



(51) International Patent Classification:

C12P 7/04 (2006.01) *C12N 1/21* (2006.01)
C12P 7/16 (2006.01) *C12M 1/06* (2006.01)
C12P 7/22 (2006.01)

(21) International Application Number:

PCT/US2017/031807

(22) International Filing Date:

09 May 2017 (09.05.2017)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/333,415 09 May 2016 (09.05.2016) US
 62/423,187 16 November 2016 (16.11.2016) US

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(81) Designated States (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: MICROBIAL SYSTEM FOR BIOSYNTHESIS OF PRODUCTS, AND IN SITU EXTRACTION IN SUPERCRITICAL CO₂

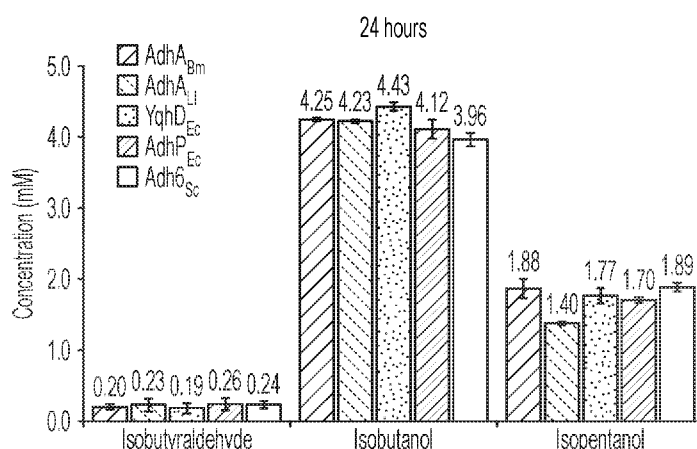


FIG. 27A

(57) Abstract: Provided herein are methods of producing a bioproduct by culturing a cell in a multiphase reactor that comprises supercritical CO₂, near critical CO₂, or liquid CO₂.



MICROBIAL SYSTEM FOR BIOSYNTHESIS OF PRODUCTS, AND IN SITU EXTRACTION IN SUPERCRITICAL CO₂

RELATED APPLICATIONS

5 This application claims the benefit under 35 U.S.C. § 119(e) of U.S. provisional application number 62/423,187, filed November 16, 2016 and U.S. provisional application number 62/333,415, filed May 9, 2016, each of which is incorporated by reference herein in its entirety.

FIELD OF INVENTION

10 The invention relates to methods of producing a bioproduct involving culturing a cell in a multiphase reactor comprising a solvent phase comprising supercritical CO₂, near critical CO₂, or liquid CO₂.

BACKGROUND

15 Supercritical carbon dioxide (scCO₂) is generally regarded as a sterilizing agent of vegetative cells and a high-level disinfectant of most bacterial endospores (White et al., 2006; Ortuño et al., 2012, Mitchell et al., 2008, Zhang et al., 2006). When scCO₂ is introduced to a system, significant pH decreases (*e.g.*, to pH ~3 in unbuffered systems, pH ~5-6 in buffered
20 systems) occur on a timescale of several days (Kharaka et al., 2006). Because of these properties, scCO₂ has been studied within the context of sterilization for the food and drug industries.

SUMMARY OF THE INVENTION

25 Aspects of the present disclosure provide methods of producing a bioproduct, comprising culturing a cell in a multiphase reactor comprising an aqueous phase and a solvent phase, wherein the solvent phase comprises supercritical CO₂ (scCO₂), near critical CO₂, or liquid CO₂. In some embodiments, the cell is a viable cell. In some embodiments, the solvent phase comprises scCO₂. In some embodiments, the solvent phase comprises near critical CO₂. In
30 some embodiments, the solvent phase comprises liquid CO₂. In some embodiments, the cell is a bacterial cell. In some embodiments, the bacterial cell is resistant to scCO₂. In some

embodiments, the bacterial cell is a spore-forming bacterium belonging to the genus *Firmicutes*. In some embodiments, the bacterial cell is a *Bacillus* spp. In some embodiments, the bacterial cell is selected from the group consisting of *Bacillus cereus*, *Bacillus subterraneus*, *Bacillus amyloliquefaciens*, *Bacillus safensis*, *Bacillus licheniformis*, and *Bacillus megaterium*. In some
5 embodiments, the bacterial cell is *Bacillus megaterium* SR7.

In some embodiments, the cell is in spore-form or has been acclimated to scCO₂. In some embodiments, the cell has been acclimated to scCO₂ by previous exposure to scCO₂.

In some embodiments, the bioproduct is selected from the group consisting of isobutanol, butanol, isopentanol, phenyl-ethyl-alcohol and 4-methyl-pentanol. In some
10 embodiments, the cell is engineered to recombinantly express one or more genes. In some embodiments, the bioproduct is partitioned into scCO₂, near critical CO₂, or liquid CO₂. In some embodiments, the bioproduct is a hydrocarbon, oxygenated hydrocarbon, aldehyde, alcohol, fatty acid, or ketone. In some embodiments, the bioproduct is isobutanol and the cell is engineered to recombinantly express an isoketovalerate decarboxylase and an alcohol dehydrogenase. In some
15 embodiments, the isoketovalerate decarboxylase is a *Lactococcus lactis* gene. In some embodiments, the alcohol dehydrogenase is an *E. coli* gene. In some embodiments, the bioproduct is 4-methyl-pentanol and the cell is engineered to recombinantly express one or more enzymes selected from the group consisting of an acetolactate synthase, a ketol-acid reductoisomerase, a dihydroxy-acid dehydratase, an alpha-ketoisovalerate decarboxylase, an
20 aldehyde dehydrogenase, a propionyl-CoA transferase, a beta-keto-thiolase, a beta-keto-acyl-CoA reductase, an enoyl-CoA hydratase, a trans-enoyl-CoA reductase, a carboxylic acid reductase, a 4'-phosphopantenheiny transferase, and an alcohol dehydrogenase.

In some embodiments, the bioproduct is isolated from the solvent phase. In some
25 embodiments, the bioproduct is isolated from the aqueous phase. In some embodiments, the solvent phase further comprises less than or equal to about 3% inert helium. In some embodiments, the aqueous phase comprises a growth media. In some embodiments, the growth media comprises a spore germination inducer. In some embodiments, the spore germination inducer is D-alanine.

In some embodiments, the culturing comprises incubating the cell in the multiphase
30 reactor for at least 5 hours. In some embodiments, the culturing comprises incubating the cell in the multiphase reaction at a temperature between 35°C – 40°C.

Other aspects provide a *Bacillus megaterium* cell that recombinantly expresses an isoketovalerate decarboxylase and an alcohol dehydrogenase. In some embodiments, the cell is a *Bacillus megaterium* SR7 cell.

Yet other aspects provide a reactor comprising (a) (i) supercritical CO₂ or a mixture of
5 supercritical CO₂ and one or more other solvents, (ii) near critical CO₂ or a mixture of near critical CO₂ and one or more other solvents, or (iii) liquid CO₂ or a mixture of near critical CO₂ and one or more other solvents; and (b) at least one viable cell. In some embodiments, the viable cell is a bacterial cell. In some embodiments, the bacterial cell is resistant to scCO₂. In some
10 embodiments, the bacterial cell is a spore-forming bacterium belonging to the genus *Firmicutes*. In some embodiments, the bacterial cell is a *Bacillus* spp. In some embodiments, the bacterial cell is selected from the group consisting of *Bacillus cereus*, *Bacillus subterraneus*, *Bacillus amyloliquefaciens*, *Bacillus safensis*, *Bacillus lichenformis*, and *Bacillus megaterium*. In some
15 embodiments, the bacterial cell is *Bacillus megaterium* SR7.

In some embodiments, the viable cell is in spore-form or has been acclimated to scCO₂.
20 In some embodiments, the viable cell has been acclimated to scCO₂ by previous exposure to scCO₂. In some embodiments, the viable cell is engineered to recombinantly express one or more genes.

In some embodiments, the reactor further comprises a bioproduct. In some embodiments, the bioproduct is partitioned into the scCO₂, near critical CO₂, or liquid CO₂. In some
25 embodiments, the bioproduct is a hydrocarbon, oxygenated hydrocarbon, aldehyde, alcohol, fatty acid, or ketone. In some embodiments, the bioproduct is selected from the group consisting of isobutanol, butanol, isopentanol, phenyl-ethyl-alcohol and 4-methyl-pentanol. In some
30 embodiments, the bioproduct is isobutanol and the viable cell is engineered to recombinantly express an isoketovalerate decarboxylase and an alcohol dehydrogenase. In some embodiments, the isoketovalerate decarboxylase is a *Lactococcus lactis* gene. In some embodiments, the alcohol dehydrogenase is an *E. coli* gene. In some embodiments, the bioproduct is 4-methyl-pentanol and the cell is engineered to recombinantly express. In some embodiments, the bioproduct is isolated from the scCO₂ phase.

In some embodiments, the reactor comprises one or more other solvent, wherein the one
35 or more other solvent is inert helium. In some embodiments, the reactor further comprises a

growth media. In some embodiments, the growth media comprises a spore germination inducer. In some embodiments, the spore germination inducer is D-alanine.

These and other aspects of the invention, as well as various embodiments thereof, will become more apparent in reference to the drawings and detailed description of the invention.

5 Each of the limitations of the invention can encompass various embodiments of the invention. It is, therefore, anticipated that each of the limitations of the invention involving any one element or combinations of elements can be included in each aspect of the invention. This invention is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The invention is capable of other
10 embodiments and of being practiced or of being carried out in various ways.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings are not intended to be drawn to scale. For purposes of clarity, not every component may be labeled in every drawing. In the drawings:

15 FIG. 1 shows the expected and observed DNA staining patterns of differentially germinated *Bacillus megaterium* (*B. megaterium*) SR7 spores.

FIG. 2 shows a 16S rRNA phylogenetic tree of McElmo Dome isolates, isolates, and
20 additional closely related Bacilli.

FIG. 3 shows a schematic of the *B. megaterium* SR7 5.51 Mbp genome, including the closed 5.45 Mbp chromosome. Concentric circles (outside in) are RAST ORFs, rRNA and tRNA, GC content, and GC skew. Asymmetry in GC skew indicates proper chromosome
25 assembly. Circles at the right represent five putative plasmids native to SR7.

FIGs. 4A and 4B show the effect of mixing rates on *B. megaterium* SR7 spore germination in LB under 1 atm CO₂ as measured by CFU/mL (FIG. 4A) and OD600 (FIG. 4B).

FIGs. 5A and 5B show the effect of mixing rates on passaged *B. megaterium* SR7 vegetative growth in LB under 1 atm CO₂ as measured by CFU/mL (FIG. 5A) and OD600 (FIG. 5B).

FIGs. 6A and 6B show *B. megaterium* SR7 growth under 1 atm CO₂ at 37°C as a function of trace metals solution concentration (FIG. 6A) and in the presence (filled diamonds) and absence (open diamonds) of trace metals in M9 media types and unamended LB (black filled triangles) (FIG. 6B).

FIGs. 7A and 7B show *B. megaterium* SR7 growth dynamics under 1 atm CO₂, 37°C, 250 rpm and glucose consumption in M9+ media (FIG. 7A) and LB (FIG. 7B).

FIGs. 8A and 8B show *B. megaterium* SR7 cultures in LB under 1 atm CO₂, 37°C, 250 rpm show a lag phase when glucose-amended, as measured by OD600 (FIG. 8A) and incomplete glucose consumption after 24 hours (FIG. 8B).

FIGs. 9A and 9B show phase contrast light microscopy of SR7 vegetative cultures. PHB-filled (bright cells, black arrow) and membrane-degraded SR7 cells (transparent, gray arrows with asterisk) in glucose-amended LB grown under 1 atm CO₂ (FIG. 9A). Cells grown in LB under 1 atm CO₂ without glucose accumulate observable, but smaller amounts of PHB granules, often distributed at the cellular poles (FIG. 9B).

FIGs. 10A and 10B show the concentration of *B. megaterium* SR7 fermentation products produced under 1 atm CO₂ in LB medium (FIG. 10A) and M9+ medium (FIG. 10B) as detected and measured by HPLC. The columns are, from left to right, samples obtained at 6 hours, 24 hours, and 48 hours.

FIG. 11 shows germination of SR7 spores under 1 atm CO₂ in LB media, 100 mM L-alanine-amended LB, and LB heated at 65°C for 10 minutes. Open triangles represent viable spore counts based on heat-killed (80°C for 15 minutes) CFU counts.

FIGs. 12A and 12B show assays tracking the extent of population-level germination progress in PBS buffer by cell stain pattern by fluorescence microscopy (FIG. 12A) and culture bulk fluorescence (FIG. 12B).

FIGs. 13A and 13B show a schematic illustrating the physiological process of endospore germination (FIG. 13A) and the defining traits and hypothesized corresponding physiological state of three detected spore populations (FIG. 13B).

FIGs. 14A-14D show flow cytometry results. FIG. 14A shows fluorescence distribution of all counts previously gated by side/forward scatter gates. FIG. 14B shows a subset of those counts were then gated by GFP and PI fluorescence intensity (low/dormant; intermediate/activated; high/germinated. FIG. 14C shows the distribution and intensity of Syto16 fluorescence in Population 1 and FIG. 14D shows the same in Population 2. The top panel shows SR7 spores incubated in PBS and the bottom panel shows SR7 spores incubated in PBS amended with 25 mM L-alanine.

FIG. 15 shows a summary of flow cytometry signatures of *B. megaterium* SR7 spores incubated in PBS or L-alanine-amended (2.5, 25, 250 mM) media. Summed distribution of Population 1 and Population 2 counts within each of the three Syto16/PI fluorescence gates are shown in top panel. Values presented in plot, as well as spore stock distributions, are shown in the bottom panel.

FIG. 16 shows *B. megaterium* SR7 germination and growth frequencies under scCO₂ in respective media based on cumulative results pooled from multiple individual experiments, as summarized in Table 14. In each column, the frequencies are presented, from top to bottom, dormant, germinated, low growth, and high growth.

FIG. 17 shows nominal logistic regression of SR7 scCO₂ growth frequency (Z axis) as a function of inocula spore density ($p = 0.0057$; Y axis) and incubation time ($p = 0.003$; X axis).

FIG. 18 shows concentrations of fermentative products generated in M9A+ medium by SR7 cultures under scCO₂ showing growth, as detected by HPLC. The columns are, from left to right, samples obtained from 6 days, 12 days, and 18 days. No fermentation products were detected in cell-free control incubations or scCO₂ cultures not demonstrating growth.

5

FIG. 19 shows an isobutanol biosynthesis pathway from α -KIV encompassed by the dashed line.

FIG. 20 shows vector maps for pJBxL (left) and pJBxKY (right) using scaffold pRBBm34.

10

FIG. 21 shows a xylose promoter system endogenous to *B. megaterium*.

FIG. 22 shows LacZ specific activity of duplicate cultures using IPTG and xylose-inducible promoters under 1 atm CO₂, with B-PER as the negative control.

