximum UV/vis absorption	MRM m/z
4-CA	7.186	309 nm	163.00 > 119.10
4-HBA	3.732	254 nm	136.90 > 93.10
Catechol	3.049	275 nm	109.20 > 91.10
ccMA 1	2.400	263 nm	141.15 > 97.10
ccMA 2	3.367	263 nm	141.15 > 97.10
Ferulic Acid	8.283	322 nm	193.00 > 134.10
PCA	2.277	263 nm	153.00 > 109.10
Vanillic acid	5.473	263 nm	167.00 > 152.10
Vanillin	6.647	309 nm	151.00 > 136.00

The percent yields for ccMA were obtained by using the equation below, and the initial aromatic concentrations refers to the aromatic carbon that was used in the growth experiments.

$$\text{percent yield} = \frac{[ccMA]_{final}}{[\text{aromatic}]_{initial}} \times 100$$

Alkaline Pre-Treatment Liquor Preparation

[0102] The line 15.1 of QsuB poplar was obtained and treated to create APL as previously described (51, 52). The total phenolics in APL were calculated as the sum of the free phenolics and the glycosylated phenolics released after acid treatment of the APL (52). Growth experiments in the presence of APL were performed after adjusting to pH to 7.0 with hydrochloric acid and supplementing with glucose (1 g/L) and ammonium sulfate (1 g/L) (52). The percent yield of ccMA production was calculated as indicated above.

Construction of Plasmids for Generating Deletions of Saro_3877-8 and Saro_3873-8

[0103] Regions of *N. aromaticivorans* genomic DNA containing ~1000 base pairs (bp) upstream and downstream of the genes to be deleted were amplified via PCR using the primers listed in Table 4. Plasmid pK18mobsacB was linearized via PCR as previously described (60). The upstream and downstream flanking regions for each gene were combined with linearized pK18mobsacB using the NEBuilder HiFi Assembly system (New England Biolabs, Ipswich, MA) to produce a plasmid in which the upstream and downstream DNA sequences are adjacent, with no intervening coding region. For the strain in which Saro_3873-8 was deleted, the deleted region begins two bp before the start codon of Saro_3873. For the strain in which Saro_3877-8 was deleted, the deleted region begins one bp before the start codon of Saro_3877. In both strains, the deletion extends to the same point near the end of Saro_3878. The final 26 bp of Saro_3878 remain in the *N. aromaticivorans* genome.

Construction of a Plasmid to Insert Saro_3877-8 into the Saro_2812-3 Locus of the *N. aromaticivorans* Genome

[0104] Plasmid pK18mobsacB/ΔSaro2812/3 (32) which contains ~1,000 bp genomic regions upstream and downstream of Saro_2812-3, was linearized via PCR using primers shown in Table 4. Concurrently, Saro_3877-8 was amplified from *N. aromaticivorans* genomic DNA via PCR using primers containing upstream regions complementary to linearized pK18mobsacB/ΔSaro2812/3 (Table 4). The linearized pK18mobsacB/ΔSaro2812/3 and the Saro_3877-8 fragment were combined using the NEBuilder HiFi Assembly system (New England Biolabs, Ipswich, MA) to produce plasmid pK18mobsacB-3877-8ΔligAB, in which Saro_3877-8 was placed between the DNA regions that naturally flank Saro_2812-3. The Saro_3877 start codon is in the natural position for the Saro_2813 start codon, and the Saro_3878 stop codon is in the natural position of the Saro_2812 stop codon, with an additional C following the Saro_3878 stop codon that does not naturally follow the Saro_2812 stop codon. The Saro_2814 stop codon (UGA), which naturally overlaps with the Saro_2813 start codon, remains intact and now overlaps with the Saro_3877 start codon.

Construction of a Plasmid to Insert Saro_3873 and Saro_3877-8 into the Saro_2812-3 Locus of the *N. aromaticivorans* Genome

[0105] Plasmid pK18mobsacB-3877-8ΔligAB was linearized via PCR using primers in Table 4. Concurrently, Saro_3873 was amplified from *N. aromaticivorans* genomic DNA via PCR using primers containing upstream regions complementary to linearized pK18mobsacB-3877-8ΔligAB (Table 4). The linearized pK18mobsacB-3877-8ΔligAB and the Saro_3873 fragments were combined using the NEBuilder HiFi Assembly system (New England Biolabs, Ipswich, MA) to produce plasmid pK18mobsacB-3873/7-8ΔligAB, in which Saro_3873 and Saro_3877-8 formed an artificial operon between the DNA regions that naturally flank Saro_2812-3. The Saro_3873 start codon is in the natural position for the Saro_2813 start codon. The 2 bp intergenic region normally between Saro_3873 and Saro_3874 follows the Saro_3873 stop codon, and is now followed by the Saro_3877 start codon.

Plasmids for Inserting Genes Encoding the EcdB, EcAroY, and EcdD Proteins from *Enterobacter cloacae* into the Saro_2812-3 Locus of the *N. aromaticivorans* Genome

[0106] DNA fragments containing the genes coding for EcdB (NCBI Accession: ADF63617), EcAroY (NCBI Accession: ADF61496), and EcdD (NCBI Accession: ADF63615) from *Enterobacter cloacae* were ordered as gBlocks from Integrated DNA Technologies (Coralville, IA). Genes were constructed to have codon usage frequencies similar to those of other genes in *N. aromaticivorans* (calculated from several genes in the genome), but without making the GC content of the fragments too high for the gBlock synthesis process. The DNA sequence of the operon was split between two gBlocks (EcdB-aroY-D-NaligAB_up and EcdB-aroY-D-NaligAB_down); each gBlock contained a sequence at one end that matches one of the ends of linearized pK18mobsacB-ΔSaro2812/3; the other ends of

the gBlocks match each other. The two gBlocks were combined with linearized pK18mobsacB-ΔSaro2812/3 using the NEBuilder HiFi Assembly system. The ecdB start codon is in the natural position for the Saro_2813 start codon, and the ecdD stop codon is in the natural position of the Saro_2812 stop codon.

Construction of Plasmids ΔxylE_pK18mobsacB and ΔcatBC_pK8mobsacB

[0107] Primers for deletion of catBC and xylE (Table 4) were designed to amplify two ~1000 bp regions both downstream and upstream of the desired gene deletion in the genome of *N. aromaticivorans*. For each plasmid, these two amplified regions were combined with linearized pK18mobsacB-MCS1 as described above using NEBuilder® Hifi DNA assembly Master mix (New England Biolabs). For ΔcatBC, the deletion begins 1 bp upstream of the start codon of saro_3828 with the final 133 bp of saro_3829 remaining in the genome. The deleted region of ΔxylE begins 9 bps upstream of the start codon of saro_3857 (xylE) with the final 62 bp of saro_3857 remaining in the genome.

Construction of Plasmids ΔxylE eccatA and ΔxylE nacatA pK18mobsacB

[0108] For insertion of catA into the xylE locus of *N. aromaticivorans*, the dxylE_pK18mobsacB plasmid was first linearized using XylE_catA_ATW_F and XylE_catA_ATW_R. The XylE_CatA_ATW_F primer contained a ~20 bp region that is located upstream of the native nacatA consistent with the ribosomonal binding site (RBS) for nacatA. Primers listed in Table 4 were used to amplify either eccatA or nacatA was out of the pVP302K vector with overhangs corresponding to the linearized ΔxylE_pK18mobsacB. Next, either fragment eccatA or nacatA was combined with the linearized ΔxylE_pK18mobsacB using Hifi DNA assembly reaction.

Conjugation Procedure

[0109] Bacterial conjugations using either *E. coli* S17 or WM6026 donor cells with *N. aromaticivorans* recipients were performed essentially as previously described (26, 31) with slight modifications. Cultures of *E. coli* WM6026 cells were grown in LB kan and 0.3 mM DAP while *N. aromaticivorans* strains were grown in 10 mM glucose SMB media. Both strains were grown overnight at 30° C. in 5 mL of media in an 18×150 mm culture tube. The overnight cultures were then diluted 1:1 and grown to mid-log phase. The cell density of the donor and recipient cultures was then measured and the cultures were diluted such that the cell density of both the recipient and the donor were equal. Next, 2 mL of the donor and 1 mL of the recipient culture was subsequently washed and pelleted prior to mixing in a 2:1 donor to recipient cell ratio. The mixed cells were then suspended into 0.1 mL of LB DAP and incubated at 30° C. for 4 hr. After incubation, the cells were then pelleted by centrifugation and resuspended into 0.5 mL SMB supplemented with 10 mM glucose before incubating and shaking for an additional hour at 30° C. The cells were plated onto SMB Kan and transconjugant colonies formed within 3 to 4 days. Double crossover variants were selected as previously described (31) from SMB 10% sucrose plates. PCR amplified regions of the targeted genes were sequenced to confirm the mutation.

Results

PCA Catabolism in *N. aromaticivorans*

[0110] The genome sequence of *N. aromaticivorans* predicts that it encodes enzymes which can convert both H- and G-biomass aromatics into PCA. Therefore, in order to test if we could engineer a strain that produces ccMA from H and G lignin aromatics, we first sought to develop further understanding of PCA catabolism in *N. aromaticivorans*. It was previously shown that the PCA 4,5 extradiol cleavage pathway is the major pathway in *N. aromaticivorans* when these cultures are supplied only an aromatic substrate (32). Additionally, it was shown that there are two 4,5 PCA dioxygenase homologues (LigAB1 and LigAB2) of the PCA extradiol cleavage pathway that can convert PCA to 4-carboxy-2-hydroxy-cis,cis-muconate-6-semialdehyde (CHMS) (FIG. **1**) (32). Deletions of these genes predicted to encode enzymes in the PCA 4,5 extradiol cleavage pathway (FIG. **1**) resulted in a *N. aromaticivorans* strain that accumulates extracellular PDC from H and G aromatics with yields greater than 70% when supplied an aromatic and glucose. These results agree with published data and demonstrate that this is a major pathway for the catabolism of H and G aromatics (31, 32). However, previous work also found less than stoichiometric yields of PDC when cells were grown with PCA as a carbon source (31). These results suggest that LigAB1 and LigAB2 play a significant role in PCA metabolism, but that the existence of another PCA-consuming pathway could be the cause of the decreased yield of PDC when this strain is grown with PCA. Based on these results, we hypothesized that blocking the *N. aromaticivorans* PCA extradiol cleavage pathway in the parent strain (12444, Table 3) would result in a strain that metabolizes PCA but at a rate slower than observed in cells that contain LigAB1 and LigAB2.

[0111] To test this hypothesis, we determined the effect of deletions in ligAB1 and ligAB2 on the ability to metabolize PCA in the presence of glucose as an auxiliary carbon source. This strain 12444_ΔligABΔligAB2 (32) (hereafter called 12444_ΔligAB1/2) and the parent strain 12444 (Table 3) were grown in media containing 2 mM vanillic acid, a G aromatic, and 10 mM glucose. Both strains grew to similar cell densities and fully eliminated the vanillic acid from the medium. As predicted, there was no extracellular accumulation of PCA in the 12444 parent strain (FIG. **4**C, FIG. **5**). However, the 12444_ΔligAB1/2 mutant transiently accumulated detectable levels of PCA in the media (FIG. **4**C, FIG. **5**B). The observation of the transient formation of PCA when the 12444_ΔligAB1/2 strain is grown with vanillic acid is consistent with the hypothesis that another route for metabolism of this predicted pathway intermediate is present (31, 32).

[0112] To test how PCA might be metabolized in the 12444_ΔligAB1/2 strain, we analyzed the genome of *N. aromaticivorans* for genes that encode homologues of enzymes known to catalyze PCA ring opening reactions in other organisms. This analysis failed to identify genes that encode proteins with >25% amino acid sequence identity to the *Pseudomonas putida* 3,4 dioxygenase (PcaHG) which catalyzes intradiol cleavage of PCA to form 3-carboxy-cis, cis-muconate or to a 2,3 PCA dioxygenase (PraA) that produces 5-carboxy-2-hydroxymuconate-6-semialdehyde (35-37). However, this analysis showed that *N. aromaticivorans* contained genes (Saro_3873, Saro_3877, Saro_3878; hereafter referred to as nadB, nadC, and nadD, respectively)

that encoded proteins with at least some amino acid sequence identity to the known B, C and D gene products involved in PCA decarboxylation by *Klebsiella pneumoniae, Enterobacter cloacae* and other bacteria (Table 10) (5, 6, 38-40).