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FIG. 23 shows LacZ-specific activity in scCO₂ cultures in the absence and presence of 0.5% xylose, with B-PER negative control. SR7xL cultures demonstrating robust growth are highlighted with stars. Cultures without stars germinated but did not demonstrate robust outgrowth.

20

FIG. 24 shows growth of biofuel and empty vector control strains under 1 atm CO₂

FIG. 25 shows SR7xKA6 bioproduct concentrations under 1 atm CO₂ and aerobic conditions 4 and 24 hours after induction. Bars, left to right: 4 hr 1 atm CO₂, 24 hr 1 atm CO₂, 4 hr Aerobic, and 24 hr Aerobic. Measurements of biofuel production from aerobically-incubated cultures may be underestimated due to the removal of caps during subsampling of aerobic cultures, which may have resulted in some volatile product losses.

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FIGs. 26A-26D show bioproduct concentrations generated under aerobic conditions.

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FIG. 26A shows production of isobutryaldehyde by SR7 pXyl kivD_{LI} and in tandem with one of

five alcohol dehydrogenase variants after 4 hours. FIG. 26B shows production of isobutanol by SR7 pXyl kivD_{LI} and in tandem with one of five alcohol dehydrogenase variants after 24 hours. The columns in FIGs. 26A and 26B are, from left to right: KivD only, KivD ADH6_{Sc}, KivD AdhA_{LI}, KivD AdhP_{Ec}, KivD YqhD_{Ec}, KivD AdhA_{Bm}. FIG. 26C shows production of isobutanol and isopentanol using SR7 strains expressing a partial or full isobutanol pathway. For each strain, the left column represents the isobutanol titer and the right column represents the isopentanol titer. FIG. 26D shows production of isobutanol and isopentanol by a SR7 strain containing a redesigned full isobutanol pathway resulting in an increase in the conversion of isobutanol to isopentanol. For each bioproduct, the left column represents the titer when cultured in the presence of 10 g/L glucose, and the right column represents the titer when cultured in the presence of 25 g/L glucose.

FIGs. 27A-27D show bioproduct concentrations generated under 1 atm CO₂ by SR7 PXyl kivDLI in tandem with five alcohol dehydrogenase variants at 24 hours presented as raw (FIG. 27A), OD-normalized values (FIG. 27B), 48 hours as raw (FIG. 27C) and OD-normalized values (FIG. 27D). From left to right: AdhA_{Bm}, AdhA_{LI}, YqhD_{Ec}, AdhP_{Ec}, Adh6_{Sc}.

FIGs. 28A and 28B show standard curves based on abiotic scCO₂ phase (FIG. 28A) and aqueous phase extraction of isobutanol (FIG. 28B)

FIGs. 29A to 29B show natural fermentative products generated by SR7xL and SR7xKY cultures under scCO₂ showing growth, as detected by HPLC. FIG. 29A shows total titers and FIG. 29B shows filter count-normalized per cell metabolite productivity. Final cell concentrations for each sample are listed at the right. No metabolites were detected in media-only reactors and reactors without cell growth.

FIGs. 30A to 30B show examples of GC-FID traces detecting biofuel products isobutanol and isopentanol in the media phase (FIG. 30A) and scCO₂-extracted phase (FIG. 30B) of scCO₂-incubated SR7xKY cultures.

FIG. 31 shows recovered bioproduct concentrations from cultures showing growth in xylose-induced and uninduced cultures, and media only negative controls. “Bulk” refers to scCO₂ phase-extracted headspace volumes from all induced, uninduced, or negative control reactors. Glucose consumption designated as zero, low/zero (0-5% consumed), low (5-20%), medium (20-40%), and high (>40%). “LOD” = limit of detection for scCO₂-extracted isobutanol (0.13 mM). Isobutanol is shown for each culture in gray bars; and isopentanol is shown in white above the isobutanol bars. Independent replicates are identified by the letter beneath each column.

FIG. 32 shows a schematic of an example of a continuously CO₂-stripped bioreactor. The reactor includes two distinct phases: a scCO₂ phase and a media phase containing the cells producing the bioproduct (*e.g.*, biofuels). The media/substrate and scCO₂ are continuously provided to the reactor, and scCO₂, bioproducts, media, and cells are removed.

FIG. 33 presents n-butanol extraction profiles at varying scCO₂ flow rates (12.5 mL/min, 9 mL/min, 5.4 mL/min, 3.2 mL/min, and 1.26 mL/min).

FIG. 34 presents isobutanol extraction profiles at varying scCO₂ flow rates (9 mL/min, 5.4 mL/min, 3.2 mL/min, and 1.26 mL/min).

FIG. 35 is a graph comparing the n-butanol extraction profile to the isobutanol extraction profile using a scCO₂ flow rate of 5.4 mL/min.

FIG. 36 presents n-pentanol extraction profiles at varying scCO₂ flow rates (9 mL/min, 5.4 mL/min, 3.2 mL/min, and 1.26 mL/min).

FIG. 37 presents n-hexanol extraction profiles at varying scCO₂ flow rates (9 mL/min, 5.4 mL/min, and 1.28 mL/min).

FIGs. 38A and 38B show energy demand for butanol extraction. FIG. 38A shows a schematic for butanol extraction. FIG. 38B shows an energy consumption for butanol extraction using scCO₂.

DETAILED DESCRIPTION OF SOME EMBODIMENTS

Described herein are methods of producing bioproducts by culturing cells in a multiphase reactor in which the solvent phase comprises supercritical CO₂ (scCO₂), near critical CO₂, or liquid CO₂. Also provided herein are cells, including cells that have been genetically engineered to express heterologous enzymes and produce bioproducts, and reactors for use in the methods described herein. The invention is based, at least in part, on the identification and isolation of microorganisms that are resistant to scCO₂ and are capable of replicating and producing bioproducts when cultured in the presence of scCO₂. Although scCO₂ is frequently used as a solvent for compound extraction and *in vitro* biocatalysis, it is considered to be inaccessible for *in vivo* microbial bioproduct stripping due to lethal effects on microorganisms. Therefore, the methods described herein unexpectedly provide active bioproduct synthesis and extraction in a scCO₂-exposed multiphase reactor. As liquid CO₂ and near critical CO₂ have similar solvation strength as scCO₂, liquid CO₂, and near critical CO₂ may alternatively be used in the solvent phase.

In vivo bioproduct production using scCO₂ provides a number of benefits, including: reducing contamination in the reactor as scCO₂ is toxic to the majority of microorganisms; providing a high concentration reactant for CO₂ consuming reactions; relieving end product toxicity effects by *in situ* extraction (stripping); and alleviating the need for additional product dehydration due to the desiccating effects of scCO₂. Additionally, scCO₂ may be decanted from the culture, leaving the bioproducts at a high concentration.

This invention is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments and of being practiced or of being carried out in various ways. Also, the phraseology and terminology used herein is for the purpose of description and should not be regarded as limiting. The use of “including,” “comprising,” or “having,” “containing,” “involving,” and variations of thereof herein, is meant to encompass the items listed thereafter and equivalents thereof as well as additional items.

The methods described herein involve culturing a cell (or population of cells) in a multiphase reactor comprising an aqueous phase and a solvent phase, which comprises supercritical CO₂, near critical CO₂, or liquid CO₂. As used herein, the term “supercritical CO₂” or “scCO₂” refers to carbon dioxide (CO₂) that is maintained in a state above its critical
5 temperature and critical pressure. For example, supercritical CO₂ may be maintained at a temperature of at least about 31°C and a pressure of at least 72.8 atm.

The presence of scCO₂ may acidify the environment (culture) as well as introduce a range of potentially toxic stresses on microbial cells. Due to its predominantly non-polar solvent chemistry, scCO₂ penetrates bacterial cell walls and membranes, extracting fatty acids, lipids,
10 and other intracellular materials that preferentially partition into the scCO₂ from the cytosol (Ulmer et al., 2002). Inside the cell, scCO₂ may decrease intracellular pH, disable enzymes, disrupt protein synthesis, and cause cellular desiccation, ultimately resulting in cell death (Spilimbergo and Bertucco, 2003; Kirk, 2011; Zhang et al., 2006).

As used herein, the term “near critical CO₂” refers to carbon dioxide (CO₂) that is
15 maintained in a state that is substantially near the critical point (near the critical temperature and critical pressure).

As also used herein, the term “liquid CO₂” refers to carbon dioxide (CO₂) that is maintained in the liquid state.

In some embodiments, the cells that are cultured under scCO₂, near critical CO₂, or liquid
20 CO₂ headspace are viable cells, meaning the cells are metabolically active. In some embodiments, the cells are bacterial cells. In some embodiments the cells are resistant to scCO₂, near critical CO₂, or liquid CO₂. As used herein, a cell is considered to be “resistant” to scCO₂ if the cell experiences fewer deleterious effects upon exposure to scCO₂ than other cells. In some embodiments, a population of cells that is resistant to scCO₂ undergoes less cell death than cells
25 in a population of cells that are not resistant to scCO₂. Alternatively or in addition, a cell that is resistant to scCO₂ may be described as being “tolerant” to scCO₂.

Cells that are resistant to scCO₂ may exhibit any of a number of characteristics that may provide for the resistance to scCO₂ and/or may employ any adaptive mechanism to defend
30 against the lethal effects of scCO₂. For example, the rigidity of gram-positive cell walls afforded by dense layers of peptidoglycan (comprising up to 90% of the thickness) may confer enhanced tolerance to exposure by reducing the rate of scCO₂ penetration into the cell (Oulé et al., 2010).

Examples of adaptive mechanisms used by cells in the presence of scCO₂ to maintain viability include the dense matrix of extrapolymeric substances (EPS) composed of carboxylic acids, polysaccharides, amino acids, and other components that are commonly found in biofilms, thought to limit scCO₂ cellular envelope penetration through chemical interaction with CO₂

(Mitchell et al., 2008; Braissant et al., 2003); modifications of microbial membrane structure (e.g., branching and chain length, fatty acid saturation) which may enable a cell to calibrate its membrane fluidity and permeability in response to solvent, environmental and nutrient conditions (Spilimbergo et al., 2009; Isenschmid et al., 1995; Mitchell et al., 2008; Spilimbergo and Bertucco, 2003; Klein et al., 1999; Mangelsdorf et al., 2009; Kieft et al., 1994; Mukhopadhyay et al., 2006); and expression of alternative transcription factors which trigger the general stress response, acid stress response, and sporulation cascade, each of which induces physiological adaptations to offset scCO₂-related stresses (Ogasawara et al., 2012; Liao et al., 2011; Martin-Galiano et al., 2001; Richard and Foster, 2004; Foster, 1999; Gaidenko and Price, 1998).

Peet et al., (2015) investigated the extent to which scCO₂-resistant *Bacillus* strains alter their protein expression and cell wall and membrane compositions in response to culturing under headspaces of 1 and 100 atm of CO₂ and N₂. Changes in the proteomic profiles of cells when exposed to scCO₂ may indicate that scCO₂ acclimation can be induced or enhanced, for example, by acclimating a cell to scCO₂ prior to inoculation of a culture (media) or reactor comprising scCO₂. In some embodiments, a cell, such as a cell in the vegetative state or a cell in spore form, may be acclimated to scCO₂ by exposing the cell to scCO₂ for a short period prior to inoculating the culture or reactor.

As described herein, the inventors have surprisingly identified bacterial strains that are resistant or tolerant to scCO₂. In some embodiments, scCO₂ resistant cells are provided that are capable of growth in supercritical CO₂. In some embodiments, the cell is a bacterial cells, such as a bacterial cell belonging to the genus *Firmicutes*. In some embodiments the bacterial cell is capable of forming spores. In some embodiments, the cell belongs to the genus *Bacillus*. In some embodiments, the bacterial cell is a *Bacillus cereus*, *Bacillus subterraneus*, *Bacillus amyloliquefaciens*, *Bacillus safensis*, or a *Bacillus megaterium* cell, such as *Bacillus megaterium* strain SR7. In some embodiments, the supercritical-CO₂ tolerant strain has a bacterial 16S rRNA gene sequence that has at least 99.5% sequence identity with the 16S rRNA of *B. megaterium*

SR7, *B. megaterium* DSM319, or *B. licheniformis* MR4. The identity may be higher or lower than this percent identity, such as about 98.0%, 98.1%, 98.2%, 98.3%, 98.4%, 98.5%, 98.6%, 98.7%, 98.8%, 98.9%, 99.0%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, or 99.9% identity.

5

The 16S rRNA gene sequence of *B. megaterium* SR7 is provided by SEQ ID NO: 18:

gatgaacgctggcggcgtgcctaatacatgcaagtcgagcgaactgattagaagcttgccttatgacgttagcggcggacgggtgagtaa
cacgtgggcaacctgcctgtgagactgggataacttcgggaaaccgaagctaataccggataggatcttctcctcatgggagatgattgaa
agatggtttcggctatcacttacagatgggcccgcggtgcattagctagttggtaggtaacggctcaccaaggcaacgatgcatagccga
10 cctgagagggtgatcgccacactgggactgagacacggcccagactcctacgggaggcagcagtagggaaatcttccgcaatggacga
aagtctgacggagcaacgccgcgtgagtgatgaaggcttccgggtcgtaaaactctgttggtagggaagaacaagtacgagagtaactgct
cgtacctgacgggtacctaaccagaaagccacggctaactacgtgccagcagccgcggtaatacgtagggtggcaagcgttatccggaatta
ttgggcgtaaagcgcgcgagggcgtttcttaagtctgatgtgaaagcccacggctcaaccgtggagggtcattggaaactggggaacttg
agtgcagaagagaaaagcgggaattccacgtgtagcgggtgaaatgcgtagagatgtggaggaacaccagtggcgaaggcggcgttttggc
15 tgtactgacgctgagggcgcgaaagcgtggggagcaaacaggattagataccctggtagtcacgccgtaaacgatgagtgctaagtgtt
agagggtttccgccctttagtgctgcagctaacgcattaagcactccgcctggggagtagcgtcgcaagactgaaactcaaaggaattgac
ggggggccgcacaagcgggtggagcatgtggttaattcgaagcaacgcgaagaaccttaccaggtcttgacatcctctgacaactctagag
atagagcgttcccccttcgggggacagagtacaggtggtgcatggtgtcgtcagctcgtgctgtagatgttgggttaagtcccgcaacga
gcgcaaccttgatcttagttgccagcatttagttgggcaactctaaggtgactgccggtgacaaaccggaggaaggtggggatgacgtcaa
20 atcatcatgcccttatgacctgggctacacacgtgctacaatggatggtacaaagggtgcaagaccgcgaggtcaagccaatcccataa
aaccattctcagttcggattgtaggtgcaactgcctatatgaagctggaatcgctagtaatcgcgatcagcatgcccggtggaatacgtt
cccgggccttgtagacaccgccgtcacaccacgagagtttgaacacccgaagtcggtggagtaaccgtaaggagctagccgcctaag
gtgggacagatgattggggtgaagtcgtaacaa