TABLE 10

Amino acid sequence identity of *N. aromaticivorans* gene products with amino acid similarity to known PCA decarboxylases. Genes from either *Klebsiella pneumoniae* or *Enterobacter cloacae* have been used previously in bacterial engineering for ccMA production.

Amino Acid Sequence Identity	Saro_3873 (NadB)	Saro_3877 (NadC)	Saro_3878 (NadD)
Enterobacter cloacae (EcdB)	47%		
Klebsiella pneumoniae (KpdB)	44%		
Enterobacter cloacae (EcdC)		45%	
Klebsiella pneumoniae (KpdC)		46%	
Enterobacter cloacae (EcAroY)		31%	
Klebsiella pneumoniae (KpAroY)		32%	
Enterobacter cloacae (EcdD)			25%
Klebsiella pneumoniae (KpdD)			27%

[0113] We propose that a previously uncharacterized *N. aromaticivorans* PCA decarboxylase was responsible for metabolizing the PCA that transiently accumulated in the media of the 12444_ΔligAB1/2 strain. We performed a set of in vitro and in vivo experiments to test this proposal.

Characterization of *N. aromaticivorans* PCA Decarboxylase Enzyme

[0114] The amino acid sequence of the predicted *N. aromaticivorans* PCA decarboxylase enzyme (hereafter called NadBCD) predicts that it is most similar to a family of hydroxyarylic acid decarboxylases (38) that typically require 3 proteins, BCD, for activity. The B gene product encodes a predicted prenyltransferase that produces a prenylated flavin mononucleotide (prFMN) cofactor, while C catalyzes decarboxylation and D encodes a protein of unknown function (41, 42). Analysis of the most extensively studied decarboxylases in this family (*Escherichia coli* UbiD and *Enterobacter cloacae* EcAroY) have shown that the prFMN cofactor is required for decarboxylase activity (39, 43). Therefore, we tested if the *N. aromaticivorans* NadCD had PCA decarboxylase activity and whether it required a prFMN cofactor for catalysis (39).

[0115] To test this hypothesis, Saro_3877 (nadC) and Saro_3878 (nadD) were amplified from the genome of *N. aromaticivorans*, cloned into expression vectors, and purified recombinant enzymes (FIG. **6**A) were tested for PCA decarboxylase activity in vitro. Previously, researchers demonstrated that a source of prFMN can be produced by overexpression of the *E. coli* prenyltransferase when this bacterium is grown under anaerobic conditions in the presence of riboflavin (FMN precursor) and prenol (as a prenyl source) (44). In this study, we used a similar procedure, based on heterologous expression of the *E. cloacae* EcdB prenyltransferase in *E. coli*, to obtain crude cell extracts that contained prFMN. We then tested purified recombinant NadCD for PCA decarboxylase activity in the presence and absence of the crude cell extracts containing the predicted prFMN cofactor. As expected, we failed to observe detectable loss of PCA or production of catechol when purified NadCD was supplied 1 mM PCA with no source of prFMN (FIG. **7**). However, when the same purified NadCD was mixed with PCA and with the crude *E. coli* lysate that contained prFMN, the PCA was converted into catechol (FIG. **7**). We propose that the lack of stoichiometric conversion of PCA to catechol in these longer incubation assays reflects catechol degradation via its well-known abiotic oxidation reaction (21). A control reaction in which PCA was mixed with the prFMN-containing *E. coli* lysate and no purified recombinant NadCD failed to detect any loss of PCA or production of catechol over the same incubation period (FIG. **7**). From these results, we conclude that NadCD has PCA decarboxylase activity and that, like most previously studied hydroxyarylic acid decarboxylases, requires prFMN for catalysis.

[0116] We also sought to compare the activity of NadCD to EcAroY, which is an extensively studied PCA decarboxylase from *E. cloacae* (39, 45). Temporal analysis of PCA decarboxylation by recombinant NadCD and EcAroY indicated that, under identical reaction conditions, EcAroY produced stoichiometric catechol from PCA within 15 min whereas 1 hour was needed for NadCD to produce stoichiometric levels of catechol (FIG. **8**A). These results suggest that the recombinant *N. aromaticivorans* protein performs PCA decarboxylation slower than EcAroY in vitro. Overall, these results confirm the predicted function of *N. aromaticivorans* NadCD as a PCA decarboxylase that converts PCA to catechol. It also predicts that the NadCD decarboxylase activity was responsible for the observed PCA metabolism in the 12444_ΔligAB1/2 strain (FIG. **4**C) and that a derivative of this mutant which also lacks the nadBCD genes should exhibit a defect in PCA metabolism.

Loss of Both nadBCD and ligAB1/2 is Sufficient to Accumulate Extracellular PCA

[0117] To test this hypothesis, we generated a strain which lacks both ligAB1/2 and the Saro_3873-8 gene cluster, which contains nadBCD (12444_PCA; Table 3). We grew the parent strain 12444, the 12444_ΔligAB1/2 and 12444_PCA mutants in media containing 2 mM vanillic acid and 10 mM glucose as an additional carbon source. We found all strains fully consumed vanillic acid but that strain 12444_PCA reproducibly grew to a lower cell density than 12444 strain or the 12444_ΔligAB1/2 mutant (FIG. **4**A). We hypothesized that the lower cell density was due to failure of 12444_PCA to assimilate the products of vanillic acid metabolism. Analysis of the extracellular metabolites of 12444_PCA revealed that the vanillic acid was consumed at the end of the incubation and that the media contained 1.4±0.1 mM of PCA, representing an almost complete (78±5%) recovery of extracellular PCA from vanillic acid.

We propose that the less than stoichiometric recovery of PCA in the media at the end of the incubation is due to the previously reported abiotic oxidation of PCA (46-48). From these results we concluded that NadBCD contributes to PCA metabolism of 12444 *N. aromaticivorans*. We further propose that the combined deletion of the nadBCD and ligAB1/2 genes generates a *N. aromaticivorans* strain (12444_PCA) that excretes PCA because it has a defect in assimilation of this intermediate in aromatic metabolism.

Converting PCA to Catechol

[0118] Extracellular accumulation of PCA by strain 12444_PCA predicts that there are no other major pathways for PCA metabolism in *N. aromaticivorans*. This finding enabled us to use 12444_PCA as a platform strain to test if we could divert the PCA derived from H- and G-family aromatics towards ccMA production. Since bacterial production of ccMA often proceeds through the intradiol aromatic ring cleavage of catechol (FIG. **1**) (5, 6, 23), we sought to divert PCA towards this ring-opening pathway. However, conversion of PCA to catechol has been observed as a metabolic bottleneck in other engineered bacterial strains (5, 6). Since the activity of the *N. aromaticivorans* NadCD enzyme was lower than that of the well-studied PCA decarboxylase EcAroY, we generated two strains which contained either nadBCD (LigAB1_NaDec) or ecdB/ecaroY/ecdD (LigAB1_EcDec) in the ligAB1 (Saro_2812-13) locus of 12444_PCA. We chose to place genes encoding either of the PCA decarboxylase genes in the ligAB1 locus as this locus is highly transcribed when *N. aromaticivorans* is grown in the presence of G-family aromatic compounds (33). Based on the activity of these two decarboxylases in vitro, we predicted that insertion of these PCA decarboxylase genes into the ligAB1 locus of the 12444_PCA strain should allow conversion of PCA to catechol in vivo and reduce or block extracellular PCA accumulation.

[0119] To test this hypothesis, we grew strains LigAB1_EcDec and LigAB1_NaDec in media containing 2 mM vanillic acid and 10 mM glucose as an auxiliary carbon source. We found that both the LigAB1_EcDec and LigAB1_NaDec strains reached similar final cell densities and completely consumed vanillic acid within 12 h (FIGS. **9**A and **9**B). We observed transient accumulation of extracellular PCA in each strain, reaching a maximum of 1.4±0.1 mM in the LigAB1_NaDec at 10 h and 0.8±0.2 mM at 6 h for LigAB1_EcDec (FIG. **9**C). However, extracellular PCA was undetectable in both cultures after 12 h (FIGS. **10**A and **10**B). These results indicate that placement of either the Ec or Na PCA decarboxylase genes at the ligAB1 locus of the 12444_PCA strain restored the strain's ability to assimilate this pathway intermediate. In comparison to the 12444_ΔligAB1/2 strain, which has the genes for nadBCD in its native locus, both of the above strains consume PCA faster with full consumption of this pathway intermediate within 12 h compared to 24 h. This is consistent with the observation of higher transcript levels from the ligAB1 locus versus the nadBCD locus when cells are grown in the presence of G-family aromatics (33).

N. aromaticivorans Genes Predicted to be Involved in Catechol Metabolism

[0120] While both LigAB1_NaDec and LigAB1_EcDec were able to metabolize PCA, neither of these strains accumulated detectable levels of extracellular catechol or ccMA (FIGS. **10**A and **10**B). To explain this observation, we propose that these strains contain one or more previously undescribed pathways for catechol assimilation into cellular material. Therefore, we sought to identify potential enzymes involved in catechol metabolism in *N. aromaticivorans*.

[0121] Bacterial catechol catabolism can be initiated via extradiol cleavage by a 2,3 dioxygenase, XylE, producing 2-hydroxymuconate semialdehyde or through intradiol cleavage by a 1,2-catechol dioxygenase, CatA, to generate ccMA (FIG. **1**). To predict the potential *N. aromaticivorans* pathways for catechol metabolism, we analyzed its genome for homologs of genes that encode enzymes that function in the extradiol and intradiol pathways. This analysis predicted the presence of catBCA and xylEGHIJKQ (26) transcription units that encode *N. aromaticivorans* proteins with >40% amino acid identity to known enzymes in the intradiol and extradiol cleavage pathways, respectively (Tables 1 and 11), suggesting that *N. aromaticivorans* can potentially metabolize catechol by both pathways.

TABLE 11

The amino acid sequence identity of the CatBCA from *Pseudomonas putida* or *Enterobacter cloacae* compared to homologues encoded by *Novosphingobium aromaticivorans*.

Amino Acid Sequence Identity	Saro_3828 (NaCatB)	Saro_3829 (NaCatC)	Saro_3830 (NaCatA)
Enterobacter cloacae (EcCatB)	63%		
Pseudomonas putida (PpCatB)	62%		
Enterobacter cloacae (EcCatC)		69%	
Pseudomonas putida (PpCatC)		60%	
Enterobacter cloacae (EcCatA)			43%
Pseudomonas putida (PpCatA)			49%

[0122] Of these two potential catechol cleavage pathways, only CatA is predicted to generate ccMA. Unlike some other aromatic metabolizing bacteria (49), the *N. aromaticivorans* genome is not predicted to contain a second copy of a gene that encodes a protein with amino acid sequence identity to known CatA enzymes (Table 11). Thus, we sought to test the activity of the predicted, but previously uncharacterized *N. aromaticivorans* CatA enzyme (NaCatA), and to compare it to the CatA of *E. cloacae* (EcCatA). To do this, the catechol 1,2 dioxygenase activity of purified recombinant NaCatA and EcCatA proteins (FIGS. **11**A and **11**B) was monitored by following the absorbance at 260 nm corresponding to ccMA by UV/visible absorption spectroscopy (50). Under identical assay conditions, both EcCatA and NaCatA produced stoichiometric (FIG. **12**) amounts of ccMA and the data best fit to a linear equation yielding zeroth order rate constants on

the same order of magnitude of each other (FIGS. **13**A and **13**B). From this we conclude that NaCatA catalyzes the intradiol cleavage of catechol to produce ccMA in vitro and exhibits a similar rate of activity as EcCatA under these assay conditions.

Engineering a *N. aromaticivorans* Strain to Divert Catechol to ccMA

[0123] Based on the genomic, bioinformatic and in vitro analysis of *N. aromaticivorans* enzymes that are predicted to be involved in catechol metabolism, we reasoned that several genetic modifications could be employed to engineer a strain that accumulated extracellular ccMA from pathway intermediates like PCA. First, the existence of a catBCA operon for intradiol cleavage of catechol predicted deletion of catBC genes would block metabolism of the ccMA generated by CatA activity. To do this, we generated ΔcatBC derivatives of strains LigAB1_NaDec and LigAB1_EcDec, which produced the strains, NaDec_cat and EcDec_cat respectively (Table 3). We also reasoned that inactivation of the xylE-dependent pathway for extradiol cleavage of catechol would divert catechol through the intradiol CatA-dependent pathway. Therefore, we replaced the native *N. aromaticivorans* xylE with either eccatA or the nacatA producing EcDec_ccMA or NaDec_ccMA, respectively (Table 3). We inserted catA into the native xylE locus since the transcript abundance of the xylE gene in *N. aromaticivorans* is higher than catA transcript levels when wild-type cells are grown in the presence of aromatics (FIG. **14**) (33). We reasoned that placement of eccatA or nacatA at the xylE locus should help ensure that these engineered strains were expressing sufficient levels of CatA to completely metabolize catechol to ccMA. We chose to generate strains expressing either EcCatA or NaCatA from the same locus in order to test whether the two versions of CatA led to production of significantly different levels of extracellular ccMA.

[0124] To test the impact of these genomic alterations on aromatic metabolism, we evaluated the ability of EcDec_ccMA and NaDec_ccMA to convert PCA into ccMA by growing these strains in media containing 2 mM PCA and 10 mM glucose as an auxiliary carbon source. Both strains exhibited transient accumulation of catechol (FIG. **15**C) and completely converted PCA to ccMA within 30 h with ccMA yields of 100±5% for NaDec_ccMA or 97±3% for EcDec_ccMA (FIGS. **15**B, **15**D, **16**A and **16**B). These results illustrate that these alterations to the genome of *N. aromaticivorans* allowed for funneling of PCA to the catechol intradiol branch and subsequent ccMA production. This finding provides new evidence in support that *N. aromaticivorans* can be used as a chassis for the metabolic conversion of G and H aromatics into ccMA.

Synthesis of ccMA from Aromatics in Poplar APL

[0125] To further evaluate the use of *N. aromaticivorans* as a chassis for ccMA production, we tested the ability of the NaDec_ccMA and EcDec_ccMA strains to produce this compound from biomass-derived aromatics. It is known that transgenic plants expressing the quinate and shikimate utilization B (qsuB) gene increase the accumulation of aromatics, notably PCA, found in biomass (51). Previously we have shown that these QsuB transgenic poplar plants can be used as a source of aromatics for the conversion of biomass aromatics to PDC (52). Thus, we tested the ability of the NaDec_ccMA and EcDec_ccMA strains to produce ccMA from aromatics derived from a transgenic poplar QsuB plant.

[0126] An aqueous solution containing both phenolic monomers and glycosylated forms of PCA and vanillic acid was obtained from QsuB poplar biomass using a mild alkaline pretreatment that cleaves ester linkages (FIGS. **17**A and **17**B) (52). After acid treatment of the alkaline pretreatment liquor (APL) to release the glycosylated phenolic compounds (52), the major identifiable phenolics in this material were PCA, vanillic acid, and 4-hydroxybenzoic acid (4-HBA) with trace amounts of ferulic acid and 4-coumaric acid (Table 12). *N. aromaticivorans* is able to metabolize both free and glycosylated phenolics (52), so the concentration of known phenolics derived from the Qsub poplar biomass was calculated to include the concentration of the glycosylated phenolics (52). This calculation showed that we obtained 0.42±0.03 mM of total aromatics in the Qsub APL with PCA accounting approximately 60% of the total aromatics (FIGS. **18**, **17**A, and **17**B).

TABLE 12

Concentration of aromatics in Qsub Poplar APL before and after acid hydrolysis. ND represents that the phenolic was not detected and trace indicates that a compound that co-migrated with a commercial standard was detected but at too low of levels to be quantified.

Aromatic	Before acid hydrolysis	After acid hydrolysis
PCA	0.00 ± 0.000 mM	0.31 ± 0.025 mM
Vanillic Acid	0.04 ± 0.006 mM	0.10 ± 0.028 mM
4-HBA	0.04 ± 0.001 mM	0.03 ± 0.014 mM
Vanillin	ND	ND
4-CA	Trace	ND
Ferulic Acid	Trace	ND

To test for microbial production of ccMA from the aromatics in QsuB poplar APL, we added glucose as an auxiliary carbon source and ammonium sulfate as a nitrogen source to cultures of EcDec_ccMA or NaDec_ccMA. After 48 h, both strains produced ccMA from QsuB poplar APL with calculated yields of 157±26% for EcDec_ccMA and 163±25% NaDec_ccMA (FIG. **18**). After 48 h of incubation, we did not observe any 4-HBA or PCA in the medium and only trace amounts of vanillic acid (FIGS. **17**A and **17**B), which indicates that these APL aromatics were metabolized and possibly converted to ccMA. From this, we conclude that the EcDec_ccMA and NaDec_ccMA strains were both capable of production of ccMA from Qsub APL poplar. Possible explanations for the greater than 100% yield of ccMA from components of the Qsub poplar APL are provided in the discussion.

Discussion

[0127] Lignin is the second most abundant renewable polymer on Earth (8) and represents a potential source of phenolics for conversion into industrial chemicals and materials (12). Despite this, the heterogeneity of aromatic monomers and their inter-subunit linkages have presented challenges in producing sources of valuable chemicals from this abundant resource. In recent years, the ability of some microbes to funnel a diverse set of aromatics to common intermediates has catalyzed interest in using genome-enabled strain engineering to generate one or more valuable compounds from these phenolic mixtures (7, 15, 37). This study investigated the utility of the aromatic metabolizing bacterium *N. aromaticivorans* as a chassis for ccMA pro-

duction from biomass aromatics by combining an expanded knowledge base of its aromatic metabolic pathways with metabolic engineering.

[0128] Microbial production of ccMA from glucose has been reported in bacteria that either lack or have a limited ability to metabolize the mixed aromatics that are abundant in plant biomass (53, 54). Many efforts to produce ccMA from aromatics have been limited by the accumulation of pathway intermediates (6, 22-24). We chose *N. aromaticivorans* as a potential host for ccMA production as it has the native ability to metabolize the major aromatic monomers found in plant cells walls, to transport and cleave low molecular weight aromatic oligomers with different intersubunit linkages, and to convert modified phenolics that are formed by some methods of biomass or lignin deconstruction (11, 30, 55). Below, we summarize how we combined bioinformatics, enzymology, and the genetic tractability of *N. aromaticivorans* to gain knowledge about its pathways for aromatic metabolism and use this information to engineer strains that produce ccMA from aromatics present in biomass.

Diverting PCA to Catechol

[0129] Previous studies that engineered *N. aromaticivorans* to produce PDC from aromatics suggested that the extradiol cleavage of PCA by 4,5 PCA dioxygenase homologs (LigAB1 and LigAB2) represented a major route for metabolism of this pathway intermediate (31, 32). However, the results of these studies also predicted the possibility of another minor route for PCA metabolism since yields of PDC were significantly less than 100% when a PDC-producing strain was grown in the presence of PCA (31, 32). Furthermore, work with a derivative of the PDC producing strain that had deletions of both the ligAB1 and ligAB2 gene sets found that, when grown with vanillic acid, the cells accumulated only ~50% of expected PCA and failed to accumulate PDC (32). Combined, these results suggest that LigAB1 and LigAB2 both play a significant role in PCA metabolism but that another PCA metabolic pathway exists. In this study, we identified a previously uncharacterized PCA decarboxylase as the enzyme that is responsible for consumption of PCA in cells that lack the two 4,5 PCA dioxygenase homologs (LigAB1 and LigAB2).

[0130] As predicted from previous work (31), we found that inactivation of both LigAB1 and LigAB2 in the 12444_ΔligAB1/2 strain resulted in only transient PCA accumulation (FIG. **4**C), leading us to conclude that this mutant was able to consume PCA by another catabolic pathway. Analysis of both the *N. aromaticivorans* genome sequence and of the transcript levels when cells are grown in the presence of G-family aromatics suggested that a previously uncharacterized PCA decarboxylase could be converting PCA to catechol when genes encoding both LigAB homologues were deleted. In addition, a published analysis of a genome-wide transposon insertion library suggested a connection between PCA and catechol metabolism in *N. aromaticivorans* (26). Our genetic data and in vitro enzyme assays of the previously uncharacterized *N. aromaticivorans* NadCD led us to propose that NadCD is the PCA decarboxylase that is responsible for conversion of PCA to catechol (FIG. **1**). As predicted by this hypothesis, we found that deletion of the genes encoding NadCB in the 12444_ΔligAB1/2 strain resulted in the accumulation of extracellular PCA. We think it is unlikely that the less than 100% PCA recovery (78±5%) in this strain reflects the function of another unknown PCA catabolic pathway in *N. aromaticivorans*. In support of this hypothesis, we could not identify genes that encode proteins with >25% amino acid identity to the intradiol 3,4 or to the extradiol 2,3 PCA dioxygenases found in other reported PCA catabolic pathways (35-37). Therefore, we suggest that the lack of 100% stoichiometry of PCA in these strains is due to the well-known abiotic oxidation of this compound (46-48). Instead, our data suggests that a heretofore uncharacterized *N. aromaticivorans* decarboxylase (NadCD) is responsible for PCA metabolism through conversion to catechol.

[0131] The prediction that *N. aromaticivorans* contains a PCA decarboxylase (NadCD) was not expected as there are only a few characterized homologs of this enzyme in aromatic-metabolizing bacteria (39, 45, 56). Indeed, while most metabolic engineering strategies for ccMA production using aromatic-metabolizing bacteria take advantage of the intradiol cleavage pathway of aromatic metabolism for ccMA production, they often utilize a PCA decarboxylase from another bacterium (often *E. cloacae* or *K. pneumoniae*) to convert PCA into catechol (4, 20, 57). In addition, the conversion of PCA to catechol is often a bottleneck in ccMA production; one that has been circumvented in *P. putida* KT2440 by using a foreign promoter to increase expression of a foreign PCA decarboxylase that is comprised of the EcdB/EcAroY/EcdD proteins. For these reasons, we sought to compare the PCA decarboxylase activity of NadCD to that of the commonly used EcAroY/EcdD decarboxylase.

[0132] Our in vitro analysis of recombinant NadCD and EcAroY showed that under identical conditions, the *N. aromaticivorans* enzyme was active, albeit slower, than the *E. cloacae* homologue. The lower activity of NadCD compared to EcAroY in vitro suggested that the conversion of PCA to catechol in vivo could be slower for engineered strains that depend on the native PCA decarboxylase. Indeed, the LigAB1_NaDec strain showed a higher transient extracellular level of PCA in vivo than the strain containing genes encoding the *E. cloacae* decarboxylase at the same locus (LigAB1_EcDec). However, a more detailed analysis of enzyme activity in vitro is needed to confirm this correlation. Nevertheless, only transient accumulation of PCA was found when either LigAB1_EcDec or LigAB1_NaDec are grown with vanillic acid, indicating active PCA decarboxylation in either strain and full consumption of PCA by the end of the experiment.

[0133] This study also demonstrated that the insertion of the nadBCD genes into the ligAB1 locus resulted in faster PCA consumption as compared to the 12444_ΔligAB1/2 strain, which has nadBCD in its native locus. The faster PCA consumption observed in the LigAB1_NaDec strain is consistent with the previous transcript analysis of *N. aromaticivorans* that showed higher abundance of ligAB1 transcripts than those from nadBCD when cells are grown in the presence of G-family aromatics (33). These results demonstrated that placement of either naBCD or ecdB/ecaroY/ecdD into the ligAB1 locus provided sufficient decarboxylase activity for PCA consumption. From this, we conclude that the predicted PCA decarboxylase transcript levels in the LigAB1_NaDec strain are higher than those which express the PCA decarboxylase only from its native locus and contributes to faster PCA decarboxylation by this strain in vivo.