25 The 16S rRNA gene sequence of *B. licheniformis* MR4 is provided by SEQ ID NO: 19:

agagtttgatcctggctcaggacgaacgctggcggcgtgcctaatacatgcaagtcgagcggaccgacgggagcttgctcccttaggtca
gcggcgacgggtgagtaaacacgtgggtaacctgcctgtaagactgggataactccgggaaaccggggctaataccgatgcttgattga
accgcatggttcaatcataaaaggtggcttttagctaccacttacagatggaccgcggcgcaatagctagttggtaggtaacggctcacca
aggcgacgatgcgtagccgacctgagagggtgatcgccacactgggactgagacacggcccagactcctacgggaggcagcagtag
30 ggaatcttccgcaatggacgaaagtctgacggagcaacgccgcgtgagtgatgaagggtttcggatcgtaaaactctgttggtagggaagaa
caagtaccgttcgaataggcgccaccttgacgggtacctaaccagaaagccacggctaactacgtgccagcagccgcggtaatagtag
gtggcaagcgttgcgggaattattgggcgtaaagcgcgcgagggcgtttcttaagtctgatgtgaaagccccggctcaaccggggag
ggtcattggaaactggggaacttgagtgagaaaggagagtggaattccacgtgtagcgggtgaaatgcgtagagatgtggaggaacac
cagtggcgaaggcgactctctggtctgtaactgacgtgaggcgcgaaagcgtggggagcgaacaggattagataccctggtagtcac
35 gccgtaaacgatgagtgctaagtgttagagggtttccgccctttagtgctgcagcaaacgcattaagcactccgcctggggagtagcgtcgc
aagactgaaactcaaaggaattgacggggccgcacaagcgggtggagcatgtggttaattcgaagcaacgcgaagaaccttaccaggt
cttgacatcctctgacaaccttagagatagggttcccccttcgggggacagagtacaggtggtgcatggtgtcgtcagctcgtgctgtag
atgttgggttaagtcccgcaacgagcgaaccttgatcttagttgccagcattcagttgggcaactctaaggtgactgccggtgacaaaccg
gaggaaggtggggatgacgtcaaatcatcatgcccttatgacctgggctacacacgtgctacaatgggcagaacaaagggcagcgaag
40 ccgcgaggtgaagccaatcccaaaatctgttctcagttcggatcgagctgcaactgcactgcgtgaagctggaatcgctagtaatcgcg
gatcagcatgcccggtgaaatcgttccgggcttgtagacaccgccgtcacaccacgagagtttgaacacccgaagtcggtgaggtga

acccttttgagccagccgccgaaggtgggacagatgattggggtgaagtcgtaacaaggtagccgtatcggaaggtgcggctggatcacc
tcctttct

In general, *B. megaterium* SR7 may be distinguished from other industrial *B. megaterium*
5 strains by analyzing the nucleotide sequence of any of a number of genes that differ between the
strains. For example, *B. megaterium* SR7 may be distinguished from *B. megaterium* QM B1551
and DSM319 by analyzing the nucleotide sequence of *atpD*, *dnaK*, and/or *groEL*.

The nucleotide sequence of *atpD* of *B. megaterium* SR7 is provided by SEQ ID NO: 20.

10 atgagtcaaccagctgtagccaagcgtatgcactagctcttttcaattagcaacagaaaaacagatgatcgatgaaatgcaagaccagcta
caaatcgttgaagaggtgttggctaaaacacctgaattaatggatgtattaactcatcaaaaaattacaattgagcgaaaaaacagtttgaag
tgaggcatttgcgtgaactttaccaactgttcaacatacggttcttattattagagcgtcaccgcattcaaatgttagccaaatggtacaaga
gtatcgtttcctagcgaacgaagtacgtggcgtggcagatgcaactgtttattctgtcaaacctttaagcgcagatgagaaaagagcaatctc
gcaatcatttgcctcaaaagttgaaaacatacgttaaatatttcaaatgtagtagataaaagcctaatacggcggtgaagcttcgattggta
15 atcgtatctatgatggcagcattagcagcaaatagaaacgatccaccgaggacttctgcacacagatcgtag (SEQ ID NO: 20)

The nucleotide sequence of *dnaK* of *B. megaterium* SR7 is provided by SEQ ID NO: 21.

atggctcctgtgcgtcaagctttaaagatgcaggtctttctgcaagcgaacttgataaagtaatcttagttggtggttcaactcgatcccagc
ggtacaagatgcaatcaaaaaagaaactgggtcaagatcctcataaaggtgaaatcctgatgaagtagttgcaacttggcgcagcaattcaag
20 gtggcgtgttaactgggtgatgtaaaaagacgtgtattactagacgtaacgcctttatcactaggtatcgaacaatgggtggcgtatttacaag
ctaattgagcgtatacgaatccaacaagtaaatcacaagtattctcaacggctgcagatagccaaacagctgtagatattcacgttttc
aaggtgagcgcccaatgtctgcagacaacaaacgctaggacgtttccaattaacagacattcctcctgcaccacgcggagtacctcaaat
cgaagtatcattcgatattgataagaatggatcgtaaacgttcgtgcaaaagatttaggtacaaacaaagagcaggctattacaattaaatctt
caactggtttatcagatgacgaaatcgaccgatggtaaaagaagcgggaagaaaacgcagatgctgataagcaacgtaaaagaagaagt
25 gaactacgcaacgaagcagatcaattagtgttcacaactgaaaaacattaaaagatcttgaaggaaaagtagaagaagctgaagtaacaa
aagctaacgaagcaaaagatgctttaaagctgcgattgaaaagaatgaccttgaagaaatcaaaagcaaaaaagatgaacttcaagaaat
cgttcaagcgttaactgtaaaattgtatgagcaagctcaacaagctcagcaagcaggtgaacaaggcgtcaaatgatgatgtttagatg
cagagtttgaggaagtaaacgacgacaaaaataa (SEQ ID NO: 21)

30 The nucleotide sequence of *groEL* of *B. megaterium* SR7 is provided by SEQ ID NO: 22.

atggcaaaagacattaaatttagcgaagaagcacgtcgcgcaatgtactggtgtagatacattggcaaatgctgtaaaagtaacgcttgg
acaaaaggtcgtaacgttgattagaaaagaaattcgggtcaccgcttattacaaatgatggtgaacaattgcaaaagaaatcgaattagaa
gacgcatttgaaaacatgggtgctaaattagtagccgaggttctagcaaaacaaacgacgttgctggtgacggtacaactactgcaacagt
tttagcgcaagcaatgatcagagaaggtcttaaaaacgtaacggctggtgctaaccaatgggtatccgtaaaaggtatggaaaaagcagta
35 gctgtagcgggtgaagaactaaaagcaatctctaaaccaattcaaggtaaagattcaattgctcaagtagcggctatctcagcagctgacgaa
gaagtaggtcaactaatcgctgaagcaatggagcgcgttggtaacgacggcggttatcacactgaagaatcaaaaggtttcacaactgaatt
agaagtggtagaaggtatgcagtttgaccgtggatgcatctccttacctggttaactgattcagataaaatggaagctgtattagatgatccat
acatcttaacacagacaaaaaaatcgtaagattgaagaaatctaccgggtattagagcaaggtgttcaacaaggcaagcctctattaatcatc
gctgaagacgtagaaggcgaagctttgacaacattagttgtgaacaaacttcgtggtacattttagcagctgtagctgtttaaagctcctggtttgg
40 tgatcgtcgtaaagcaatgctacaagacgttgcgatcttaacaggcggagaagtaatacactgaagagcttggctttagctttaaacaacagcag
gcatcgatcaattaggtcgcgttctaaaattgttgaacaaaagaaaatacaacagttgtaaacggtgcaggaaacgcagaagatatcctag

5 caccgctaaaccaaatacaagctcagcttgaagaaacaacttcagagtttgaccgtgaaaaattacaagagcgttagcaaaaacttgcgtgt
 ggcgtagctgtaatacaagttggtgcggcaactgaaactgagttaaaagaacgtaaattacgtattgaagatgcattaaactctacgcgtgct
 gcggttgaagaaggatcgtagctgggtggtggtactgcattagtaataatctataataaagtagcaagcatcgaagctgacgggtgacactgct
 acaggtatcaacatcgtattacgtgcgattgaagagcctgtacgtcaaatcgctcacaatgctggtttagaaggatcagtaatcgttgagcgtc
 10 taaaaggcgaagcagttggaactggattcaacgctgcaactggcgagtgggtaaatatgctagacactgggtatcgttgaccaacaaaagt
 aacgcgttcagctcttcaaatgcttcttctgtagcggctatgttcttaacaactgaagcagttgttctgacaagccagaagaaggcggagc
 acctgcaatgcctgacatgggcggcatgggtggaatgggcggcatgatgtaa (SEQ ID NO: 22)

10 In some embodiments, the supercritical-CO₂ tolerant strain has a *atpD* gene having at
 least 99.5% identity to SEQ ID NO: 20. In some embodiments, the supercritical-CO₂ tolerant
 strain has a *dnaK* gene having at least 99.5% identity to SEQ ID NO: 21. In some embodiments,
 the supercritical-CO₂ tolerant strain has a *groEL* gene having at least 99.5% identity to SEQ ID
 NO: 22. The identity may be higher or lower than this percent identity, such as about 98.0%,
 98.1%, 98.2%, 98.3%, 98.4%, 98.5%, 98.6%, 98.7%, 98.8%, 98.9%, 99.0%, 99.1%, 99.2%,
 15 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, or 99.9% identity.

The supercritical-CO₂ tolerant strain in one preferred embodiment is *B. megaterium* strain
 SR7. A strain deposit has been submitted to the American Tissue Culture Collection (ATCC) for
 this strain. In another embodiment, the supercritical-CO₂ tolerant strain is *B. megaterium* strain
 DSM319 (Vary et al. *Appl. Microbiol. Biotechnol.* (2007) 76(5): 957-67; genome RefSeq
 20 number is NC_041403.1). In some embodiments, the scCO₂ resistant cell is capable of growth at
 a pressure of greater than about 71 atm, such as about 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81,
 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105,
 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124,
 125, 126, 127, 128, 129, 130 atm or more.

25 In some embodiments, the supercritical CO₂ resistant microbial strain is capable of
 growth below an acidity of about pH 4.6, such as below about pH 4.5, pH 4.4, pH 4.3, pH 4.2,
 pH 4.1, pH 4.0, pH 3.9, pH 3.8, pH 3.7, pH 3.6, pH 3.5, pH 3.4, pH 3.3, pH 3.2, pH 3.1, pH 3.0,
 pH 2.9, pH 2.8, pH 2.7, pH 2.6, pH 2.5, pH 2.4, pH 2.3, pH 2.2, pH 2.1, pH 2.0, or lower.

30 Supercritical CO₂ resistant cells can in some embodiments be identified by the following
 non-limiting characteristics and/or methods. In some embodiments, a supercritical CO₂ tolerant
 microbial strain is a microbial strain in which cell survival is maintained for at least 6 hours
 under supercritical CO₂ conditions as measured by membrane integrity probing. Membrane
 integrity probing can be performed, for example, by contacting the strain (*e.g.*, cells of the strain)

with a dye that is excluded by live cells and that can cross the cell membrane and/or cell wall in cells that are not alive. Methods and dyes used for membrane integrity probing are known in the art. Total cell counts can be compared to counts of cells that exclude dye to determine if the microbial strain has cells surviving under supercritical CO₂ conditions and therefore is a
5 supercritical CO₂ tolerant microbial strain. In some embodiments, a scCO₂ resistant cell or strain is one that has more than 0.1% live cells after 6 hours under a supercritical CO₂ headspace, preferably more than 0.5%, 1%, 2%, 5%, 10%, 15%, 20%, 25%, 30%, or more live cells after 6 hours under supercritical CO₂ conditions such as a supercritical CO₂ headspace.

In some embodiments, a scCO₂ resistant cell is a microbial strain that demonstrates cell
10 growth under continued exposure to scCO₂ environments as measured by change over time of total cell count. Methods to determine change over time of total cell count are known to the person of skill in the art, and include such methods as epifluorescent DNA staining, membrane integrity probing and/or a method to quantify biomass. Samples of the culture can be taken at predetermined times or intervals and the samples analyzed for the characteristics described
15 above. For example, samples can be taken each day for two or more days, such as at any two of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 days after inoculation of the culture. A comparison of the values of total cell count on the two or more days can be made to determine if the microbial strain demonstrates cell growth under continued exposure to supercritical CO₂ and thus is a scCO₂ resistant cell. In some embodiments, the supercritical CO₂ tolerant microbial strains has a
20 doubling time of about 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, or 2.0 days, such as from 1.2 to 1.8 days, or 1.4 to 1.6 days.

Aspects of the present disclosure provide methods for producing a bioproduct. As used herein, a “bioproduct” refers to any molecule that may be produced by a cell associated with the invention and may be collected or recovered from the culture. In some embodiments, the
25 bioproduct comprises a carbon chain that contains 3, 4, 5, 6, or 7 carbon molecules. In some embodiments, the bioproduct has properties that allow the bioproduct to partition into the scCO₂, near critical CO₂, or liquid CO₂. In some embodiments, the bioproduct has hydrophobic characteristics. In some embodiments, the bioproduct is a fuel product. In some embodiments, the bioproduct is a non-fuel product. In some embodiments, the bioproduct is a hydrocarbon. In
30 some embodiments, the bioproduct is an oxygenated hydrocarbon. In some embodiments, the bioproduct is a carboxylic acid. In some embodiments, the bioproduct is an alcohol, such as a

primary alcohol, a secondary alcohol, or a tertiary alcohol. In some embodiments, the bioproduct is an aldehyde. In some embodiments, the bioproduct is a fatty acid. In some embodiments, the bioproduct is a ketone.

In some embodiments, the bioproduct is a natural product. As used herein, a “natural product” refers to any bioproduct that is produced by the cell and does not require recombinant expression of an enzyme that is not naturally expressed in the cell. In some embodiments, the cell has been genetically modified to produce enhanced titers of the natural product. Examples of natural products include, without limitation, ethanol, succinate, lactate, and acetate. In some embodiments, the cell has not been genetically modified and produces a natural product.

Also encompassed by the term bioproduct are heterologous molecules, for example molecules that are not natural products of the cells. In some embodiments, the bioproduct is a high value molecule, for example a biofuel. In some embodiments, the bioproduct is a substrate that may be used to produce a high value molecule. In some embodiments, the bioproduct is an alcohol, such as a short or medium chain alcohol. In some embodiments, the bioproduct is an aldehyde. Non-limiting examples of bioproducts that may be produced using the cells and methods described herein are 1-propanol, 1-butanol, 1-pentanol, 1-hexanol, 1-methyl-ethanol (isopropanol), 2-methyl-propanol (isobutanol), 3-methyl-butanol (isopentanol), 4-methyl-pentanol (isohexanol), 2-phenylethanol, acetaldehyde, 1-butanal, 1-pentanal, 1-hexanal, 2-methyl-propanal (isobutanal), 3-methyl-butanal (isopentanal), 4-methyl-pentanal (isohexanal), and 2-phenylethanal.