Diverting Catechol to ccMA

[0134] Previous analysis of *N. aromaticivorans* indicated high transcript levels of the catechol 2,3-dioxygenase (xylE) when cells are grown in the presence of PCA or one of several G-family aromatics (33). These results suggest that catechol can be metabolized through the extradiol pathway (FIG. **1**). However, evidence was lacking for the function of a CatA homologue in the intradiol cleavage pathway of catechol to ccMA in this bacterium. While the *N. aromaticivorans* genome encodes a protein with significant amino acid sequence identity to other CatA enzymes (Table 11), nacatA transcripts are lower than those encoding enzymes of other known aromatic metabolizing enzymes when cells are grown in the presence of G-family aromatics (33). There is also no published evidence for metabolism of catechol via the intradiol branch in *N. aromaticivorans*. Therefore, to generate additional knowledge of *N. aromaticivorans* aromatic metabolism we tested a purified recombinant NaCatA for catechol 1,2-dioxygenase activity.

[0135] Our in vitro results indicated that recombinant NaCatA was active for catechol 1,2-dioxygenase activity and that it has comparable activity to recombinant EcCatA enzyme. Other catechol 1,2-dioxygenases typically follow Michaelis-Menten kinetics (58), so the zeroth order plot obtained with either CatA from *N. aromaticivorans* or *E. cloacae* suggest substrate saturation by catechol of both enzymes. While further kinetic analysis of NaCatA is needed to confirm that both enzymes exhibit typical Michaelis-Menten kinetics, our results suggest that *N. aromativorans* encodes a catechol 1,2-dioxygenase that is capable of converting catechol to ccMA. Overall, the results of these experiments are the first report that *N. aromativorans* has the ability to metabolize aromatics via the intradiol branch of catechol catabolism.

ccMA Production from Biomass Aromatics by *N. aromaticivorans*

[0136] Comparison between the transcript abundance of catA and xylE suggested that the extradiol (xylE) dependent pathway is the major pathway for catechol catabolism in *N. aromaticivorans*. The relatively low catA transcript abundance when cells were grown in the presence of aromatics suggested there might be little to no flux through this intradiol CatA-dependent pathway in these cultures. Additionally, the presence of only one gene encoding a protein with amino acid sequence similarity to known CatA enzymes, suggested there was a potential bottleneck in the conversion of catechol to ccMA when relying on the expression of catA from its native locus. Therefore, the genes for nacatA and eccatA were separately placed into the xylE locus, a region that is highly transcribed when cells are grown in the presence of aromatics (33). We also inactivated xylE and catBC in order to generate a strain which is predicted to only metabolize catechol via the intradiol pathway and unable to metabolize ccMA. This generated two *N. aromaticivorans* strains to compare for ccMA accumulation using genes derived from either *N. aromaticivorans* (NaDec_ccMA) or *E. cloacae* (EcDec_ccMA). As predicted by the similar rates observed in vitro for EcCatA or NaCatA, when cells were grown with PCA, both strains accumulated minimal amounts of catechol and produced stoichiometric ccMA at a similar rate. Stoichiometric conversion of ccMA from PCA indicated that the knowledge gained from these experiments on the aromatic metabolism of *N. aromaticivorans* was successfully implemented to direct PCA metabolism to the catechol intradiol pathway using either native genes or genes derived from *E. cloacae*.

[0137] The industrial conversion of abundant renewable aromatics by metabolically engineered microorganisms requires the ability of these strains to generate commodity chemicals from deconstructed biomass. To date, strains tested for ccMA production from crude biomass aromatics produced ccMA yields ranging from 5-100%, with most strains producing less than 50% yields of ccMA from deconstructed lignin (22). To test the feasibility of our engineered *N. aromaticivorans* strains to produce ccMA from biomass aromatics, we chose to use QsuB poplar biomass (51) because (1) our strains were engineered to use PCA as a precursor for ccMA production and (2) our results showed they were capable of stoichiometric conversion of PCA to ccMA. Therefore, we predicted that when our strains grow with this APL source, we would get high yields of ccMA. Indeed, at the end of the culture period, LCMS analysis showed one major peak corresponding to ccMA in the extracellular media from the NaDec_ccMA and EcDec_ccMA cultures (FIGS. **19**A and **19**B). The aromatics that were quantified in the APL included PCA, vanillic acid, and 4-HBA (FIGS. **17**A and **17**B), with the majority of PCA and vanillic acid found in glycosylated forms. All the PCA and 4-HBA were transformed during incubation, with only trace amounts of vanillic acid remaining after the incubation. Umana et al. demonstrated that the glycosylated forms of PCA and vanillic acid are degraded by *N. aromaticivorans*, and as PCA is an intermediate in the degradation of vanillic acid and 4-HBA, this observation leads us to conclude that these strains were producing ccMA from all the aromatics quantified in the biomass APL. However, the greater than 100% ccMA yield in the NaDec_ccMA and EcDec_ccMA cultures grown in the presence of APL suggests that both of these engineered strains are capable of metabolizing other unidentified aromatics present in the APL. Furthermore, since the mild alkaline pretreatment process used to generate the APL cleaves the ester bonds and liberates soluble metabolites without breaking down the lignin backbone (52), it is likely that these unidentified aromatics are not oligomeric forms of deconstructed lignin. Nevertheless, the diversity of aromatic catabolic pathways available in *N. aromaticivorans* is a valuable characteristic of *N. aromaticivorans* and important for host selection as many lignocellulosic biomass deconstruction methods are more aggressive than the mild alkaline pretreatment used in this study and would produce a range of aromatic monomers, dimers and oligomers, as well as sugar conjugates and other organic materials that could be used to support growth of engineered strains.

[0138] Overall, this work increases our knowledge on the diversity of aromatic metabolic routes available in *N. aromaticivorans*. We identified unreported *N. aromaticivorans* metabolic pathways that are involved in the conversion of PCA to ccMA. In vitro characterization of newly-identified PCA decarboxylase (NadCD) and catechol 1,2-dioxygenase (NaCatA) enzymes predicted the existence of formerly unknown metabolic routes for aromatic metabolism. We confirmed the function of these metabolic pathways through creation of defined mutants that demonstrated a new route for PCA catabolism to catechol, as well as the function of an intradiol pathway for catechol metabolism in *N. aromaticivorans*. The existence of a native PCA decarboxylase in *N. aromaticivorans* is somewhat unique in comparison to other

reported ccMA producing hosts which do not naturally possess a PCA decarboxylase capable of converting PCA to catechol (22). The pathways for PCA catabolism in *N. aromaticivorans* are also different in comparison to other sphingomonads such as *Sphingobium* sp. SYK-6, since we were unable to identify genes in this well-studied aromatic metabolizing bacterium that encode proteins with significant amino acid sequence identity to known PCA decarboxylases (38). In addition, while we could identify a *Sphingobium* sp. SYK-6 catA homologue that encoded a protein with ~40% amino acid sequence identity to *E. cloacae* CatA, the genome is not predicted to encode proteins with amino acid sequence identity to the typical CatBC enzymes of the catechol intradiol catabolic pathway. These observations increase our knowledge of the number and diversity of *N. aromaticivorans* aromatic catabolic pathways and further highlight the potential of this bacterium as a host for converting aromatics into commodity chemicals.

[0139] Our biochemical and genetic characterization of previously uncharacterized *N. aromaticivorans* gene products allowed for the generation of an engineered ccMA-producing microbe that is completely derived from native genes and transcriptional units. The use of native enzymes is potentially advantageous as it likely bypasses problems associated with folding or stability of foreign proteins, availability of required cofactors, and the accumulation of unusual intermediates that are part of a pathway that is not normally used by the host. In addition, we found that the *N. aromaticivorans* PCA decarboxylase and CatA proteins have activity that is comparable to that of well-studied enzymes from other hosts that have been used to build other ccMA production strains. Indeed, comparison of NaDec_ccMA and EcDec_ccMA demonstrated that both strains produced ccMA at similar yields and rates from either pure aromatics or biomass-derived aromatics. These results suggest that it will be possible to further engineer *N. aromaticivorans* strains for improved ccMA productivity using solely native genes.

[0140] In conclusion, our findings have expanded the knowledge of aromatic catabolic pathways in *N. aromaticivorans* and demonstrated the utility of this bacterium as a chassis for ccMA production from phenolic mixtures derived from lignocellulosic biomass. Our studies provide a proof of concept for stoichiometric ccMA production from *N. aromaticivorans* and generates a host that can be used for future studies to optimize ccMA production rates, titers and yields in bioreactors. The new findings reported herein also illustrate the value of the genetic and metabolic tractability of the abundant aromatic catabolism pathways in *N. aromaticivorans* as engineering of these ccMA-producing strains did not require the use of synthetic promoters and additional genomic alterations to produce stoichiometric yields of ccMA from deconstructed biomass. Overall, this work provides new insights in the aromatic metabolism of *N. aromaticivorans* and highlights the potential for using this bacterium as a host for producing additional valuable products from biomass aromatics.

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EMBODIMENTS

[0203] 1. A recombinant microorganism comprising:

[0204] one or more modifications with respect to a corresponding microorganism not comprising the one or more modifications, wherein the one or more modifications comprise at least one of:

[0205] a modification that increases flavin prenyltransferase activity with respect to the corresponding microorganism;

[0206] a modification that increases protocatechuate decarboxylase activity with respect to the corresponding microorganism;

[0207] a recombinant protocatechuate decarboxylase D gene encoding a protocatechuate decarboxylase D protein;

[0208] a modification that increases catechol 1,2-dioxygenase activity with respect to the corresponding microorganism;

[0209] a modification that decreases muconate lactonizing enzyme activity with respect to the corresponding microorganism;

[0210] a modification that decreases muconolactone isomerase activity with respect to the corresponding microorganism;

[0211] a modification that decreases catechol 2,3-dioxygenase activity with respect to the corresponding microorganism; and

[0212] a modification that decreases protocatechuate 4,5-dioxygenase activity with respect to the corresponding microorganism.

[0213] 2. The recombinant microorganism of embodiment 1, wherein the one or more modifications comprise one or more genetic modifications in the recombinant microorganism with respect to the corresponding microorganism.

[0214] 3. The recombinant microorganism of any prior embodiment, wherein the one or more modifications comprise the modification that increases flavin prenyltransferase activity with respect to the corresponding microorganism.

[0215] 4. The recombinant microorganism of any prior embodiment, wherein the modification that increases flavin prenyltransferase activity with respect to the corresponding microorganism, if present in the recombinant microorganism, comprises a recombinant gene encoding a flavin prenyltransferase.

[0216] 5. The recombinant microorganism of any prior embodiment, wherein the modification that increases flavin prenyltransferase activity with respect to the corresponding microorganism, if present in the recombinant microorganism, comprises a recombinant gene encoding a flavin prenyltransferase comprising a sequence having at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO:2.

[0217] 6. The recombinant microorganism of any prior embodiment, wherein the one or more modifications comprise the modification that increases protocatechuate decarboxylase activity with respect to the corresponding microorganism.

[0218] 7. The recombinant microorganism of any prior embodiment, wherein the modification that increases protocatechuate decarboxylase activity with respect to the corresponding microorganism, if present in the recombinant microorganism, comprises a recombinant gene encoding a protocatechuate decarboxylase.

[0219] 8. The recombinant microorganism of any prior embodiment, wherein the modification that increases protocatechuate decarboxylase activity with respect to the corresponding microorganism, if present in the recombinant microorganism, comprises a recombinant gene encoding a protocatechuate decarboxylase comprising a sequence having at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO:4.

[0220] 9. The recombinant microorganism of any prior embodiment, wherein the one or more modifications comprise the recombinant protocatechuate decarboxylase D gene.