In some embodiments, the cell has been genetically engineered to express one or more heterologous enzymes. The terms “heterologous enzyme” or “heterologous polypeptide” refer to any enzyme or polypeptide that is expressed recombinantly in the host cell. For example, a host cell may be genetically engineered to express an enzyme or polypeptide involved in a biosynthetic pathway that is not present in the host cell. In some embodiments, a host cell may be genetically engineered to express an enzyme or polypeptide that has one or more desired properties (*e.g.*, activity level, specificity, etc). In some embodiments, the one or more heterologous enzymes or heterologous polypeptides are obtained from another organism. For example, in some embodiments, the cell is a bacterial cell and the heterologous enzyme or heterologous polypeptide is a yeast enzyme or protein. In some embodiments, the one or more heterologous enzyme or heterologous polypeptide are obtained from another species. For

example, in some embodiments, the cell is a strain belonging to *Bacillus megaterium* and the heterologous enzyme or heterologous polypeptide are obtained from another bacterial species, for example *E. coli*. As will be appreciated by one of ordinary skill in the art, any of the heterologous enzymes described herein may be obtained from any source known in the art.

5 In some embodiments, the bioproduct is 1-propanol. In some embodiments, production of 1-propanol may involve expressing a threonine synthesis and/or an aldehyde-alcohol dehydrogenase, such as an aldehyde-alcohol dehydrogenase from *Clostridium acetobutylicum*.

In some embodiments, the bioproduct is 1-butanol. In some embodiments, production of 1-butanol may involve expressing a beta-keto-thiolase, an enoyl-CoA hydratase, a 3-

10 hydroxybutyryl-CoA dehydrogenase, a Trans-enoyl-CoA reductase, and/or an Aldehyde-alcohol dehydrogenase. In some embodiments, the beta-keto-thiolase may be from *E. coli*. In some embodiments, the enoyl-CoA hydratase may be from *Clostridium acetobutylicum*. In some embodiments, the 3-hydroxybutyryl-CoA dehydrogenase may be from *Clostridium acetobutylicum*. In some embodiments, the trans-enoyl-CoA reductase may be from *T.*

15 *denticola*. In some embodiments, the aldehyde-alcohol dehydrogenase may be from *Clostridium acetobutylicum*.

In some embodiments, the bioproduct is 1-pentanol. In some embodiments, production of 1-pentanol may involve expressing a threonine synthetase, a beta-keto-thiolase, an enoyl-CoA hydratase, a 3-hydroxybutyryl-CoA dehydrogenase, trans-enoyl-CoA reductase, and/or an

20 aldehyde-alcohol dehydrogenase. In some embodiments, beta-keto-thiolase may be from *C. necator*. In some embodiments, the enoyl-CoA hydratase may be from *Clostridium acetobutylicum*. In some embodiments, the 3-hydroxybutyryl-CoA dehydrogenase may be from *Clostridium acetobutylicum*. In some embodiments, the trans-enoyl-CoA reductase may be from *T. denticola*. In some embodiments, the aldehyde-alcohol dehydrogenase may be from

25 *Clostridium acetobutylicum*.

In some embodiments, the bioproduct is 1-hexanol. In some embodiments, production of 1-hexanol may involve expressing a beta-keto-thiolase, Beta-keto-acyl-CoA reductase, an enoyl-CoA hydratase, a trans-enoyl-CoA reductase, and/or an aldehyde-alcohol dehydrogenase.

In some embodiments, beta-keto-thiolase may be from *C. necator*. In some embodiments, the beta-keto-acyl-CoA reductase may be from *C. necator*. In some embodiments, the enoyl-CoA hydratase may be from *C. necator*. In some embodiments, the trans-enoyl-CoA reductase may

be from *T. denticola*. In some embodiments, the aldehyde-alcohol dehydrogenase may be from *S. cerevisiae*.

In some embodiments, the bioproduct is 1-methyl-ethanol. In some embodiments, production of 1-methyl-ethanol may involve expressing a beta-keto-thiolase, an acetoacetyl-CoA transferase, an acetoacetate decarboxylase, and/or an aldehyde-alcohol dehydrogenase. In some
5 embodiments, the beta-keto-thiolase is from *C. necator*. In some embodiments, the acetoacetyl-CoA transferase is from *C. necator*. In some embodiments, the acetoacetate decarboxylase is from *C. necator*. In some embodiments, the aldehyde-alcohol dehydrogenase is from *Clostridium acetobutylicum*.

10 In some embodiments, the bioproduct is 2-methyl-propanol. In some embodiments, production of 2-methyl-propanol involves expressing an acetolactate synthase, a ketol-acid reductoisomerase, a dihydroxy-acid dehydratase, an alpha-ketoisovalerate decarboxylase and/or an alcohol dehydrogenase. In some embodiments, the ketol-acid reductoisomerase is from *E. coli*. In some embodiments, the dihydroxy-acid dehydratase is from *E. coli*. In some
15 embodiments, the alpha-ketoisovalerate decarboxylase is from *E. coli*. In some embodiments, the alcohol dehydrogenase is from *E. coli*.

In some embodiments, the bioproduct is 3-methyl-butanol. In some embodiments, production of 3-methyl-butanol involves expressing an alpha-ketoisovalerate decarboxylase and/or an alcohol dehydrogenase. In some embodiments, the alpha-ketoisovalerate
20 decarboxylase is from *L. lactis*. In some embodiments, the alcohol dehydrogenase is from *E. coli*.

In some embodiments, the bioproduct is 4-methyl-pentanol. In some embodiments, production of 4-methyl-pentanol involves expressing an acetolactate synthase, a ketol-acid reductoisomerase, a dihydroxy-acid dehydratase, an alpha-ketoisovalerate decarboxylase, an aldehyde dehydrogenase, a propionyl-CoA transferase, a beta-keto-thiolase, a beta-keto-acyl-
25 CoA reductase, an enoyl-CoA hydratase, a trans-enoyl-CoA reductase, a carboxylic acid reductase, a 4'-phosphopantetheinyl transferase, and/or an alcohol dehydrogenase. In some embodiments, the acetolactate synthase is from *B. subtilis*. In some embodiments, the ketol-acid reductoisomerase is from *E. coli*. In some embodiments, the dihydroxy-acid dehydratase is from *E. coli*. In some embodiments, the alpha-ketoisovalerate decarboxylase is from *L. lactis*. In
30 some embodiments, the aldehyde dehydrogenase is from *F. johnsoniae*. In some embodiments, the propionyl-CoA transferase is from *C. propionicum*. In some embodiments, the beta-keto-

thiolase is from *C. necator*. In some embodiments, the beta-keto-acyl-CoA reductase is from *C. necator*. In some embodiments, the enoyl-CoA hydratase is from *C. necator*. In some embodiments, the trans-enoyl-CoA reductase is from *T. denticola*. In some embodiments, the carboxylic acid reductase is from *N. iowensis*. In some embodiments, the 4'-

5 phosphopantetheinyl transferase is from *B. subtilis*. In some embodiments, the alcohol dehydrogenase is from *Leifsonia* sp strain S749.

In some embodiments, the bioproduct is 2-phenylethanol. In some embodiments, production of 2-phenylethanol involves expressing alpha-ketoisovalerate decarboxylase and/or an alcohol dehydrogenase. In some embodiments, the alpha-ketoisovalerate decarboxylase is
10 from *L. lactis*. In some embodiments, the alcohol dehydrogenase is from *E. coli*.

In some embodiments, the bioproduct is acetaldehyde. In some embodiments, production of Acetaldehyde involves expressing an acetaldehyde dehydrogenase, such as an acetaldehyde dehydrogenase from *E. coli*.

In some embodiments, the bioproduct is 1-butanal. In some embodiments, production of
15 1-butanal involves expressing a beta-keto-thiolase, an enoyl-CoA hydratase, 3-hydroxybutyryl-CoA dehydrogenase, a trans-enoyl-CoA reductase, a thioesterase, a carboxylic acid reductase, and/or a 4'-phosphopantetheinyl transferase. In some embodiments, the beta-keto-thiolase is from *E. coli*. In some embodiments, the enoyl-CoA hydratase is from *Clostridium acetobutylicum*. In some embodiments, the 3-hydroxybutyryl-CoA dehydrogenase is from
20 *Clostridium acetobutylicum*. In some embodiments, the trans-enoyl-CoA reductase is from *T. denticola*. In some embodiments, the thioesterase is from *E. coli*. In some embodiments, the carboxylic acid reductase is from *N. iowensis*. In some embodiments, the 4'-phosphopantetheinyl transferase is from *B. subtilis*.

In some embodiments, the bioproduct is 1-pentanal. In some embodiments, the
25 production of 1-pentanal involves expressing a threonine synthesis, a beta-keto-thiolase, an enoyl-CoA hydratase, a 3-hydroxybutyryl-CoA dehydrogenase, a trans-enoyl-CoA reductase, a thioesterase, a carboxylic acid reductase, and/or a 4'-phosphopantetheinyl transferase. In some embodiments, the beta-keto-thiolase is from *C. necator*. In some embodiments, the enoyl-CoA hydratase is from *Clostridium acetobutylicum*. In some embodiments, the 3-hydroxybutyryl-CoA dehydrogenase is from *Clostridium acetobutylicum*. In some embodiments, the trans-enoyl-CoA reductase is from *T. denticola*. In some embodiments, the thioesterase is from *E.*
30

coli. In some embodiments, the carboxylic acid reductase is from *N. iowensis*. In some embodiments, the 4'-phosphopantetheinyl transferase is from *B. subtilis*.

In some embodiments, the bioproduct is 1-hexanal. In some embodiments, the production of 1-hexanal involves expressing a beta-keto-thiolase, a beta-keto-acyl-CoA reductase, an enoyl-CoA hydratase, a trans-enoyl-CoA reductase, a thioesterase, a carboxylic acid reductase, and/or a 4'-phosphopantetheinyl transferase. In some embodiments, the beta-keto-thiolase is from *C. necator*. In some embodiments, the beta-keto-acyl-CoA reductase is from *C. necator*. In some embodiments, the enoyl-CoA hydratase is from *C. necator*. In some embodiments, the trans-enoyl-CoA reductase is from *T. denticola*. In some embodiments, the thioesterase is from *E. coli*. In some embodiments, the carboxylic acid reductase is from *N. iowensis*. In some embodiments, the 4'-phosphopantetheinyl transferase is from *B. subtilis*.

In some embodiments, the bioproduct is 2-methyl-propanal. In some embodiments, the production of 2-methyl-propanal involves expressing an acetolactate synthase, a ketol-acid reductoisomerase, a dihydroxy-acid dehydratase, and/or an alpha-ketoisovalerate decarboxylase. In some embodiments, the acetolactate synthase is from *B. subtilis*. In some embodiments, the ketol-acid reductoisomerase is from *E. coli*. In some embodiments, the dihydroxy-acid dehydratase is from *E. coli*. In some embodiments, the alpha-ketoisovalerate decarboxylase is from *L. lactis*.

In some embodiments, the bioproduct is 3-methyl-butanal. In some embodiments, the production of 3-methyl-butanal involves expressing an alpha-ketoisovalerate decarboxylase, such as an alpha-ketoisovalerate decarboxylase from *L. lactis*.

In some embodiments, the bioproduct is 4-methyl-pentanal. In some embodiments, the production of 4-methyl-pentanal involves expressing an acetolactate synthase, a ketol-acid reductoisomerase, a dihydroxy-acid dehydratase, an alpha-ketoisovalerate decarboxylase, an aldehyde dehydrogenase, a propionyl-CoA transferase, a beta-keto-thiolase, a beta-keto-acyl-CoA reductase, an enoyl-CoA hydratase, a trans-enoyl-CoA reductase, a carboxylic acid reductase and/or 4'-phosphopantetheinyl transferase from *B. subtilis*. In some embodiments, the acetolactate synthase is from *B. subtilis*. In some embodiments, the ketol-acid reductoisomerase is from *E. coli*. In some embodiments, the dihydroxy-acid dehydratase is from *E. coli*. In some embodiments, the alpha-ketoisovalerate decarboxylase is from *L. lactis*. In some embodiments, the aldehyde dehydrogenase is from *F. johnsoniae*. In some embodiments, the propionyl-CoA

transferase is from *C. propionicum*. In some embodiments, the beta-keto-thiolase is from *C. necator*. In some embodiments, the beta-keto-acyl-CoA reductase is from *C. necator*. In some embodiments, the enoyl-CoA hydratase is from *C. necator*. In some embodiments, the trans-enoyl-CoA reductase is from *T. denticola*. In some embodiments, the carboxylic acid reductase is from *N. iowensis*. In some embodiments, the 4'-phosphopantetheinyl transferase is from *B. subtilis*.

In some embodiments, the bioproduct is 2-phenylethanol. In some embodiments, the production of 2-phenylethanol involves expressing an alpha-ketoisovalerate decarboxylase, such as an alpha-ketoisovalerate decarboxylase from *L. lactis*.

In some embodiments, the bioproduct is isobutanol. In some embodiments, the production of isobutanol involves expressing an isoketovalerate decarboxylase and an alcohol dehydrogenase. In some embodiments, the isoketovalerate decarboxylase is from *L. lactis*. In some embodiments, the alcohol dehydrogenase is from *E. coli*.

According to aspects of the invention, cells that recombinantly express one or more genes associated with the invention, and the use of such cells in bioproducts, are provided. It should be appreciated that the genes associated with the invention can be obtained from a variety of sources. For example, homologous genes for use in methods associated with the invention can be obtained from other species and can be identified by homology searches, for example through a protein BLAST search, available at the National Center for Biotechnology Information (NCBI) internet site 10 (www.ncbi.nlm.nih.gov). In some embodiments, genes associated with the invention are synthetic. Any means of obtaining a gene encoding the enzymes associated with the invention are compatible with the instant invention. For example, genes and/or operons associated with the invention can be cloned, such as by PCR amplification and/or restriction digestion, from DNA from any source of DNA which contains the given gene. In some embodiments, a gene and/or operon associated with the invention is synthetic. Any means of obtaining a gene and/or operon associated with the invention is compatible with the instant invention.

In general, homologs and alleles typically will share at least 75% nucleotide identity and/or at least 90% amino acid identity to the sequences of nucleic acids and polypeptides, respectively, in some instances will share at least 90% nucleotide identity and/or at least 95% amino acid identity and in still other instances will share at least 95% nucleotide identity and/or

at least 99% amino acid identity. The homology can be calculated using various, publicly available software tools developed by NCBI (Bethesda, Maryland) that can be obtained through the NCBI internet site. Exemplary tools include the BLAST software, also available at the NCBI internet site (www.ncbi.nlm.nih.gov). Pairwise and ClustalW alignments (BLOSUM30 matrixsetting) as well as Kyte-Doolittle hydropathic analysis can be obtained using the MacVector sequence analysis software (Oxford Molecular Group). Watson-Crick complements of the foregoing nucleic acids also are embraced by the invention.

The invention also includes degenerate nucleic acids which include alternative codons to those present in the native materials. For example, serine residues are encoded by the codons TCA, AGT, TCC, TCG, TCT and AGC. Each of the six codons is equivalent for the purposes of encoding a serine residue. Thus, it will be apparent to one of ordinary skill in the art that any of the serine-encoding nucleotide triplets may be employed to direct the protein synthesis apparatus, in vitro or in vivo, to incorporate a serine residue into an elongating polypeptide. Similarly, nucleotide sequence triplets which encode other amino acid residues include, but are not limited to: CCA, CCC, CCG and CCT (proline codons); CGA, CGC, CGG, CGT, AGA and AGG (arginine codons); ACA, ACC, ACG and ACT (threonine codons); AAC and AAT (asparagine codons); and ATA, ATC and ATT (isoleucine codons). Other amino acid residues may be encoded similarly by multiple nucleotide sequences. Thus, the invention embraces degenerate nucleic acids that differ from the biologically isolated nucleic acids in codon sequence due to the degeneracy of the genetic code. The invention also embraces codon optimization to suit optimal codon usage of a host cell.

In some embodiments, modification of a gene before it is recombinantly expressed in a cell involves codon optimization for expression in a bacterial, yeast, or plant cell. Codon usages for a variety of organisms can be accessed in the Codon Usage Database (www.kazusa.or.jp/codon/). Codon optimization, including identification of optimal codons for a variety of organisms, and methods for achieving codon optimization, are familiar to one of ordinary skill in the art, and can be achieved using standard methods. It should be appreciated that any of the nucleic acids and/or polypeptides described herein can be codon-optimized and expressed recombinantly in a codon-optimized form. In some embodiments, modifying a gene before it is recombinantly expressed in a cell involves making one or more mutations in the gene before it is recombinantly expressed in a cell. For example, a mutation can involve a substitution

or deletion of a single nucleotide or multiple nucleotides. In some embodiments, a mutation of one or more nucleotides in a gene will result in a mutation in the protein produced from the gene, such as a substitution or deletion of one or more amino acids.

The invention also provides modified nucleic acid molecules which include additions,
5 substitutions and deletions of one or more nucleotides. In preferred embodiments, these modified nucleic acid molecules and/or the polypeptides they encode retain at least one activity or function of the unmodified nucleic acid molecule and/or the polypeptides, such as enzymatic activity. In certain embodiments, the modified nucleic acid molecules encode modified polypeptides, preferably polypeptides having conservative amino acid substitutions as are
10 described elsewhere herein. The modified nucleic acid molecules are structurally related to the unmodified nucleic acid molecules and in preferred embodiments are sufficiently structurally related to the unmodified nucleic acid molecules so that the modified and unmodified nucleic acid molecules hybridize under stringent conditions known to one of skill in the art.

For example, modified nucleic acid molecules which encode polypeptides having single
15 amino acid changes can be prepared. Each of these nucleic acid molecules can have one, two or three nucleotide substitutions exclusive of nucleotide changes corresponding to the degeneracy of the genetic code as described herein. Likewise, modified nucleic acid molecules which encode polypeptides having two amino acid changes can be prepared which have, e.g., 2-6 nucleotide changes. Numerous modified nucleic acid molecules like these will be readily envisioned by one
20 of skill in the art, including for example, substitutions of nucleotides in codons encoding amino acids 2 and 3, 2 and 4, 2 and 5, 2 and 6, and so on. In the foregoing example, each combination of two amino acids is included in the set of modified nucleic acid molecules, as well as all nucleotide substitutions which code for the amino acid substitutions. Additional nucleic acid molecules that encode polypeptides having additional substitutions (*e.g.*, 3 or more), additions or
25 deletions (*e.g.*, by introduction of a stop codon or a splice site(s)) also can be prepared and are embraced by the invention as readily envisioned by one of ordinary skill in the art. Any of the foregoing nucleic acids or polypeptides can be tested by routine experimentation for retention of structural relation or activity to the nucleic acids and/or polypeptides disclosed herein.

The invention also encompasses variants of polypeptides. As used herein, a “variant” of
30 a polypeptide is a polypeptide which contains one or more modifications to the primary amino acid sequence of the polypeptide. Modifications which create a variant can be made to a

polypeptide 1) to reduce or eliminate an activity of a polypeptide; 2) to enhance a property of a polypeptide; 3) to provide a novel activity or property to a polypeptide, such as addition of an antigenic epitope or addition of a detectable moiety; or 4) to provide equivalent or better binding between molecules (*e.g.*, an enzymatic substrate). Modifications to a polypeptide are typically made to the nucleic acid which encodes the polypeptide, and can include deletions, point mutations, truncations, amino acid substitutions and additions of amino acids or non-amino acid moieties. Alternatively, modifications can be made directly to the polypeptide, such as by cleavage, addition of a linker molecule, addition of a detectable moiety, such as biotin, addition of a fatty acid, and the like. Modifications also embrace fusion proteins comprising all or part of the amino acid sequence. One of skill in the art will be familiar with methods for predicting the effect on protein conformation of a change in protein sequence, and can thus “design” a variant of a polypeptide according to known methods. One example of such a method is described by Dahiyat and Mayo (1997) *Science* 278:82-87.

In general, variants include polypeptides which are modified specifically to alter a feature of the polypeptide unrelated to its desired physiological activity. For example, cysteine residues can be substituted or deleted to prevent unwanted disulfide linkages. Similarly, certain amino acids can be changed to enhance expression of a polypeptide by eliminating proteolysis by proteases in an expression system (*e.g.*, dibasic amino acid residues in yeast expression systems in which KEX2 protease activity is present).

Mutations of a nucleic acid which encode a polypeptide preferably preserve the amino acid reading frame of the coding sequence, and preferably do not create regions in the nucleic acid which are likely to hybridize to form secondary structures, such a hairpins or loops, which can be deleterious to expression of the variant polypeptide.

Mutations can be made by selecting an amino acid substitution, or by random mutagenesis of a selected site in a nucleic acid which encodes the polypeptide. Variant polypeptides are then expressed and tested for one or more activities to determine which mutation provides a variant polypeptide with the desired properties. Further mutations can be made to variants (or to non-variant polypeptides) which are silent as to the amino acid sequence of the polypeptide, but which provide preferred codons for translation in a particular host. The preferred codons for translation of a nucleic acid in, *e.g.*, *E. coli*, are well known to those of ordinary skill in the art. Still other mutations can be made to the oncoding sequences of a gene or

cDNA clone to enhance expression of the polypeptide. The activity of variant polypeptides can be tested by cloning the gene encoding the variant polypeptide into a bacterial or mammalian expression vector, introducing the vector into an appropriate host cell, expressing the variant polypeptide, and testing for a functional capability of the polypeptides as disclosed herein.

5 The skilled artisan will also realize that conservative amino acid substitutions may be made in polypeptides to provide functionally equivalent variants of the foregoing polypeptides, *i.e.*, the variants retain the functional capabilities of the polypeptides. As used herein, a “conservative amino acid substitution” refers to an amino acid substitution which does not alter the relative charge or size characteristics of the protein in which the amino acid substitution is
10 made. Variants can be prepared according to methods for altering polypeptide sequence known to one of ordinary skill in the art such as are found in references which compile such methods, *e.g.*, *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Fourth Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2012, or *Current Protocols in Molecular Biology*, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. Exemplary
15 functionally equivalent variants of polypeptides include conservative amino acid substitutions in the amino acid sequences of proteins disclosed herein. Conservative substitutions of amino acids include substitutions made amongst amino acids within the following groups: (a) M, I, L, V; (b) F, Y, W; (c) K, R, H; (d) A, G; (e) S, T; (f) Q, N; and (g) E, D.

 In general, it is preferred that fewer than all of the amino acids are changed when
20 preparing variant polypeptides. Where particular amino acid residues are known to confer function, such amino acids will not be replaced, or alternatively, will be replaced by conservative amino acid substitutions. Preferably, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 residues can be changed when preparing variant polypeptides. It is generally preferred that the fewest number of substitutions is made.

25 Thus, one method for generating variant polypeptides is to substitute all other amino acids for a particular single amino acid, then assay activity of the variant, then repeat the process with one or more of the polypeptides having the best activity.

 Conservative amino-acid substitutions in the amino acid sequence of a polypeptide to produce functionally equivalent variants of the polypeptide typically are made by alteration of a
30 nucleic acid encoding the polypeptide. Such substitutions can be made by a variety of methods known to one of ordinary skill in the art. For example, amino acid substitutions may be made by

PCR-directed mutation, site-directed mutagenesis according to the method of Kunkel (Kunkel, Proc. Nat. Acad. Sci. U.S.A. 82: 488-492, 1985), or by chemical synthesis of a gene encoding a polypeptide.

In some embodiments “rational design” is involved in constructing specific mutations in proteins such as enzymes. As used herein, “rational design” refers to incorporating knowledge of the enzyme, or related enzymes, such as its three dimensional structure, its active site(s), its substrate(s) and/or the interaction between the enzyme and substrate, into the design of the specific mutation. Based on a rational design approach, mutations can be created in an enzyme which can then be screened for increased production of a bioproduct relative to control levels or levels of the bioproduct produced using enzymes that do not contain the mutation(s). In some embodiments, mutations can be rationally designed based on homology modeling. As used herein, “homology modeling” refers to the process of constructing an atomic resolution model of one protein from its amino acid sequence and a three-dimensional structure of a related homologous protein.

In some embodiments, random mutations can be made in a gene, such as a gene encoding for an enzyme, and these mutations can be screened for increased production of a bioproduct relative to control levels or levels of the bioproduct produced using enzymes that do not contain the mutation(s). For example, screening for mutations in components of a biosynthetic pathway that lead to enhanced production of a desired bioproduct may be conducted through a random mutagenesis screen, or through screening of known mutations. In some embodiments, shotgun cloning of genomic fragments could be used to identify genomic regions that lead to an increase in production of a desired compound, through screening cells or organisms that have these fragments for increased production of the compound. In some cases one or more mutations may be combined in the same cell or organism.

In some embodiments, production of a desired compound (*e.g.*, any of the bioproducts described herein) in a cell can be increased through manipulation of enzymes that act in the same pathway as the enzymes associated with the invention. For example, in some embodiments it may be advantageous to increase expression of an enzyme or other factor that acts upstream of a target enzyme such as an enzyme associated with the invention. This could be achieved by over-expressing the upstream factor using any standard method.

Optimization of protein expression can also be achieved through selection of appropriate promoters and ribosome binding sites. In some embodiments, this may include the selection of high-copy number plasmids, or low or medium-copy number plasmids. The step of transcription termination can also be targeted for regulation of gene expression, through the introduction or
5 elimination of structures such as stem-loops. Further aspects of the invention relate to screening for bacterial cells or strains that exhibit optimized production of a desired compound (*e.g.*, any of the bioproducts described herein).

As described above, methods associated with the invention involve generating cells that recombinantly express one or more genes of a synthetic pathway. Production of a desired
10 compound for culturing such cells can be measured and compared to another cell. The cell can be further modified by increasing or decreasing expression of one or more genes or recombinantly expressing one or more additional genes. Production of a desired compound for culturing such cells can be measured again, leading to the identification of an improved cell.

In some embodiments, methods associated with the invention involve generating cells
15 that express or overexpress one or more genes involved in the biosynthesis of a bioproduct.

The invention also encompasses nucleic acids that encode for any of the polypeptides described herein, libraries that contain any of the nucleic acids and/or polypeptides described herein, and compositions that contain any of the nucleic acids and/or polypeptides described herein. In some embodiments, one or more of the genes associated with the invention is
20 expressed in a recombinant expression vector. As used herein, a “vector” may be any of a number of nucleic acids into which a desired sequence or sequences may be inserted, for example by restriction and ligation, for transport between different genetic environments or for expression in a host cell. Vectors are typically composed of DNA, although RNA vectors are also available. Vectors include, but are not limited to: plasmids, fosmids, phagemids, virus
25 genomes and artificial chromosomes.

An expression vector is one into which a desired DNA sequence may be inserted by restriction and ligation such that it is operably joined to regulatory sequences and may be expressed as an RNA transcript. Vectors may further contain one or more marker sequences suitable for use in the identification of cells which have or have not been transformed or
30 transfected with the vector. Markers include, for example, genes encoding proteins which increase or decrease either resistance or sensitivity to antibiotics or other compounds, genes

which encode enzymes whose activities are detectable by standard assays known in the art (*e.g.*, β -galactosidase, luciferase or alkaline phosphatase), and genes which visibly affect the phenotype of transformed or transfected cells, hosts, colonies or plaques (*e.g.*, green fluorescent protein). Preferred vectors are those capable of autonomous replication and expression of the structural gene products present in the DNA segments to which they are operably joined.

As used herein, a coding sequence and regulatory sequences are said to be “operably” joined when they are covalently linked in such a way as to place the expression or transcription of the coding sequence under the influence or control of the regulatory sequences. If it is desired that the coding sequences be translated into a functional protein, two DNA sequences are said to be operably joined if induction of a promoter in the 5' regulatory sequences results in the transcription of the coding sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the coding sequences, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a promoter region would be operably joined to a coding sequence if the promoter region were capable of effecting transcription of that DNA sequence such that the resulting transcript can be translated into the desired protein or polypeptide.

When the nucleic acid molecule that encodes any of the enzymes of the claimed invention is expressed in a cell, a variety of transcription control sequences (*e.g.*, promoter/enhancer sequences) can be used to direct its expression. The promoter can be a native promoter, *i.e.*, the promoter of the gene in its endogenous context, which provides normal regulation of expression of the gene. In some embodiments the promoter can be constitutive, *i.e.*, the promoter is unregulated allowing for continual transcription of its associated gene. A variety of conditional promoters also can be used, such as promoters controlled by the presence or absence of a molecule.

The precise nature of the regulatory sequences needed for gene expression may vary between species or cell types, but shall in general include, as necessary, 5' non-transcribed and 5' non-translated sequences involved with the initiation of transcription and translation, respectively, such as a TATA box, capping sequence, CAAT sequence, and the like. In particular, such 5' non-transcribed regulatory sequences will include a promoter region which includes a promoter sequence for transcriptional control of the operably joined gene. Regulatory

sequences may also include enhancer sequences or upstream activator sequences as desired. The vectors of the invention may optionally include 5' leader or signal sequences. The choice and design of an appropriate vector is within the ability and discretion of one of ordinary skill in the art.

5 Expression vectors containing all the necessary elements for expression are commercially available and known to those skilled in the art. See, *e.g.*, Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Fourth Edition, Cold Spring Harbor Laboratory Press, 2012. Cells are genetically engineered by the introduction into the cells of heterologous DNA (RNA). That heterologous DNA (RNA) is placed under operable control of transcriptional elements to permit
10 the expression of the heterologous DNA in the host cell. Heterologous expression of genes associated with the invention, for production of a bioproducts, such as those described herein, is demonstrated in the Examples section.