[0221] 10. The recombinant microorganism of any prior embodiment, wherein the recombinant protocatechuate decarboxylase D gene, if present in the recombinant microorganism, encodes a protocatechuate decarboxylase D protein comprising a sequence having at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to any one of SEQ ID NOS:6, 24, and 26.

[0222] 11. The recombinant microorganism of any prior embodiment, wherein the recombinant protocatechuate decarboxylase D gene, if present in the recombinant microorganism, encodes a protocatechuate decarboxylase D protein comprising a sequence having at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO:6.

[0223] 12. The recombinant microorganism of any prior embodiment, wherein the one or more modifications comprise the modification that increases catechol 1,2-dioxygenase activity with respect to the corresponding microorganism.

[0224] 13. The recombinant microorganism of any prior embodiment, wherein the modification that increases catechol 1,2-dioxygenase activity with respect to the corresponding microorganism, if present in the recombinant microorganism, comprises a recombinant gene encoding a catechol 1,2-dioxygenase.

[0225] 14. The recombinant microorganism of any prior embodiment, wherein the modification that increases catechol 1,2-dioxygenase activity with respect to the corresponding microorganism, if present in the recombinant microorganism, comprises a recombinant gene encoding a catechol 1,2-dioxygenase comprising a sequence having at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO:8.

[0226] 15. The recombinant microorganism of any prior embodiment, wherein the one or more modifications comprise the modification that decreases muconate lactonizing enzyme activity with respect to the corresponding microorganism.

[0227] 16. The recombinant microorganism of any prior embodiment, wherein the modification that decreases muconate lactonizing enzyme activity with respect to the corresponding microorganism, if present in the recombinant microorganism, comprises a mutation to a gene in the corresponding microorganism encoding a muconate lactonizing enzyme.

[0228] 17. The recombinant microorganism of any prior embodiment, wherein the modification that decreases muconate lactonizing enzyme activity with respect to the corresponding microorganism, if present in the recombinant microorganism, comprises a mutation to a gene in the corresponding microorganism encoding a muconate lactonizing enzyme comprising a sequence having at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO:10.

[0229] 18. The recombinant microorganism of any prior embodiment, wherein the one or more modifications comprise the modification that decreases muconolactone isomerase activity with respect to the corresponding microorganism.

[0230] 19. The recombinant microorganism of any prior embodiment, wherein the modification that decreases

muconolactone isomerase activity with respect to the corresponding microorganism, if present in the recombinant microorganism, comprises a mutation to a gene in the corresponding microorganism encoding a muconolactone isomerase.

[0231] 20. The recombinant microorganism of any prior embodiment, wherein the modification that decreases muconolactone isomerase activity with respect to the corresponding microorganism, if present in the recombinant microorganism, comprises a mutation to a gene in the corresponding microorganism encoding a muconolactone isomerase comprising a sequence having at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO:12.

[0232] 21. The recombinant microorganism of any prior embodiment, wherein the one or more modifications comprise the modification that decreases catechol 2,3-dioxygenase activity with respect to the corresponding microorganism.

[0233] 22. The recombinant microorganism of any prior embodiment, wherein the modification that decreases catechol 2,3-dioxygenase activity with respect to the corresponding microorganism, if present in the recombinant microorganism, comprises a mutation to a gene in the corresponding microorganism encoding a catechol 2,3-dioxygenase.

[0234] 23. The recombinant microorganism of any prior embodiment, wherein the modification that decreases catechol 2,3-dioxygenase activity with respect to the corresponding microorganism, if present in the recombinant microorganism, comprises a mutation to a gene in the corresponding microorganism encoding a catechol 2,3-dioxygenase comprising a sequence having at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO:14.

[0235] 24. The recombinant microorganism of any prior embodiment, wherein the one or more modifications comprise the modification that decreases protocatechuate 4,5-dioxygenase activity with respect to the corresponding microorganism.

[0236] 25. The recombinant microorganism of any prior embodiment, wherein the modification that decreases protocatechuate 4,5-dioxygenase activity with respect to the corresponding microorganism, if present in the recombinant microorganism, comprises a mutation to a gene in the corresponding microorganism encoding a protocatechuate 4,5-dioxygenase subunit.

[0237] 26. The recombinant microorganism of any prior embodiment, wherein the modification that decreases protocatechuate 4,5-dioxygenase activity with respect to the corresponding microorganism, if present in the recombinant microorganism, comprises a mutation to any one, two, three, or each of:

[0238] a gene in the corresponding microorganism encoding a protocatechuate 4,5-dioxygenase subunit comprising a sequence having at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO:16;

[0239] a gene in the corresponding microorganism encoding a protocatechuate 4,5-dioxygenase subunit comprising a sequence having at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO:18;

[0240] a gene in the corresponding microorganism encoding a protocatechuate 4,5-dioxygenase subunit comprising a sequence having at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO:20; and

[0241] a gene in the corresponding microorganism encoding a protocatechuate 4,5-dioxygenase subunit comprising a sequence having at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO: 22.

[0242] 27. The recombinant microorganism of any prior embodiment, wherein the recombinant microorganism exhibits enhanced production of cis,cis-muconic acid with respect to the corresponding microorganism.

[0243] 28. The recombinant microorganism of any prior embodiment, wherein the recombinant microorganism is a bacterium.

[0244] 29. The recombinant microorganism of any prior embodiment, wherein the recombinant microorganism is a phenol-degrading microorganism.

[0245] 30. The recombinant microorganism of any prior embodiment, wherein the recombinant microorganism is from the genus *Novosphingobium.*

[0246] 31. The recombinant microorganism of any prior embodiment, wherein the recombinant microorganism is *Novosphingobium aromaticivorans.*

[0247] 32. A method for producing cis,cis-muconic acid comprising culturing the recombinant microorganism of any prior embodiment in a medium.

[0248] 33. The method of embodiment 32, wherein the medium comprises a plant-derived phenolic.

[0249] 34. The method of any one of embodiments 32-33, wherein the medium comprises a plant-derived phenolic selected from the group consisting of a syringyl phenolic, a guaiacyl phenolic, and a p-hydroxyphenyl phenolic.

[0250] 35. The method of any one of embodiments 32-34, wherein the medium comprises depolymerized lignin.

[0251] 36. The method of any one of embodiments 32-35, further comprising isolating the cis,cis-muconic acid from the medium and/or the recombinant microorganism.

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cgcgcctttt tccaggcgtt tgacgaattc gatcggcaca gcattatcct gcgcgaggcc 180
gaccaagccg ggatggacgt gatgggcttt aaggtcgtca aggatgccga cctcgaccat 240
ttcgcggagc gcctgctcga tatcggcgtc catgtcgatg tgatcccggc cggatcggac 300
cccggtgtgg gtcgcaagat ccgcttcaac acgccaaccc agcacgtgtt cgaactctac 360
gccgaaatgg aactgtcggc caccggcccg gcggtcaaga accccgatgt ctgggtggtc 420
gaaccgcgcg gcatgcgtgc cacccgattc gatcactgcg cactgaatgg cgtggacatc 480
tccgcatccg caaaaatctt cgtcgatgcg ctggatttct cggttaccga ggagctggtc 540
gatgaaggca gtgggacgcg cctcggcatc ttcctgagct gcagcaacaa ggcgcatgat 600
gttgccttcc tcggttatcc cgaagacggc aagatccacc acacctcttt caacctcgaa 660
tcctggcacg acgtcggcca tgcagccgac atcatcagcc gctacgatat ttcgctcgat 720
atcgggccga cgcgtcacgg gatcacccgc ggccagacga tctacttctt cgatccctcg 780
ggtaaccgca acgaaacctt cagcggcggt tacatctatt atcccgacaa tccgcagcgc 840
atgtggcagg cagagagcgc cggcaaggcg atcttctact acgaaaaggc gctgaacgac 900
cgcttcatga cggtgaacac ctga 924

SEQ ID NO: 14 moltype = AA length = 307
FEATURE Location/Qualifiers
source 1..307
mol_type = protein
organism = Novosphingobium aromaticivorans
SEQUENCE: 14
MALTGVIRPG YVQLRVLDLD EAIGHYRDRI GLNFVNRDGD RAFFQAFDEF DRHSIILREA 60
DQAGMDVMGF KVVKDADLDH FAERLLDIGV HVDVIPAGSD PGVGRKIRFN TPTQHVFELY 120
AEMELSATGP AVKNPDVWVV EPRGMRATRF DHCALNGVDI SASAKIFVDA LDFSVTEELV 180
DEGSGTRLGI FLSCSNKAHD VAFLGYPEDG KIHHTSFNLE SWHDVGHAAD IISRYDISLD 240
IGPTRHGITR GQTIYFFDPS GNRNETFSGG YIYYPDNPQR MWQAESAGKA IFYYEKALND 300
RFMTVNT 307

SEQ ID NO: 15 moltype = DNA length = 438
FEATURE Location/Qualifiers
source 1..438
mol_type = genomic DNA
organism = Novosphingobium aromaticivorans

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SEQUENCE: 15
gtgactgaca acagctcgac cgataagccc aagcctgtgc agaacatcca cgagtacctc 60
gccgagttcg aggacatccc cggcacgcgc atctataccg ctgcccgcgc ccgcaagggg 120
tactggatca accagttcgc gatgagcctg atgaagccgg agaaccgcga gcggttcaag 180
gcgaacgagc gggcctatct tgacgaatgg aagatcagcg aggaggccaa ggaagcgctg 240
ctcgcgcggg actacaaccg cctgctcgac cttggcggca acgtctattt cctgtcgaag 300
ctgttctcct cggacggact gccgttcgcc gaggcggtca gcacgatgac cgacatgacc 360
tggccggaat accgccagat gatgctggac ggcggacgca agcccgaagg caaccgatcg 420
atcaagggag gctattga 438

SEQ ID NO: 16 moltype = AA length = 145
FEATURE Location/Qualifiers
source 1..145
mol_type = protein
organism = Novosphingobium aromaticivorans
SEQUENCE: 16
MTDNSSTDKP KPVQNIHEYL AEFEDIPGTR IYTAARARKG YWINQFAMSL MKPENRERFK 60
ANERAYLDEW KISEEAKEAL LARDYNRLLD LGGNVYFLSK LFSSDGLPFA EAVSTMTDMT 120
WPEYRQMMLD GGRKPEGNRS IKGGY 145

SEQ ID NO: 17 moltype = DNA length = 843
FEATURE Location/Qualifiers
source 1..843
mol_type = genomic DNA
organism = Novosphingobium aromaticivorans
SEQUENCE: 17
atggcccgca taaccgctgg cgttggctcc agccacgttc cgctgctggg tgtcgctgtt 60
gatcagggca agtggcagga cgactatttc ggcccgatct tcaagggtta tgaatggacc 120
cgcgaatggg aaaagcgcga gaagcccgat gtggtcattc tggtctacaa cgaccacgct 180
tcggcattcg acgcgaacat catcccgacc ttcgcaatcg gctgcggcga gcactacaag 240
tcggccgatg aaggctgggg cccgcgcccg gtgcccgacg tggaaggcca tgccgacctt 300
gcctggcaca tcgcgcagag cctgatcctc gacgatttcg acatgaccat catcaacgag 360
atggatgtgg accacggcct gaccgtgccg ctctcgatga tgttcggcca gcccgagaag 420
tggccgtgca aggtcgtgcc gctggcggtg aacgtcgtca cctatccggt gccgtcgggc 480
aaccgctgct gggcgctggg cgaggcgatc gcccgcgcgg tggaaagctt ccccgaggac 540
ctcaacgtgc agatctgggg cacgggcggc atgagccacc agctccaggg cccgcgcgcc 600
ggcctgctca accgcgagtg ggacaacaag ttcctcgaca tgctggaatc ggacaacgac 660
gatgtccgct acattccgca tatcgaatac ctgcgcgaga ccggctcgga aggcatcgag 720
atggtcatgt ggctgatcat gcgcggcgcg ctcggcaaga aggtcaagcg cctgaaccgc 780
cattaccaca ttccctgcag caacaccgcg atcgggcaca tcgtgctcga gcccgcggac 840
tga 843