 A nucleic acid molecule that encodes an enzyme associated with the invention can be introduced into a cell or cells using methods and techniques that are standard in the art. For
15 example, nucleic acid molecules can be introduced by standard protocols such as transformation including chemical transformation and electroporation, conjugation, transduction, particle bombardment, etc. In some embodiments, the nucleic acid molecules encoding an enzyme may be introduced into a cell using transformation. In some embodiments, the method of introducing a nucleic into a cell may involve generating protoplasts, for example by subjecting the cells to a
20 cell wall-disrupting agent, *e.g.*, lysozyme, thereby generating a protoplast. In some embodiments, the protoplast is subsequently subjected to osmotic shock to incorporate the nucleic acid. In some embodiments, the cells are recovered, for example in high sucrose media, following incorporation of the nucleic acid. In some embodiments, the transformation further involves L-malic acid. Expressing the nucleic acid molecule encoding the enzymes of the
25 claimed invention also may be accomplished by integrating the nucleic acid molecule into the genome.

 In some embodiments one or more genes associated with the invention is expressed recombinantly in a bacterial cell. Bacterial cells according to the invention can be cultured in an aqueous phase comprising a growth media, such as any of the growth media described herein.
30 As would be understood by one of ordinary skill in the art, routine optimization would allow for use of a variety of types of media. As described in the Examples, a non-limiting example of an

optimized growth media was determined for culturing cells of the instant invention (*e.g.*, *Bacillus megaterium*). In some embodiments, the growth media comprises a base media, such as LB or M9 media. In some embodiments, the growth media may be supplemented with additional components, such as yeast extract, trace metals, and/or glucose. Additional non-limiting
5 examples of supplemental components include antibiotics, xylose or IPTG for gene induction, spore germination inducers, and/or ATCC Trace Mineral Supplement.

In some embodiments, the cells of the instant invention are provided in spore-form. In some embodiments, a spore germination inducer may be added to the growth media to induce germination of the cells and initiate production of a desired bioproduct. As will be appreciated
10 by one of ordinary skill in the art, cells in spore form may be induced to germinate by any of a variety of inducers. The inducer used to stimulate spore germination may depend on any of a number of factors, such as the type of cell. In some embodiments, the spore germination inducer is physical treatment of the cells, such as by heat pretreatment. In some embodiments, the spore germination inducer is a nutrient or a portion of a bacterial cell wall (*e.g.*, portion of
15 peptidoglycan). In some embodiments, the spore germination inducer is D-alanine. In some embodiments, the spore germination inducer is leucine.

Similarly, other aspects of the growth media and growth conditions of the cells of the invention may be optimized through routine experimentation. For example, pH and temperature are non-limiting examples of factors which can be optimized. In some embodiments, methods
20 described herein may involve incubating the cells (*e.g.*, in multiphase reactors) at a temperature that allows metabolic activity of the cell and production of the desired bioproduct. In some embodiments, the methods involve incubating the cells at a temperature between about 10°C – 50°C. In some embodiments, the methods involve incubating the cells at a temperature about 10°C, 11°C, 12°C, 13°C, 14°C, 15°C, 16°C, 17°C, 18°C, 19°C, 20°C, 21°C, 22°C, 23°C, 24°C,
25 25°C, 26°C, 27°C, 28°C, 29°C, 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, 41°C, 42°C, 43°C, 44°C, 45°C, 46°C, 47°C, 48°C, 49°C, or about 50°C. In some embodiments, the methods involve incubating the cells at a temperature between about 35°C – 40°C. In some embodiments, the methods involve incubating the cells at a temperature about 35°C, 35.5°C, 36°C, 36.5°C, 37°C, 37.5°C, 38°C, 38.5°C, 39°C, 39.5°C, or about 40°C.

30 In some embodiments, the methods involve incubating the cells under conditions in which the density of CO₂ is greater than 0.1 g cm³. In some embodiments, the methods involve

incubating the cells under conditions in which the density of CO₂ is greater than 0.1 g cm³, 0.2 g cm³, 0.3 g cm³, 0.4 g cm³, 0.5 g cm³, 0.6 g cm³, 0.7 g cm³, 0.8 g cm³, 0.9 g cm³, 1.0 g cm³, 1.1 g cm³, 1.2 g cm³, or higher.

In some embodiments, factors such as choice of media, media supplements, and
5 temperature can influence production levels of the desired compound (*e.g.*, bioproducts described herein). In some embodiments the concentration and amount of a supplemental component may be optimized. In some embodiments, how often the media is supplemented with one or more supplemental components, and the amount of time that the media is cultured before collecting the bioproduct, is optimized. In some embodiments, the growth media (containing the
10 cell of the instant invention) is cultured for at least 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 24, or 48 hours prior to collecting the bioproduct.

Chemical ions catalyzed by recombinant microorganisms are becoming more commonly used to replace syntheses of various chemicals, including in industrial scale reactors. Due to the presence of more complicated reaction mixtures (*e.g.*, culture media, cells, reactants and
15 products), downstream processing to isolate products can be difficult, particularly in processes requiring phase separation. Supercritical CO₂, near critical CO₂, or liquid CO₂ may be useful for both phase separation and product purification, such as by extraction of the culture medium, leading to more efficient, simple and cost effective processing.

In some embodiments, the bioproduct, once produced by the cultured cell, partitions to
20 the aqueous phase of the multiphase reactor. In some embodiments, the bioproduct can be recovered (*e.g.*, isolated) from the aqueous phase of the multiphase reactor. In some embodiments, the bioproduct, once produced by the cultured cell, partitions to the solvent phase (phase containing scCO₂) of the multiphase reactor. In some embodiments, the bioproduct can be recovered (*e.g.*, isolated) from the solvent phase of the multiphase reactor.

25 In some embodiments, the bioproduct is soluble or insoluble in the reaction mixture and may be recovered or collected from the appropriate phase of the reactor by any method known in the art, for example by filtration, pumping the fluid from the reaction vessel, or by venting the carbon dioxide from the reaction vessel for a soluble product.

According to aspects of the invention, bioproducts are produced through culturing cells,
30 including cells that recombinantly express genes associated with the invention, under conditions involving a solvent phase comprising supercritical CO₂, near critical CO₂, or liquid CO₂. In

some embodiments, the titer of the bioproduct is at least 10 mg L⁻¹. For example, the titer of the bioproduct can be at least 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 75, 80, 85, 90, 95, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 525, 550, 575, 600, 625, 650, 675, 700, 725, 750, 775, 800, 825, 850, 875, 900 or more than 900 mg L⁻¹ including any intermediate values.

In some embodiments, the titer of the bioproduct is at least 1 g L⁻¹. For example, the total titer of the bioproduct can be at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more than 50 g L⁻¹ including any intermediate values.

In some embodiments, the titer of the bioproduct is less than 10 mg L⁻¹. In some embodiments, the titer of the bioproduct is at least 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8.0, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, 9.0, 9.1, 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, 9.8 or 9.9 mg L⁻¹.

Any of the cells described herein may be cultured in a multiphase reactor. As used herein, a multiphase reactor refers to a reactor, such as a bioreactor, that comprises at least two distinct phases. In some embodiments, the multiphase reactor comprises at least an aqueous phase and a solvent phase. In some embodiments, the solvent comprises supercritical CO₂ (scCO₂). In some embodiments, the solvent phase comprises scCO₂ and one or more other solvents. In some embodiments, the solvent phase comprises scCO₂ and inert helium. In some embodiments, the solvent comprises near critical CO₂. In some embodiments, the solvent phase comprises near critical CO₂ and one or more other solvents. In some embodiments, the solvent phase comprises near critical CO₂ and inert helium. In some embodiments, the solvent comprises liquid CO₂. In some embodiments, the solvent phase comprises liquid CO₂ and one or more other solvents. In some embodiments, the solvent phase comprises liquid CO₂ and inert helium. In some embodiments, the one or more other solvents, such as inert helium, are present in the solvent phase at less than or equal to about 3%. For example, the solvent phase may

contain about 3%, 2.9%, 2.8%, 2.7%, 2.6%, 2.5%, 2.4%, 2.3%, 2.2%, 2.1%, 2%, 1.9%, 1.8%, 1.7%, 1.6%, 1.5%, 1.4%, 1.3%, 1.2%, 1.1%, 1%, 0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%, or about 0.1% inert helium.

In some embodiments, the aqueous phase comprises the growth media and a cell or
5 population of cells.

Also within the scope of the present invention are reactors comprising scCO₂, near critical CO₂, or liquid CO₂ and at least one viable cell. In some embodiments, the reactor is a multiphase reactor comprising at least two distinct phases. In some embodiments, the reactor comprises a mixture of scCO₂ and one or more other solvents, and at least one viable cell. In
10 some embodiments, the reactor comprises a mixture of near critical CO₂ and one or more other solvents, and at least one viable cell. In some embodiments, the reactor comprises a mixture of liquid CO₂ and one or more other solvents, and at least one viable cell. As described herein, in some embodiments, the one or more other solvents comprise inert helium. In some
embodiments, the scCO₂, near critical CO₂, or liquid CO₂ and the viable cell of the reactor are
15 located in different phases within the reactor.

In some embodiments, the reactor or multiphase reactor includes one or more pressurizable culture vessels for culturing microbes in fluid communication with a supply of CO₂ (*e.g.*, supercritical CO₂, near critical CO₂, or liquid CO₂). The reactor may permit regulation of CO₂ pressure, permitting the user to increase or decrease CO₂ pressure as needed. In some
20 embodiments, the reactor also permits changing the pressure conditions inside the one or more pressurizable culture vessels without interrupting the cultivation of microbes inside the one or more pressurizable culture vessels.

The cells can be grown under batch conditions or continuous flow conditions. The general parameters of batch and continuous processes are well known in the art. In one
25 embodiment of a batch process, after a suitable time for production of one or more metabolites by the cells, the culture process (*e.g.*, fermentation) is stopped by releasing the pressure in the bioreactor and collecting the CO₂ (*e.g.*, supercritical CO₂, near critical CO₂, or liquid CO₂) mixture of supercritical CO₂ and one or more other solvents from the bioreactor. In one
embodiment of a continuous process, the bioreactor is a pressurized continuous flow chemostat,
30 and preferably a portion of the growth media is removed from the continuous flow chemostat. In some embodiments, the portion of the growth media that is removed is replaced with an amount

of fresh growth media. Similarly, portions of the supercritical CO₂ or the mixture of supercritical CO₂ and one or more other solvents can be removed from the bioreactor (*e.g.*, chemostat) in a continuous flow process. In some embodiments, the portion of the supercritical CO₂ or the mixture of supercritical CO₂ and one or more other solvents that is removed is replaced with an amount of fresh (*i.e.*, unreacted) CO₂ (*e.g.*, supercritical CO₂, near critical CO₂, or liquid CO₂) or a mixture of CO₂ (*e.g.*, supercritical CO₂, near critical CO₂, or liquid CO₂) and one or more other solvents.

The present invention is further illustrated by the following Examples, which in no way should be construed as further limiting. The entire contents of all of the references (including literature references, issued patents, published patent applications, and co pending patent applications) cited throughout this application are hereby expressly incorporated by reference.

EXAMPLES

EXAMPLE 1: Development and Characterization of a scCO₂ Resistant *Bacillus megaterium* Strain

To isolate and identify microorganisms that are able to grow in biphasic scCO₂-water reactors, fluid samples were collected from the deep subsurface McElmo Dome CO₂ field in Colorado, where scCO₂ had accumulated over 40-70 million years. Metagenomic analysis of formation fluids at this site suggested existence of an anaerobic microbial ecosystem. This work contributes to the establishment of a new technology for microbial bioproduction by enabling a bacterial strain capable of bioproduct generation to access the unique properties of sustainable solvent supercritical carbon dioxide.

Methods

Subsurface fluid sample collection and storage

Formation water samples from the McElmo Dome CO₂ field were used as inocula for microbial strain isolation through scCO₂-exposed enrichment culture and passaging. Sample fluids were sourced from the deep subsurface, where CO₂ is trapped at depths of 1800 to 2600 m within the 100 m thick dolomitic Leadville Formation (Allis et al., 2001; Gilfillan et al., 2009) and exists as a supercritical fluid at a temperature and pressure of approximately 65°C and 135

atm (Allis et al., 2001). Sampled fluids from each of ten CO₂ production wells (operated by KinderMorgan CO₂) were collected from fluid-gas separators that were decanted and filled 15 hours prior to sample collection. At each separator, one liter of degassed fluid was collected in an acid-washed bottle (Nalgene; Rochester, NY) and placed immediately on ice for use as

5 enrichment culture inocula. Fluids were shipped on ice and stored at 4°C.

Supercritical CO₂-exposed enrichment passaging

Culturing media and vessels

Media for enrichment culture and passaging of McElmo Dome samples was a modified

10 version of MS media (Colwell *et al.*, 1997) with supplements targeting diverse metabolic groups as described in Peet *et al.*, 2015. Media consisted of (in g/l) 0.5 yeast extract, 0.5 tryptic peptone, 10.0 NaCl, 1.0 NH₄Cl, 1.0 MgCl₂•6H₂O, 0.4 K₂HPO₄, 0.4 CaCl₂, 0.0025 EDTA, 0.00025 CoCl₂•6H₂O, 0.0005 MnCl₂•4H₂O, 0.0005 FeSO₄•7H₂O, 0.0005 ZnCl₂, 0.0002 AlCl₃•6H₂O, 0.00015 Na₂WO₄•2H₂O, 0.0001 NiSO₄•6H₂O, 0.00005 H₂SeO₃, 0.00005 H₃BO₃,

15 and 0.00005 NaMoO₄•2H₂O. MS medium supplements (g/l) consisted of 0.5 glucose (MS-FM); or 1.3 MnO₂, 2.14 Fe(OH)₃, and 1.64 sodium acetate (MS-MR); or 0.87 K₂SO₄, 0.83 FeSO₄, 0.82 sodium acetate (MS-SR). Following enrichment culturing and three passages using MS medium, Luria-Bertani Broth (LB) (Difco™; Becton, Dickinson, and Company; Franklin Lakes, NJ) was included as an additional growth media for scCO₂ culturing. Phosphate buffered LB (P-

20 LB) is amended with 50 mM K₂HPO₄. During all rounds of culturing, CO₂-incubated media was amended with 0.25 g/l of reducing agent Na₂S (at 0.25 g/l) and 0.001 g/l of the redox indicator resazurin. A summary of all media utilized during enrichment passaging and subsequent culturing is presented in Table 1.

High-pressure culturing vessels were constructed of ¾ inch 316 stainless steel tubing for

25 a 10 ml total capacity, and fitted with quarter turn plug valves (Swagelok (Solon, OH) or Hylok (Houston, TX)). Between uses, all vessel components were cleaned and soaked for at least two hours with 10% bleach and detergent, then autoclaved prior to assembly. All tubing in the pressurization manifold was filled before use with 10% bleach for 30 minutes, flushed with MilliQ-H₂O, rinsed with 70% ethanol for 20 minutes, and dried with CO₂ gas. Prior to reactor

30 loading, culture media was added to 100 ml serum bottles and degassed with a stream of 100% CO₂ for 30 minutes. Vessels were then filled to ½ capacity (5 ml) with inocula and degassed

media, after which the headspace was pressurized with extraction grade CO₂ gas at a rate of 2-3 atm min⁻¹ until reaching a final pressure of 100 atm. Since the CO₂ tank used for reactor pressurization contained a helium (He) cushion (in order to reach elevated pressures) the gas tank mixture contained 97-99% CO₂. Unless stated otherwise, after pressurization, reactors were incubated in a 37°C warm room (to reach supercritical conditions) with shaking at 100 rpm to increase the extent of inocula and media mixing.

As described previously (Peet et al., 2015) prior to depressurization, culturing vessels were connected via 316 stainless steel tubing and fittings to a pressure gauge (Hunter) to measure the final vessel pressure. All reported vessel incubation data maintained pressures above the CO₂ critical point (>72.9 atm) when mixed with ≤3% inert Helium at 37°C (Roth, 1998). Reactors were depressurized at a rate of 3-5 atm min⁻¹ over approximately 30 min, at which point the vessels were transferred to the anaerobic chamber for sub-sampling, glycerol stock preparation and passaging.

Table 1: Summary of microbial culturing media

Use	Name	Base	Supplements	Base Reference
Enrichment Passaging	MS-MR	MS: Yeast Extract, Trypticase Peptone, Salts	^a Metals (Mn, Fe) + Acetate	Colwell et al., 1997
	MS-SR		^b Sulfates + Acetate	
	MS-FM		0.5 g/L Glucose	
Pure Culture	P-LB	LB: Yeast Extract Tryptone, NaCl	50 mM Phosphate (Dibasic)	BD Difco™
	P-LBA		50 mM Phosphate (Dibasic) + 100 mM L-alanine	
	P-LBL		50 mM Phosphate (Dibasic) + 10 mM L-leucine	
	P-LBAL		50 mM Phosphate (Dibasic) +100 mM L-alanine + 10 mM L-leucine	
	M9+	M9: Phosphates, Salts	M9 + °0.1X trace metals + 50 mM YE + 0.4% Glucose	thelabrat.com*
	M9A+		M9 + °0.1X trace metals + 50 mM YE + 0.4% Glucose + 100 mM L-alanine	
*www.thelabrat.com/protocols/m9minimal.shtml				
^a 1.30 g/l MnO ₂ , 2.14 g/l Fe(OH) ₃ , 1.64 g/l Na-Acetate				
^b 0.87 g/l K ₂ SO ₄ , 0.83 g/l FeSO ₄ •7H ₂ O, 0.82 g/l Na-Acetate				
^c 0.1X trace metals solution (Boone, 1989); see methods				

Enumeration of cell density

In order to quantify biomass of CO₂ cultures, 0.5-1.0 ml samples were treated with Syto 9 stain (Life Technologies), left in a dark room for 15 minutes to allow the stain to adhere to nucleic acids, collected on 0.22 um polycarbonate filters (Whatman® Nucleopore™; GE

Healthcare Bio-sciences; Pittsburgh, PA) by vacuum pump, and washed twice with phosphate buffered saline (PBS) to remove excess stain. Each filter was mounted on glass slides for visualization by epifluorescent microscopy (Zeis Axioscope; Oberkochen, Germany) with immersion oil dropped below and above the filters, after which a cover slip was applied. Filters
5 were stored at 4°C in the dark until use. Cell densities were extrapolated by multiplying individual cell counts in a 10x10 microscope eyepiece grid by a dilution factor (if <1.0 ml of sample was filtered), and then multiplied by 3.46×10^4 , because a 10x10 grid at 1000X magnification corresponds to $1/(3.46 \times 10^4)$ of a 25 mm filter. Final cell densities represented the mean values of cell counts in 15-20 separate 10x10 grids/sample. The limit of detection was
10 considered to be one half of a cell per 15 grids, which corresponds to 1150 cells/ml. Fluorescent images were captured on a Nikon D100 camera using the NKRemote live-imaging software. Cell density calculations and morphological observations were conducted for inocula prior to pressurization as well as after incubation in order to determine the extent to which growth had occurred. CFU plating was performed using LB Agar with order of magnitude dilutions in
15 autoclaved PBS buffer prior to plating with cell-free negative controls. Plates were dried, inverted and incubated overnight at 37°C prior to colony counting.

Enrichment cultures and serial passaging

Fluids from four of the sampled wells (HB-5/Well 2, HE-1/Well 4, HF-3/Well 5, YB-
20 4/Well 7) were selected as inocula for enrichment culturing under scCO₂ because they appeared to harbor elevated cell density by fluorescent microscopy. 100 ml of fluids from the four respective wells were filtered onto 5 mm, 0.2 µm pore size, polycarbonate filters (Nucleopore; GE Healthcare Bio-sciences; Pittsburgh, PA) in order to concentrate microbial content. The filters were then placed inside an anaerobic chamber (Coy Lab Products; Grass Lake, MI)
25 containing 95% CO₂ / 5% H₂. Using sterilized tweezers, filters were then placed inside 10 ml 316 stainless steel pressure reactors with 1 ml of formation fluid from the same well used to concentrate biomass on the filter. The filters and formation fluids were then amended with 4 ml of growth media. After the initial round of growth using filter-concentrated biomass inoculum, cultures were inspected by epifluorescence microscopy to identify biomass accumulation.
30 Cultures that showed growth relative to inocula based on cell counts were serially passaged by dilution in freshly degassed growth media to achieve initial concentrations of approximately 10^4

cells/ml. The McElmo Dome enrichment culture (M1) was incubated for 46 days, while subsequent passages were incubated for 19 days (M2), 33 days (M3), and 35 days (M4).

Strain isolation and scCO₂ growth verification

5 Samples from the final round of passaging (M4) were plated on LB agar and incubated overnight at 37°C under aerobic conditions at ambient pressure. Single colonies with unique morphologies were used to inoculate liquid LB. DNA extraction from overnight grown cultures was performed using the Qiagen Blood and Tissue DNA extraction kit protocol for gram-positive cells (Qiagen; Hilden, Germany). Resulting DNA was used as template for 16S rRNA
10 PCR using universal Bacterial primers 515F (3'–GTGCCAGCMGCCGCGGTAA–5' (SEQ ID NO: 1)) and 1492R (3'–GGTTACCTTGTTACGACTT–5' (SEQ ID NO: 2); Turner et al., 1999). PCR mixtures (20 µl per reaction) included 1X Phusion High Fidelity Polymerase buffer, 0.4 µM of each primer (Integrated DNA Technologies (IDT); Coralville, IA), 0.4 µM deoxynucleotide mixture and 1 U Phusion Polymerase (New England Biolabs; Ipswich, MA). Thermal cycling
15 conditions consisted of an initial 5 minutes at 95°C followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 90 sec; followed by a final extension time of 7 min. Every PCR reaction included negative and positive controls (Peet *et al.*, 2015). PCR products were then purified using Exo-SAP IT (Affymetrix; Santa Clara, CA) and submitted for Sanger sequencing (Genewiz, Cambridge, MA). Returned sequences were
20 processed in CLC Genomics Workbench (Version 7; Qiagen Bioinformatics), including primer removal and universal sequence trimming to 918 bp for all isolates. Sequence alignment and tree building of isolates and reference sequences consisting of *Bacillus*, closely related taxa, and an *E. coli* outgroup using a 914 bp alignment was also conducted in CLC Genomics Workbench. Tree building used a bootstrapped (100X) neighbor-joining method, which was visualized in
25 FigTree v1.4.2. 16S rRNA reference sequences were downloaded from GenBank (NCBI) or generated in Peet et al., (2015; *e.g.*, *Bacillis* sp. OT1, *Bacillus* sp MIT0214).

Because *Bacillus spp.* spores were previously demonstrated to be able to germinate and grow under 1 atm CO₂ and scCO₂ headspace conditions (Peet *et al.*, 2015), spores of all *Bacillus spp.* strains isolated from McElmo Dome fluids were prepared using the protocol described in
30 Kim and Goepfert (1974). Briefly, colonies streaked from glycerol stocks were used to inoculate overnight cultures in LB medium that were incubated under aerobic conditions at 37°C while

shaking at 100 rpm. Dense, stationary-phase cultures were then diluted 1:50 into 100 ml of Modified G Medium, which is composed of (in g/l): yeast extract 2.0, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.025, K_2HPO_4 0.5, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 0.05, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.005, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.005, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.0005, $(\text{NH}_4)_2\text{SO}_4$ 2.0, adjusted to pH 7.1 after autoclaving. Modified G Medium-
 5 inoculated cells were incubated at 37°C for 72 hours to induce sporulation, then centrifuged for 10 minutes at 10,000 x g. The pellet was resuspended and centrifuged 5 times in autoclaved wash buffer containing 0.058 g/l $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ and 0.155 g/l $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ with 0.01% (v/v) Tween® 20 to prevent clumping. Spores were stored in wash buffer at 4°C until use and periodically assayed for continued viability after extended storage by LB agar colony plating.

10 Growth of *B. megaterium* SR7 (and other isolates) under scCO_2 was validated by triplicate incubation for 28-42 days inoculated in pure culture from spores loaded at $\sim 1 \times 10^4$ spores/ml using multiple media (Table 1). Cultures were scored for growth by filter counts, as previously described.

15 **Isolate *B. megaterium* strain SR7 genomics**

Understanding the genomic landscape of strain SR7 provides useful insight into endogenous physiological and metabolic capacities and will aid future development of SR7 as a strain for bioengineered product generation for *in situ* scCO_2 extraction. SR7 genomic DNA was extracted from a 10 ml overnight aerobic LB culture using the Qiagen Blood & Tissue Kit,
 20 following the Gram-positive bacteria protocol. Eluted DNA was submitted to the MIT BioMicro Center for sequencing using PacBio SMRT technology (Pacific Biosciences; Menlo Park, CA). Following sequencing, the PacBio assembler software was used to assemble SR7 contigs, which were then compared to the genome of closely related strain *B. megaterium* QM B1551 (Eppinger *et al.*, 2011) using the online tools nucmer and “double act” (hpa-bioinfotools.org.uk/pise/double_act.html), the latter of which cuts the query and reference DNA
 25 into smaller pieces to create an inter-genome Blastn comparison file that can be viewed in the Artemis Comparison Tool (ACT; Carver *et al.*, 2005). Based on the ACT comparison, the putative SR7 chromosome (longest contig) was adjusted to start at the beginning of gene *dnaA* in agreement with the reference genome. The closed chromosome was then plotted by DNA Plotter
 30 (sanger.ac.uk/resources/software/dnaplotter) and submitted to RAST (Aziz *et al.*, 2008) for gene prediction and functional annotation. Remaining contigs, potentially indicative of endogenous

plasmid based on sequenced *B. megaterium* strains, were also submitted to RAST for annotation. Shared and unique RAST-annotated genes between SR7 and *B. megaterium* reference genomes QM B1551, DSM319 (Eppinger *et al.*, 2011), and WSH-002 (Liu *et al.*, 2011) were determined using online tool Venny 2.1. Inter-strain sequence comparisons were conducted using the
5 Average Nucleotide Identity (ANI) calculator (enve-omics.ce.gatech.edu/ani).

Physiological characterization of *B. megaterium* strain SR7

To help guide optimization of growth conditions for strain SR7, physiological tests were conducted under aerobic conditions. To determine tolerance for pH, salinity and bicarbonate,
10 high throughput culturing was done in 96 well plates and scored for growth by OD₆₀₀ using a microplate reader (BioTek Synergy 2; BioTek; Winooski, VT). 200 uL LB solutions/well were inoculated in triplicate with 10⁴ spores/ml (based on SR7 spore stock filter counts) and incubated on a plate rocker at 37°C with unamended positive and cell-free negative LB controls. Tests for pH tolerance (pH 2-10) were conducted in LB medium amended with HCl or NaOH. The effect
15 of salinity and bicarbonate on growth was determined by adding NaCl (1-10%) and NaHCO₃ (0.1-0.5M), respectively, to LB media. Optimal SR7 growth temperature was tested by inoculating 10⁴ spores/ml in 5 ml of LB in triplicate at temperatures of 9-55°C. Cultures and cell-free negative controls were incubated without shaking, with subsamples taken for periodic OD₆₀₀ measurements. SR7 antibiotic sensitivity was determined by supplementing 5 ml LB with
20 ampicillin (5-50 µg/ml), chloramphenicol (3.5-35 µg/ml), kanamycin (5-50 µg/ml), spectinomycin (10-100 µg/ml), streptomycin (10-100 µg/ml), or tetracycline (1.5-15 µg/ml). 5 mL cultures inoculated with 10⁴ spores/ml were incubated in a spinning rack at 100 rpm for 24 hours at 37°C and assayed for growth by comparing OD₆₀₀ measurements to unamended positive and cell-free negative LB controls.

25 Biolog GenIII Microplates 96 well plates (Biolog; Hayward, CA)(unamended and with a trace metals solution amendment (Boone *et al.*, 1989)) were used to determine SR7 growth substrates and to test growth sensitivities relative to a positive control. Plates were inoculated with 2-4 SR7 colonies grown overnight on solid BUG media (Biolog) such that starting OD₄₉₀ transmission was 90-94%. Plates were incubated at 37°C on a plate shaker at 200 rpm and
30 assayed for growth using NADH-dependent colorimetric changes measured by OD₄₉₀ on a microplate reader (BioTek Synergy 2; BioTek; Winooski, VT). Total growth was quantified by

integrating the area under the curve of OD₄₉₀ values over the course of the incubation, and categorized as: "-" displays an area less than the negative control, "+" is greater than the negative control, but less than half of the maximum value, and "+++" is between "+" and the maximum value.

5

Process improvements for *B. megaterium* SR7 growth under 1 atm CO₂ and scCO₂

After initial physiological characterization assays, subsequent culturing improvements sought to establish consistent, replicable growth of SR7 under scCO₂ by conducting experiments under 1 atm CO₂ as a proxy for pressurized conditions (*e.g.*, Peet *et al.*, 2015). In order to improve spore germination frequencies, the effects of chemical inducers and mixing regimes (*e.g.*, culture volume and shake speed) were examined, as the literature has shown certain compounds (*e.g.*, amino acids, KNO₃, peptidoglycan, Ca-dipicolinic acid, and others; Ghosh and Setlow, 2009) and conditional treatments (temperature, pressure; Wei *et al.*, 2010) increase *Bacillus* spore germination rates. Experiments testing the role of mixing speed and modified culture media on rates of vegetative outgrowth were conducted under 1 atm CO₂ with CO₂-degassed media or buffer in 100 ml serum vials with clamped rubber stoppers.

Evaluating the effect of spore germination inducers

The effect of shake speed on spore germination was assayed by inoculating 5 ml LB medium with *B. megaterium* SR7 spores at a starting concentration of 10⁵ spores/ml. Singleton cultures were subjected to shake speeds of 150, 250, and 350 rpm and scored for growth by OD₆₀₀ and LB agar colony plating.