SEQ ID NO: 18 moltype = AA length = 280
FEATURE Location/Qualifiers
source 1..280
mol_type = protein
organism = Novosphingobium aromaticivorans
SEQUENCE: 18
MARITAGVGS SHVPLLGVAV DQGKWQDDYF GPIFKGYEWT REWEKREKPD VVILVYNDHA 60
SAFDANIIPT FAIGCGEHYK SADEGWGPRP VPDVEGHADL AWHIAQSLIL DDFDMTIINE 120
MDVDHGLTVP LSMMFGQPEK WPCKVVPLAV NVVTYPVPSG NRCWALGEAI ARAVESFPED 180
LNVQIWGTGG MSHQLQGPRA GLLNREWDNK FLDMLESDND DVRYIPHIEY LRETGSEGIE 240
MVMWLIMRGA LGKKVKRLNR HYHIPCSNTA IGHIVLEPAD 280

SEQ ID NO: 19 moltype = DNA length = 402
FEATURE Location/Qualifiers
source 1..402
mol_type = genomic DNA
organism = Novosphingobium aromaticivorans
SEQUENCE: 19
atgacacctg aaggaaaccg cgaggaccgg gcggccgtgg acaaggcgct ccgccgcgcg 60
attccgctgt tcgatggcga tctcgccacg cgcggatacg agctcaacgc gatgtgtttt 120
tccttcaacg aaaaagccaa tcgcgaggcc tttctggccg atgaagaagc ctattgccgc 180
aagttcaacc tgacgccgca gcaacgcaag gccgtggccg atcgcgatgt gctcgcgatg 240
ctcgatgcgg gcgggaacgt ctattatctg gccaagctgg ccggcatttt cggccttggc 300
gtgcaggacc tgggcgcatt gcagaccggc atgtcggtcg ctgatttcaa ggccatgctc 360
gtgcgctggg ccgacagtat tcccaacaag gagaacgcgt ga 402

SEQ ID NO: 20 moltype = AA length = 133
FEATURE Location/Qualifiers
source 1..133
mol_type = protein
organism = Novosphingobium aromaticivorans
SEQUENCE: 20
MTPEGNREDR AAVDKALRRA IPLFDGDLAT RGYELNAMCF SFNEKANREA FLADEEAYCR 60
KFNLTPQQRK AVADRDVLAM LDAGGNVYYL AKLAGIFGLG VQDLGALQTG MSVADFKAML 120

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VRWADSIPNK ENA                                                          133

SEQ ID NO: 21          moltype = DNA  length = 843
FEATURE                Location/Qualifiers
source                 1..843
                       mol_type = genomic DNA
                       organism = Novosphingobium aromaticivorans
SEQUENCE: 21
atggccaagg tcattggcgg ttatttcacc agccacgtcc cgggcattgg cggcgccatc  60
gttcgcggcg atcaggaaac gccttattgg aagccgttct tcgatggcta cccgcccatc  120
cgcgaatggc tggtggaggc ccggcctgac gtcgcaatcg tcttttccaa cgaccacggc  180
ctcaacttct tcctcgacaa gatgccgacc tttgccgtcg gtgcggcaga gcgctacgac  240
aatgccgacg agggctgggg cctgccggtc tacaagagct tcgccggtca cccggcgctt  300
tcctggcacc tgatcgacag tctggtgcgt gacgagttcg acatcacaac ctgccagaag  360
atgctggtcg atcacgcggt ttcgatcccg ttcgaactga tctacccggg tgcggagagc  420
tggccgatca agctcgtccc gatctcgatc aacaccgtgc aatatccgct gccgagtcct  480
aagcgctgcc ttgcgcttgg ccgtgcggta ggtcgcgcgc tgcaatcctg ggccggtgac  540
gaacgtgtcc tgatttgtgg taccggcggg ctttcgcatc agctggacgg tccacgcgcg  600
ggtttcatga acccggacta cgacatgttc tgccttgata atcttgcggc caatcccgac  660
gccctgaccg gccataccgc cgagcaggta gccgagcttg ccggaacgca gggcgtcgag  720
attctcaact ggatcgcggc gcgcggggca atgggcgatg tgccgctgca cgaggtcagc  780
cggaactacc atatccccat cagcaatact gcggccgcca gcctcctcct cgagcctgcc  840
tga                                                                     843

SEQ ID NO: 22          moltype = AA  length = 280
FEATURE                Location/Qualifiers
source                 1..280
                       mol_type = protein
                       organism = Novosphingobium aromaticivorans
SEQUENCE: 22
MAKVIGGYFT SHVPGIGGAI VRGDQETPYW KPFFDGYPPI REWLVEARPD VAIVFSNDHG  60
LNFFLDKMPT FAVGAAERYD NADEGWGLPV YKSFAGHPAL SWHLIDSLVR DEFDITTCQK  120
MLVDHAVSIP FELIYPGAES WPIKLVPISI NTVQYPLPSP KRCLALGRAV GRALQSWAGD  180
ERVLICGTGG LSHQLDGPRA GFMNPDYDMF CLDNLAANPD ALTGHTAEQV AELAGTQGVE  240
ILNWIAARGA MGDVPLHEVS RNYHIPISNT AAASLLLEPA                        280

SEQ ID NO: 23          moltype = DNA  length = 222
FEATURE                Location/Qualifiers
source                 1..222
                       mol_type = genomic DNA
                       organism = Enterobacter cloacae
SEQUENCE: 23
atgatctgcc cgcgctgcgc cgacgagcag atcgaggtca tggccaccag cccggtgaag  60
ggcatctgga ccgtctacca gtgccagcac tgcctgtaca cctggcggga caccgaaccg  120
cttcgtcgca cctcgcgcga gcactatccc gaagcgttcc gcatgacgca gaaggacatc  180
gatgaagcgc cgcaggtgcc cacgattcct ccgctcctgt ga                     222

SEQ ID NO: 24          moltype = AA  length = 73
FEATURE                Location/Qualifiers
source                 1..73
                       mol_type = protein
                       organism = Enterobacter cloacae
SEQUENCE: 24
MICPRCADEQ IEVMATSPVK GIWTVYQCQH CLYTWRDTEP LRRTSREHYP EAFRMTQKDI  60
DEAPQVPTIP PLL                                                      73

SEQ ID NO: 25          moltype = DNA  length = 222
FEATURE                Location/Qualifiers
source                 1..222
                       mol_type = genomic DNA
                       organism = Klebsiella pneumoniae
SEQUENCE: 25
atgatttgtc cacgttgtgc cgatgagcaa attgaggtga tggccacatc accggtgaaa  60
gggatctgga cggtttatca gtgccagcat tgtctgtata cctggcgcga taccgagccg  120
ctgcgtcgta ccagtcgcga acattaccct gaagcgttcc gcatgacgca gaaggatatt  180
gatgaggcgc cgcaggtacc gaccattcct ccattgctgt aa                     222

SEQ ID NO: 26          moltype = AA  length = 73
FEATURE                Location/Qualifiers
source                 1..73
                       mol_type = protein
                       organism = Klebsiella pneumoniae
SEQUENCE: 26
MICPRCADEQ IEVMATSPVK GIWTVYQCQH CLYTWRDTEP LRRTSREHYP EAFRMTQKDI  60
DEAPQVPTIP PLL                                                      73
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SEQ ID NO: 27 moltype = DNA length = 927
FEATURE Location/Qualifiers
source 1..927
mol_type = genomic DNA
organism = Escherichia coli
SEQUENCE: 27
atgtcgaaga acccgtcgca gcagtcggaa ctcgaaaccc tcctcgccat ctcgtcgggc 60
ctcaacaccg acggcggcaa cgaacgcgtc aagcgcgtca tccaccagct cctcaacgac 120
ctctgccaca ccatcaagac cttcgacatc tcggacgaag aattctggat cgccgtcaac 180
tacctcaacg aactcggcga acgcaaggaa gccgccctcc tcgccgccgg cctcggcctc 240
gaacactacc tcgacatgcg cgccgacgaa aaggaagccg cctcggtctc gcgcgccggc 300
accccgcgca ccatcgaagg cccgctctac gtcgccaacg ccccgctctc ggaccacttc 360
gcccgcatgg acgacggctc ggaacgcgcc gaagccatgt ggctccacgg caccgtcacc 420
gacatcgacg gcaagccggt cgccggcgcc atcgtcgaca tctggcacgc caacacccac 480
ggcggctact cgttcttcga cccgtcgcag tcggaatacа acctccgccg ccgcgtcaag 540
accgccgccg acggctcgta cgccgtccgc tcgatcgtcc cgtgcggcta cggctgcccg 600
ccggacggcc cgacccagaa gctcctcaac gaactcggcc gccacggcaa ccgcccggcc 660
cacatccact tcttcgtctc ggcccccggc ttcaagcacc tcaccaccca gatcaacctc 720
aacggcgacc gctacctctg ggacgacttc gccttcgcca cccgcgaaga actcatcgcc 780
gacccggtca aggtcaccga ctcgaccctc gcccgcgaac gcgacatcca cgaaccgcac 840
accgaagtct cgttctcgtt caccctcgtc aaggccgccg gcgccgaaga agaagcccgc 900
ggcaagcgcg cccgcgtcaa ggaatga 927

SEQ ID NO: 28 moltype = DNA length = 2299
FEATURE Location/Qualifiers
source 1..2299
mol_type = genomic DNA
organism = Novosphingobium aromaticivorans
SEQUENCE: 28
gtgaagcgca tggtcgtggg gattaccggc gcaaccggct cggtctatgg tcttcgcctg 60
cttgagctgc tgcgcgagac gggcggttgg gaaacccatc tggtaatgtc tccggctgcg 120
ctgctcaaca ttcgcgagga actgcccgaa ggcaaagccc ggctcgaagc gctggccgat 180
gtggtgcaca acgtccgcaa cgtcggcgcc tcgatcgcca gcggttcgtt cgtatgcgaa 240
ggcatggcga ttgcgccctg ttcgatgcgc acgctgggcg cggtggcgca cgccctgtcc 300
gacaacctta tcacccgcgc ggccgacgtg atgctgaagg aacggcgccg cctggtgatg 360
atcacccgcg aagcgccgct caacctggcg cacctgcgca acatgacggc ctgcaccgaa 420
atgggggcgg tgatcttccc cccggtgccg gccttctatg cgcggccgac ctcgctggcc 480
gacgtggtcg atcacacctg catgcgggta ctggatctgt tcgggcttca tgcgaagtcg 540
gagaaacgct ggcaaggcct tagcaaagag gcggcaagcc ttgttccggg tgctgggcaa 600
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gcgcgccggc gatcgtattc gacaatatca ccggttaccc cggcaagcgc ttggcggtgg 840
gcgtccatgg ttcgtgggac aacatcgccc tgctgctggg ccgacctaaa ggcacgacca 900
tccgcgagct tttcttcgag atcgccggcc gctggggcga tcaggaagcg caaatcagct 960
ttgtcccaga agcccaggcc ccggtgcacg aatgccggat cgaacaggac atcaaccttt 1020
acgatgtcct gccggtctat cggatcaacg aatacgatgg cgggttctac atcggcaagg 1080
cctcggtcgc ctcgcgcgat ccgctcgatc cagacaattt cggcaagcag aatgtcggca 1140
tctatcgcct gcagatccag gggccggaca ccttcaccct gatgacgatc ccctcccacg 1200
acatgggacg tcagatcatg gcggccgaac gggaaggcgt tccgctaaag attgcggtca 1260
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cggaatattc ctatgcctcg gcgatgatgg gcgcgccaat ccggctgacc aaatcgggca 1380
acgggatcga catcctggcc gacagcgaaa tcgtgataga ggccgaactg caaccgggtg 1440
gacgcgagct ggaagggccg ttcggcgaat tccccggttc ctacagcggc gtgcgcaagg 1500
cgccgatctt caaggtcacg gcggtgtcgc accggcgcga tccgatcttc gagaacattt 1560
acatcgggcg cggctggacc gagcacgata cgctgatcgg cctgcacacc tccgccccga 1620
tctatgccca gctgcgccag agcttccccg aagtcaccgc ggtcaacgcg ctttaccagc 1680
acggactgac cgggatcatc tcggtcaaaa accgcatggc cggctttgcc aagacggtcg 1740
cgctgcgcgc gctgagcacg ccgcacggcg tgatgtacct caagaacctg attatggtcg 1800
atgccgatgt cgatccgttc gatctcaacc aagtgatgtg ggcgctttcg acccgcaccc 1860
gtgcggacga tatcatcgtg ctgcccaaca tgcctgccgt gccgatcgat ccttcggcag 1920
tggtcccggg caaggggcac cgcctgatca tcgacgcgac cagctatctc ccgcccgatc 1980
cggtgggtga agcgcacctt gtcaccccgc cgaccgggga cgagatcgac gccctgagca 2040
agcggatccg cgaaatgcag ctgggagccc tgtcatgacc accaccgtct gcgggcgctg 2100
caaatcgagc ggcgctgtca ccgatcatca gggcaggcag gacggcgcgg tcgtgtggac 2160
gatcctgcgc tgcccgacct gcaacttttc ctggcgcgac agcgaaccgg cccgcgctat 2220
cgacccggct gtgcgctcgg ccgatttcgc cgtcgatgtc ggcgatctcc agcgttatcc 2280
caagattctc cagcaataa 2299

SEQ ID NO: 29 moltype = DNA length = 2381
FEATURE Location/Qualifiers
source 1..2381
mol_type = genomic DNA
organism = Escherichia coli
SEQUENCE: 29
atgaggctca tcgtgggcat gacgggagcc acgggcgctc cgcttggcgt ggccctcctg 60

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caggcgctcc gcgacatgcc cgaggttgaa acccatctgg tgatgtcgaa gtgggcgaag  120
accaccatcg agctggaaac gccgtatacc gcgcaggacg tcgctgccct ggccgacgtc  180
gtccacagcc ctgccgatca ggcagccacc atctcgtcgg gctcgttccg caccgatggc  240
atgatcgtca ttccctgcag catgaagacg cttgcaggca ttcgcgcggg ctatgccgaa  300
gggcttgtcg gtcgtgcggc agatgttgtg ctgaaagaag gtcgcaagct ggtgctggtc  360
ccgcgcgaaa cgccgctcag caccatccat ctggagaaca tgctcgcgct ttcccgcatg  420
ggggtggcga tggtgccgcc catgcccgcg tactacaacc atccgcaaac cgccgacgac  480
atcacccagc acatcgtgac ccgcgtcctc gaccagttcg gtctggagca caagaaggca  540
cgtcgctgga atggcctgca ggcggcgaag cacttcagcc aggagaacaa cgacggcatc  600
tgatgctggg caaatggaag ggaattgaga atgcagaacc ccatcaacga cctccgctct  660
gccatcgcgc tgctgcaacg ccatcccggt cactatatcg aaaccgacca cccggtcgat  720
cccaatgctg aactggcggg cgtctatcgc catatcggcg cgggcggtac cgtcaaacgc  780
cccacccgca cgggcccggc catgatgttc aacagcgtga agggctaccc tggctcccgc  840
atcctggtcg gtatgcatgc cagccgggaa cgcgcggcgc ttctgctggg ctgtgtcccc  900
tcgaagctgg cacagcacgt cggtcaggcg gtgaagaacc cggttgcacc ggtggtggtt  960
ccagcctcgc aggcaccgtg ccaggagcag gtcttctatg ccgacgatcc ggacttcgac 1020
ctgcgtaagc tgcttccggc cccgaccaac acgccgattg atgcaggccc gttcttctgc 1080
ctggggctgg tcctggcaag cgatccggaa gacacctcgc tgaccgatgt gaccattcac 1140
cgtctctgcg tgcaggagcg agacgaactc tcgatgttcc ttgccgccgg ccgccatatc 1200
gaagtctttc gcaagaaggc cgaagcggcg ggcaaaccgc tgccggtcac catcaacatg 1260
ggacttgacc cggctatcta cataggggcc tgcttcgaag cgcccaccac gcccttcggt 1320
tacaacgagc ttggcgttgc cggggcactc cgccagcaac cggtggagct ggtccagggc 1380
gtagcggtca aggagaaagc gatcgcgcgg gcggaaatca tcatcgaggg cgaactgctt 1440
cccggcgtgc gcgtccgcga agatcagcac accaacaccg gccacgccat gccggagttc 1500
ccgggctact gcggcgaggc gaatccgtcg ctgccggtga tcaaggtgaa agccgtgacg 1560
atgcgaaacc atgcgatcct gcagacgctg gtgggccctg gcgaagagca caccacgctt 1620
gccggtctgc cgaccgaggc cagcattcgc aacgcggtcg aagaggccat tcccggcttt 1680
ctgcagaacg tctacgccca caccgccgga ggcggtaagt tcctcggcat cctacaggtg 1740
aagaagcgcc agccgtcgga cgaaggacgt cagggccagg cggcactcat cgccctggcc 1800
acctattccg agctgaagaa catcatcctc gtggacgaag acgtggacat cttcgacagc 1860
gacgacatcc tgtgggcaat gaccacccgc atgcagggcg atgtgagcat caccacgctt 1920
ccggggatcc gcggccacca gctggatccg tcgcagtcgc cggactacag cacctcgatc 1980
cgtggaaacg gcatctcctg caagactatc ttcgactgca cggtgccgtg ggcgctgaag 2040
gcgcggttcg aacgggcgcc gttcatggag gtcgacccca caccgtgggc gccggagctg 2100
ttcagcgaca agaagtgaca gctgggagcc ctgtcatgat ctgcccgcgc tgcgccgacg 2160
agcagatcga ggtcatggcc accagcccgg tgaagggcat ctggaccgtc taccagtgcc 2220
agcactgcct gtacacctgg cgggacaccg aaccgcttcg tcgcacctcg cgcgagcact 2280
atcccgaagc gttccgcatg acgcagaagg acatcgatga agcgccgcag gtgcccacga 2340
ttcctccgct cctgtgagct gaccagacag gagtagtacc c                      2381

SEQ ID NO: 30          moltype = DNA  length = 56
FEATURE                Location/Qualifiers
source                 1..56
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 30
cgattcatta atgcagctgg cacgacagcg atctgctcgc taaattgtgg aaggag      56

SEQ ID NO: 31          moltype = DNA  length = 41
FEATURE                Location/Qualifiers
source                 1..41
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 31
gctggagaat cttgggataa ccgttcccgc ctgtcagtgt c                      41

SEQ ID NO: 32          moltype = DNA  length = 47
FEATURE                Location/Qualifiers
source                 1..47
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 32
ctgacaggcg ggaacggtta tcccaagatt ctccagcaat aaggctc                47

SEQ ID NO: 33          moltype = DNA  length = 55
FEATURE                Location/Qualifiers
source                 1..55
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 33
gtttctgcgg actggctttc tagatgttcc gtaaaggttc cagtagccta gtccg       55

SEQ ID NO: 34          moltype = DNA  length = 57
FEATURE                Location/Qualifiers
source                 1..57
                       mol_type = other DNA
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                     organism = synthetic construct
SEQUENCE: 34
cgattcatta atgcagctgg cacgacagga tgttacgaag ttcaccttca ccggttc     57

SEQ ID NO: 35          moltype = DNA  length = 45
FEATURE                Location/Qualifiers
source                 1..45
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 35
ctggagaatc ttgggataac caggagactt tcctgcgcgt tttgg                  45

SEQ ID NO: 36          moltype = DNA  length = 57
FEATURE                Location/Qualifiers
source                 1..57
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 36
cccaaaacgc gcaggaaagt ctcctggtta tcccaagatt ctccagcaat aaggctc     57

SEQ ID NO: 37          moltype = DNA  length = 55
FEATURE                Location/Qualifiers
source                 1..55
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 37
gtttctgcgg actggctttc tagatgttcc gtaaaggttc cagtagccta gtccg       55

SEQ ID NO: 38          moltype = DNA  length = 26
FEATURE                Location/Qualifiers
source                 1..26
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 38
ctgaccagac aggagtagta cccatg                                       26

SEQ ID NO: 39          moltype = DNA  length = 24
FEATURE                Location/Qualifiers
source                 1..24
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 39
cataggcccc tctccttcag cttg                                         24

SEQ ID NO: 40          moltype = DNA  length = 59
FEATURE                Location/Qualifiers
source                 1..59
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 40
cgcatgggta ctactcctgt ctggtcagct tattgctgga gaatcttggg ataacgctg   59

SEQ ID NO: 41          moltype = DNA  length = 47
FEATURE                Location/Qualifiers
source                 1..47
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 41
ccaagctgaa ggagaggggc ctatgaccat gaacgatctc cctaacc                47

SEQ ID NO: 42          moltype = DNA  length = 24
FEATURE                Location/Qualifiers
source                 1..24
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 42
cacaggcccc tctccttcag cttg                                         24

SEQ ID NO: 43          moltype = DNA  length = 47
FEATURE                Location/Qualifiers
source                 1..47
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 43
ggcaaatgga agggaattga gaatgaccat gaacgatctc cctaacc                47
```

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```
SEQ ID NO: 44          moltype = DNA  length = 47
FEATURE                Location/Qualifiers
source                 1..47
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 44
caagctgaag gagaggggcc tgtgaagcgc atggtcgtgg ggattac               47

SEQ ID NO: 45          moltype = DNA  length = 38
FEATURE                Location/Qualifiers
source                 1..38
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 45
cattctcaat tcccttccat ttgcccagca cccggaac                         38

SEQ ID NO: 46          moltype = DNA  length = 54
FEATURE                Location/Qualifiers
source                 1..54
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 46
gtttctgcgg actggctttc tacgtgttcc gttcattact tcacccagca gggc        54

SEQ ID NO: 47          moltype = DNA  length = 51
FEATURE                Location/Qualifiers
source                 1..51
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 47
ggtctcaaag gctgaacgga aagggcaagg cgatcttcta ctacgaaaag g           51

SEQ ID NO: 48          moltype = DNA  length = 51
FEATURE                Location/Qualifiers
source                 1..51
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 48
ccttttcgta gtagaagatc gccttgccct ttccgttcag cctttgagac c           51

SEQ ID NO: 49          moltype = DNA  length = 54
FEATURE                Location/Qualifiers
source                 1..54
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 49
cgattcatta atgcagctgg cacgacagcg aaggtctcat ctgatcgaag agcg        54

SEQ ID NO: 50          moltype = DNA  length = 49
FEATURE                Location/Qualifiers
source                 1..49
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 50
gtttctgcgg actggctttc tacgtgttct cgccgcaagc taggaccgc              49

SEQ ID NO: 51          moltype = DNA  length = 48
FEATURE                Location/Qualifiers
source                 1..48
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 51
tcgttgcttg ccacatcgaa gatcgacggc cacaggacta agcgttgc               48

SEQ ID NO: 52          moltype = DNA  length = 48
FEATURE                Location/Qualifiers
source                 1..48
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 52
gcaacgctta gtcctgtggc cgtcgatctt cgatgtggca agcaacga               48

SEQ ID NO: 53          moltype = DNA  length = 56
FEATURE                Location/Qualifiers
source                 1..56
                       mol_type = other DNA
```

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                     organism = synthetic construct
SEQUENCE: 53
cgattcatta atgcagctgg cacgacagcg ttatgtggtg atttcagcga tcgtcg      56

SEQ ID NO: 54          moltype = DNA  length = 51
FEATURE                Location/Qualifiers
source                 1..51
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 54
cggactactc ccgttatgtg gtgatcagcc tttgagacca ttcctaaaga a          51

SEQ ID NO: 55          moltype = DNA  length = 50
FEATURE                Location/Qualifiers
source                 1..50
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 55
caaggcgatc ttctactacg aaaaggcgct gaacgaccgc ttcatgacgg            50

SEQ ID NO: 56          moltype = DNA  length = 49
FEATURE                Location/Qualifiers
source                 1..49
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 56
tttcgtagta gaagatcgcc ttgctcaggc ctgggcgcgg gtgcgttcc             49

SEQ ID NO: 57          moltype = DNA  length = 51
FEATURE                Location/Qualifiers
source                 1..51
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 57
tcaccacata acgggagtag tccgatgcct gccaccttcg ccagttccga t          51

SEQ ID NO: 58          moltype = DNA  length = 51
FEATURE                Location/Qualifiers
source                 1..51
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 58
tttcgtagta gaagatcgcc ttgtcattcc ttgacgcggg cgcgcttgcc g          51

SEQ ID NO: 59          moltype = DNA  length = 51
FEATURE                Location/Qualifiers
source                 1..51
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 59
tcaccacata acgggagtag tccgatgtcg aagaacccgt cgcagcagtc g          51

SEQ ID NO: 60          moltype = DNA  length = 49
FEATURE                Location/Qualifiers
source                 1..49
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 60
gtattttcag agcgcgatcg caggaatgag gctcatcgtg ggcatgacg             49

SEQ ID NO: 61          moltype = DNA  length = 51
FEATURE                Location/Qualifiers
source                 1..51
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 61
ctaactttgt tattttcggc tttctgtcag atgccgtcgt tgttctcctg g          51

SEQ ID NO: 62          moltype = DNA  length = 49
FEATURE                Location/Qualifiers
source                 1..49
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 62
gtattttcag agcgcgatcg caggaatgca gaaccccatc aacgacctc             49
```

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```
SEQ ID NO: 63          moltype = DNA  length = 50
FEATURE                Location/Qualifiers
source                 1..50
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 63
ctaactttgt tattttcggc tttctggctc acaggagcgg aggaatcgtg            50

SEQ ID NO: 64          moltype = DNA  length = 42
FEATURE                Location/Qualifiers
source                 1..42
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 64
gtattttcag agcgcgatcg caatgcctgc caccttcgcc ag                    42

SEQ ID NO: 65          moltype = DNA  length = 45
FEATURE                Location/Qualifiers
source                 1..45
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 65
ctaactttgt tattttcggc tttctgtcag gcctgggcgc gggtg                 45

SEQ ID NO: 66          moltype = DNA  length = 47
FEATURE                Location/Qualifiers
source                 1..47
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 66
gtattttcag agcgcgatcg caatgaccat gaacgatctc cctaacc                47

SEQ ID NO: 67          moltype = DNA  length = 52
FEATURE                Location/Qualifiers
source                 1..52
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 67
ctaactttgt tattttcggc tttctgttat tgctggagaa tcttgggata ac          52

SEQ ID NO: 68          moltype = DNA  length = 48
FEATURE                Location/Qualifiers
source                 1..48
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 68
gtattttcag agcgcgatcg caggaatgtc gaagaacccg tcgcagca               48

SEQ ID NO: 69          moltype = DNA  length = 48
FEATURE                Location/Qualifiers
source                 1..48
                       mol_type = other DNA
                       organism = synthetic construct
SEQUENCE: 69
ctaactttgt tattttcggc tttctgttcc ttgacgcggg cgcgcttg               48
```

What is claimed is:

1. A recombinant microorganism comprising:

one or more modifications with respect to a corresponding microorganism not comprising the one or more modifications, wherein the one or more modifications comprise at least one of:

a modification that increases flavin prenyltransferase activity with respect to the corresponding microorganism;

a modification that increases protocatechuate decarboxylase activity with respect to the corresponding microorganism;

a recombinant protocatechuate decarboxylase D gene encoding a protocatechuate decarboxylase D protein;

a modification that increases catechol 1,2-dioxygenase activity with respect to the corresponding microorganism;

a modification that decreases muconate lactonizing enzyme activity with respect to the corresponding microorganism;

a modification that decreases muconolactone isomerase activity with respect to the corresponding microorganism;

a modification that decreases catechol 2,3-dioxygenase activity with respect to the corresponding microorganism; and

a modification that decreases protocatechuate 4,5-dioxygenase activity with respect to the corresponding microorganism.

2. The recombinant microorganism of claim 1, wherein:

the one or more modifications comprise the modification that increases flavin prenyltransferase activity with respect to the corresponding microorganism, and the modification that increases flavin prenyltransferase activity with respect to the corresponding microorganism comprises a recombinant gene encoding a flavin prenyltransferase comprising a sequence having at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO:2; and/or

the one or more modifications comprise the modification that increases protocatechuate decarboxylase activity with respect to the corresponding microorganism, and the modification that increases protocatechuate decarboxylase activity with respect to the corresponding microorganism comprises a recombinant gene encoding a protocatechuate decarboxylase comprising a sequence having at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO:4; and/or

the one or more modifications comprise the recombinant protocatechuate decarboxylase D gene, and the recombinant protocatechuate decarboxylase D gene encodes a protocatechuate decarboxylase D protein comprising a sequence having at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO:6; and/or

the one or more modifications comprise the modification that increases catechol 1,2-dioxygenase activity with respect to the corresponding microorganism, and the modification that increases catechol 1,2-dioxygenase activity with respect to the corresponding microorganism comprises a recombinant gene encoding a catechol 1,2-dioxygenase comprising a sequence having at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO:8.

3. The recombinant microorganism of claim 1, wherein:

the one or more modifications comprise the modification that increases flavin prenyltransferase activity with respect to the corresponding microorganism, and the modification that increases flavin prenyltransferase activity with respect to the corresponding microorganism comprises a recombinant gene encoding a flavin prenyltransferase comprising a sequence having at least 95% sequence identity to SEQ ID NO:2; and/or

the one or more modifications comprise the modification that increases protocatechuate decarboxylase activity with respect to the corresponding microorganism, and the modification that increases protocatechuate decarboxylase activity with respect to the corresponding microorganism comprises a recombinant gene encoding a protocatechuate decarboxylase comprising a sequence having at least 95% sequence identity to SEQ ID NO:4; and/or

the one or more modifications comprise the recombinant protocatechuate decarboxylase D gene, and the recombinant protocatechuate decarboxylase D gene encodes a protocatechuate decarboxylase D protein comprising a sequence having at least 95% sequence identity to SEQ ID NO:6; and/or

the one or more modifications comprise the modification that increases catechol 1,2-dioxygenase activity with respect to the corresponding microorganism, and the modification that increases catechol 1,2-dioxygenase activity with respect to the corresponding microorganism comprises a recombinant gene encoding a catechol 1,2-dioxygenase comprising a sequence having at least 95% sequence identity to SEQ ID NO:8.

4. The recombinant microorganism of claim 1, wherein:

the one or more modifications comprise the modification that increases protocatechuate decarboxylase activity with respect to the corresponding microorganism; and

the one or more modifications comprise the modification that increases catechol 1,2-dioxygenase activity with respect to the corresponding microorganism, and the modification that increases catechol 1,2-dioxygenase activity with respect to the corresponding microorganism comprises a recombinant gene encoding a catechol 1,2-dioxygenase comprising a sequence having at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO:8.

5. The recombinant microorganism of claim 4, wherein the recombinant microorganism is a phenol-degrading bacterium.

6. The recombinant microorganism of claim 4, wherein the recombinant microorganism is from the genus *Novosphingobium*.

7. The recombinant microorganism of claim 4, wherein:

the one or more modifications comprise the modification that decreases catechol 2,3-dioxygenase activity with respect to the corresponding microorganism; and

the one or more modifications comprise the modification that decreases protocatechuate 4,5-dioxygenase activity with respect to the corresponding microorganism.

8. The recombinant microorganism of claim 7, wherein the recombinant microorganism is a phenol-degrading bacterium.

9. The recombinant microorganism of claim 7, wherein the recombinant microorganism is from the genus *Novosphingobium*.

10. The recombinant microorganism of claim 7, wherein:

the one or more modifications comprise the modification that increases flavin prenyltransferase activity with respect to the corresponding microorganism;

the one or more modifications comprise the recombinant protocatechuate decarboxylase D gene encoding a protocatechuate decarboxylase D protein;

the one or more modifications comprise the modification that decreases muconate lactonizing enzyme activity with respect to the corresponding microorganism; and

the one or more modifications comprise the modification that decreases muconolactone isomerase activity with respect to the corresponding microorganism.

11. The recombinant microorganism of claim 10, wherein the recombinant microorganism is a phenol-degrading bacterium.

12. The recombinant microorganism of claim 10, wherein the recombinant microorganism is from the genus *Novosphingobium*.

13. The recombinant microorganism of claim 10, wherein:

the modification that increases flavin prenyltransferase activity with respect to the corresponding microorganism comprises a recombinant gene encoding a flavin prenyltransferase comprising a sequence having at least 95% sequence identity to SEQ ID NO:2;

the modification that increases protocatechuate decarboxylase activity with respect to the corresponding

microorganism comprises a recombinant gene encoding a protocatechuate decarboxylase comprising a sequence having at least 95% sequence identity to SEQ ID NO:4;

the recombinant protocatechuate decarboxylase D gene encodes a protocatechuate decarboxylase D protein comprising a sequence having at least 95% sequence identity to any one of SEQ ID NOS:6, 24, and 26;

the modification that increases catechol 1,2-dioxygenase activity with respect to the corresponding microorganism comprises a recombinant gene encoding a catechol 1,2-dioxygenase comprising a sequence having at least 95% sequence identity to SEQ ID NO:8;

the modification that decreases muconate lactonizing enzyme activity with respect to the corresponding microorganism comprises a mutation to a gene in the corresponding microorganism encoding a muconate lactonizing enzyme comprising a sequence having at least 95% sequence identity to SEQ ID NO:10;

the modification that decreases muconolactone isomerase activity with respect to the corresponding microorganism comprises a mutation to a gene in the corresponding microorganism encoding a muconolactone isomerase comprising a sequence having at least 95% sequence identity to SEQ ID NO:12;

the modification that decreases catechol 2,3-dioxygenase activity with respect to the corresponding microorganism comprises a mutation to a gene in the corresponding microorganism encoding a catechol 2,3-dioxygenase comprising a sequence having at least 95% sequence identity to SEQ ID NO:14; and

the modification that decreases protocatechuate 4,5-dioxygenase activity with respect to the corresponding microorganism comprises a mutation to any one, two, three, or each of:

a gene in the corresponding microorganism encoding a protocatechuate 4,5-dioxygenase subunit comprising a sequence having at least 95% sequence identity to SEQ ID NO:16;

a gene in the corresponding microorganism encoding a protocatechuate 4,5-dioxygenase subunit comprising a sequence having at least 95% sequence identity to SEQ ID NO:18;

a gene in the corresponding microorganism encoding a protocatechuate 4,5-dioxygenase subunit comprising a sequence having at least 95% sequence identity to SEQ ID NO:20; and

a gene in the corresponding microorganism encoding a protocatechuate 4,5-dioxygenase subunit comprising a sequence having at least 95% sequence identity to SEQ ID NO: 22.

14. The recombinant microorganism of claim **13**, wherein the recombinant microorganism is a phenol-degrading bacterium.

15. The recombinant microorganism of claim **13**, wherein the recombinant microorganism is from the genus *Novosphingobium*.

16. The recombinant microorganism of claim **13**, wherein the recombinant microorganism exhibits enhanced production of cis,cis-muconic acid with respect to the corresponding microorganism.

17. A method for producing cis,cis-muconic acid comprising culturing the recombinant microorganism of claim **1** in a medium comprising a plant-derived phenolic.

18. The method of claim **17**, wherein the medium comprises a plant-derived phenolic selected from the group consisting of a syringyl phenolic, a guaiacyl phenolic, and a p-hydroxyphenyl phenolic.

19. The method of claim **17**, wherein the medium comprises depolymerized lignin.

20. The method of claim **17**, further comprising isolating the cis,cis-muconic acid from the medium and/or the recombinant microorganism.

* * * * *