The ability to induce spore germination based on literature precedent was tested by inoculating triplicate 10 ml cultures of SR7 spores at OD₆₀₀ 0.01 in LB amended with 100 mM L-alanine, LB subjected to a heat activation (65°C for 15 minutes) upon inoculation, or unamended LB as a control. Growth was scored by OD₆₀₀ and LB agar CFU plate counts. In addition, subsamples were heat-killed by exposure to 80°C for 10 minutes (Setlow, 2006) prior to plating, to ascertain remaining spore concentrations, as heat exposure is lethal to vegetative cells.

The role of candidate germination inducers was subsequently investigated in PBS buffer rather than growth medium to decouple the germination process from outgrowth. *B. megaterium* SR7 spores were loaded in triplicate 10 ml PBS amended with 100-250 mM L-alanine, 100 mM

L-alanine with heat treatment, 25 mM L-leucine, or an unamended PBS control. The extent of germination was measured by fluorescence microscopy staining patterns (*e.g.*, the degree of cell membrane penetration by DNA stain), bulk fluorescence, OD₆₀₀, and flow cytometry (FCM). A total of 100-300 cells per filter were counted and categorized as either “dormant” or

5 “germinated” if the spore stain was localized to the cell membrane or diffused within the interior of the cell, respectively (Cronin and Wilkinson, 2007). Cells displaying an intermediate degree of stain dispersal (“activated”) were categorized as germinated (FIG. 1).

Sub-sample bulk fluorescence (Syto9[®]) was measured by microplate reader (BioTek Synergy 2; 485/20 excitation, 528/20 emission) and OD₆₀₀ was measured by microplate reader.

10 OD should decrease in germinated cells (the index of refraction decreases due to hydration upon spore coat degradation) while bulk fluorescence should increase as the nucleic acid stain progressively penetrates and permeates the cell (Magge *et al.*, 2009). To test for germination after a delayed inducer spike rather than at the moment of inoculation, *B. megaterium* SR7 spores loaded at OD₆₀₀ 0.01 were incubated overnight in 30 ml of PBS, passaged into PBS
15 amended with L-alanine (25-250 mM) or L-leucine (10-25 mM) and then assayed for germination by bulk fluorescence and OD₆₀₀ during incubation.

FCM was employed as a high-throughput germination assay based on Baier *et al.*, (2010). Triplicate cultures of *B. megaterium* SR7 spores loaded at OD₆₀₀ 0.01 were incubated overnight in PBS and PBS amended with 2.5-250 mM L-alanine, along with cell-free PBS controls. Prior
20 to loading on the flow cytometer (BD FACS Canto II HTS-1; Becton Dickinson; Franklin Lakes, NJ) cultures were diluted 1/50 in PBS and stained with Syto16[®] and propidium iodide (PI) in the dark for at least 30 minutes prior to analysis. After spore and media-only samples were used to set forward scatter, side-scatter, Syto16 and PI gates, sample data was collected and analyzed using FACSDIVA[™] software (BD Biosciences; Franklin Lakes, NY).

25 *Testing the effect of mixing on vegetative growth*

After testing for the potential to induce germination in *B. megaterium* SR7, bacterial growth rates were evaluated in order to increase metabolic activity for eventual bioproduct pathway expression. Experiments testing the role of shake speed on vegetative growth rate were
30 inoculated with passaged cells of spore-loaded overnight cultures grown under 1 atm CO₂.

Triplicate 25 ml LB cultures of vegetative cells loaded at OD₆₀₀ 0.01 were subjected to shake speeds of 150, 250, and 350 rpm, with growth assayed by OD₆₀₀ and LB agar colony plating.

Minimal media development to improve growth

- 5 Development of a minimal medium enables individual chemical components to be tuned in order to establish optimized growth from a sole carbon source under 1 atm CO₂. Initial attempts to generate *B. megaterium* SR7 growth in triplicate cultures tested various amendments to M9 base medium (thelabrat.com; Table 1), including 0.4% glucose or 0.4% xylose amendments as sole carbon sources, with or without trace metals solution (Boone *et al.*, 1989).
- 10 The 1X concentration trace metals solution consisted of (in g/l): 0.005 Na₂(EDTA), 0.0002 NiSO₄•6H₂O, 0.0005 CoCl₂•6H₂O, 0.0001 H₂FeO₃, 0.001 FeSO₄•7H₂O, 0.0001 H₃BO₃, 0.001 ZnCl₂, 0.0001 NaMoO₄•2H₂O, 0.0004 AlCl₃•6H₂O, 0.001 MnCl₂•4H₂O, 0.0003 Na₂NO₄•2H₂O, 0.0002 CaCl₂. To further boost growth, triplicate cultures of M9 + 0.4% glucose were amended with dilute LB (0.001-0.01X) or yeast extract (YE; 0.001-0.01X) as de facto vitamin and co-
- 15 factor solutions, and/or NaNO₃ (5 mM) as an alternative electron acceptor. All M9 incubations were scored for growth by OD₆₀₀ and designated as robust above OD₆₀₀ >0.600, low level between 0.2-0.6, and no growth below 0.2. Passaged vegetative cultures were also assayed in duplicate for growth (by OD₆₀₀) amended with a range (0.1X, 0.25X, 1X) of trace metals solutions in M9 + 0.4% glucose + 0.01X YE media, including in the presence and absence of 5
- 20 mM NaNO₃.

- Growth curves under optimized shaking conditions were generated to establish baseline metabolic characteristics of strain *B. megaterium* SR7. Vegetative SR7 cells were passaged in quadruplicate at OD₆₀₀ 0.01 in minimal or LB media and assayed for growth by OD₆₀₀, LB agar colony plating, and glucose consumption (for minimal medium cultures only) measured on the
- 25 YSI 2900 with 2814 glucose starter kit. Doubling times were calculated using a log-linear fit of CFU and OD₆₀₀ data points during exponential growth.

Analysis of *B. megaterium* SR7 fermentation products under 1 atm CO₂ and scCO₂

- Following optimization of growth conditions under 1 atm CO₂ and scCO₂, identification
- 30 of fermentation products would establish potential target pathways for redirecting carbon flux and would demonstrate the ability to generate extracellular natural products. Metabolite

identification and quantification was conducted by high performance liquid chromatography (HPLC). Triplicate cultures of *B. megaterium* SR7 vegetative cells inoculated in M9+ or LB at OD₆₀₀ 0.01 were scored for growth by OD₆₀₀. 500 µl of supernatant from each spun down sample (5 mins at 21,000 x g) was loaded on the HPLC (Agilent 1100 series; Agilent

Technologies; Santa Clara, CA) for analysis. Compound separation was achieved using an Aminex HPX-87H anion exchange column; Bio-Rad Laboratories, Hercules, CA) according to the protocol established by Buday *et al.* (1990) using 5 mM H₂SO₄ as the mobile phase. Analyte concentrations were established using standard curves for fermentative substrates and products, including glucose, succinate, lactate, formate, acetate, and ethanol. Though retention times were known for pyruvate, malic acid, propionate, 2-3 butanediol, butyrate, propanol, crotonate, butyraldehyde, valerate, butanol, and pentanol, standard curves were not generated because no apparent peaks were detected for these compounds.

Evaluating SR7 scCO₂ growth using 1 atm CO₂-optimized conditions

SR7 growth outcomes were investigated under scCO₂ headspace (90-100 atm; 37°C) while shaking at 250 rpm. SR7 spores were inoculated at starting concentrations of ~3x10⁴ spores/ml (unless otherwise specified) in either 50 mM K₂HPO₄-buffered LB (P-LB) or M9+ media (Table 1). Experiments assaying the effect of germination induction included 100 mM L-alanine and 10 mM L-leucine media amendments and heat treatment upon reactor pressurization (70°C for 10 minutes). Incubations were conducted in 316 stainless steel vessels and gradually pressurized to supercritical conditions using a CO₂/He cylinder, as previously described. SR7 germination was verified by the identification of vegetative cell morphologies using fluorescence microscopy of Syto9-stained cultures. Growth was defined by an increase of at least 10-fold growth in cell counts relative to t₀.

In order to ascertain whether L-alanine, L-leucine, and heat treatment induce germination under scCO₂ headspace, three replicate experiments were conducted comparing growth for SR7 spores when loaded in media P-LB, P-LBL, P-LBAL, or P-LBA ± heat treatment (Table 1; Table 2). Reactors were depressurized and scored for germination and growth by fluorescence microscopy, as previously described.

Cell densities of P-LB and L-PBA incubations from the three experiments (Table 3; Incubations A-C) were subjected to statistical analysis to establish the significance of 100 mM L-

alanine on spore growth outcomes. A non-parametric Wilcoxon/Kruskal-Wallis Test was performed on the dataset (JMP® Pro v.12; SAS Cary, NC) where growth outcome (growth/no growth) and cell density fold change (relative to t_0) were dependent variables and incubation time and inducer presence/absence (± 100 mM L-alanine) were independent variables.

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Table 2: Incubation conditions assaying chemical germination induction and heat treatment effects on SR7 growth under scCO₂

Incubation	Duration	Media	# Columns
A	18 days	P-LB	7
		P-LBA	7
		P-LBA (+ Heat)	6
		P-LBL	7
		P-LBAL	7
		Neg Ctrl	4
B	20 days	P-LB	7
		P-LBA	7
		P-LBL	6
		P-LBAL	6
		Neg Ctrl	4
C	18 days	P-LB	6
		P-LBA	5
		Neg Ctrl	4

10 Table 3: L-alanine-amended scCO₂ incubations in P-LBA and M9A+

Media	Duration	# Cultures	# Neg Ctrl
P-LBA	18 days	7	4
	20 days	7	4
	18 days	5	4
	Total	19	12
M9A+	18 days	18	6
	20 days	7	4
	Total	25	10

Growth was compared for spore-inoculated cultures in L-alanine-amended M9+ (M9A+; Table 1) and P-LBA to determine whether either medium facilitates superior growth under scCO₂ when
 15 controlling for the presence of L-alanine. Buffering capacity was comparable for both media

based on similar phosphate content. A summary of the M9A+ vs. P-LBA incubations is provided in Table 4. A non-parametric Wilcoxon/Kruskal-Wallis Test (JMP® Pro v. 12) was run on the P-LBA and M9A+ datasets, where growth outcome (growth/no growth) and cell density fold change (relative to starting concentrations) were the dependent variables and incubation time and inducer presence/absence (± 100 mM L-alanine) were the independent variables.

To establish whether increasing starting spore concentrations and incubation time improves the likelihood of growth, replicate cultures in M9A+ loaded with four starting spore concentrations (5×10^5 , 5×10^3 , 5×10^1 , 5×10^{-1} cells/ml) were run over an 18-day time course. Samples were prepared for cell counts by fluorescence microscopy according to previously described protocols. Because reactors inoculated with 5×10^1 and 5×10^{-1} cells/ml are below the limit of detection by direct counts, their concentrations are recorded as one half the detection limit (1.15×10^3 cells/ml, as previously discussed). M9A+ time course data was combined with prior M9A+ scCO₂ results to develop a logistic regression model (JMP Pro® v. 12) for growth frequency where outcome (growth/no growth) was the dependent variable, and inocula concentration and incubation time were independent variables.

Results

Isolation of scCO₂-tolerant strains from McElmo Dome fluids

Enrichment cultivation and serial passaging of McElmo Dome formation fluids with microbial growth media in high-pressure reactors under supercritical CO₂ headspace enabled the isolation of six different microbial strains, all of which are taxonomically classified within the *Bacillus* genus. Cultures were assayed for growth after the enrichment (M1 = 45 days) and each of three subsequent passages (M2 = 19 days, M3 = 33 days, M4 = 35 days) by epifluorescence microscopy methods (Table 4). Cell density from enrichment cultures was regularly observed to be greater than 10^5 cells/ml. The second (M2) and third (M3) round of culturing winnowed down the number of reactors demonstrating growth, with passaging of most media-inocula combinations discontinued due to lack of growth (or in some cases loss of pressure in reactors). The media-inocula combinations that were incubated during the fourth round (M4) of culturing showed maximum biomass accumulations of at least 7×10^5 cells/ml (Table 6), including Well 2 + MS-MR media (7.4×10^5 cells/ml), Well 4 + MS-MR (1.2×10^8 cells/ml), Well 7 + MS-MR (3.1×10^7 cells/ml), and Well 7 + MS-SR (6.9×10^6 cells/ml).

Table 4: Enrichment passaging diversity, biomass, and isolate strain summary

Passage	M1	M2	M3	M4	Isolate Strain(s)	
Duration (d)	45	19	33	35		
WELL 2 / HB-5						
MS-FM	+++	++	n.d.	n.d.	No Isolate	
MS-MR	+++	++	+++	++	MR2	
MS-SR	+++	++	+	n.d.	No Isolate	
WELL 4 / HE-1						
MS-FM	++	n.d.	n.d.	n.d.	FM4	
MS-MR	+++	+	+++	+++	MR4	
MS-SR	+++	+	-	n.d.	No Isolate	
WELL 5 / HF-3						
MS-MR	+++	+	+	n.d.	No Isolate	
WELL 7 / YB-4						
MS-FM	+++	+	n.d.	n.d.	No Isolate	
MS-MR	+++	+	+++	+++	MR7C	MR7B
MS-SR	+++	+++	+++	+++	SR7	

Biomass Concentration (direct filter counts)	
-	cells below detection limit (<1.2E3 cells/ml)
+	biomass observed at <5E4 cells/ml
++	biomass observed at 5E4 to 1E6 cells/ml
+++	biomass observed at >1E6 cells/ml
n.d.	no data

After the fourth passage (M4), individual strains were isolated by plating on LB agar.

- 5 Colonies with unique morphologies were identified by 16S rRNA gene sequencing and taxonomic annotation (Table 5). In most cases (except Well 7 + MS-MR, which enabled isolation of two strains of the same species), a single dominant strain was able to be isolated from specific combinations of media and inocula. One additional strain was isolated by LB agar colony culturing after M1 in MS + FM media with Well 4 fluids. 16S rRNA Blastn annotations of isolated strains are presented in Table 5. A 16S rRNA phylogenetic tree of McElmo Dome CO₂-passaged isolates and several closely related Bacilli is presented in FIG. 2.
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Table 5: Summary of passaged isolate morphologies and taxonomic annotations

Well Inocula	Passage Media	Colony Morphology	16S rRNA Blastn Top Hit	Blastn ID%	Designated Strain Name
2	MS-MR	Circular, entire, umbonate, dull, cream, opaque	<i>Bacillus safensis</i>	99	<i>B. safensis</i> MR2
4	MS-MR	Circular, filamentous, flat, dull, nonpigmented, translucent	<i>Bacillus licheniformis</i>	99	<i>B. licheniformis</i> MR4
4	MS-FM	Circular, entire, umbonate, dull, cream, opaque	<i>Bacillus safensis</i>	99	<i>B. safensis</i> FM4
7	MS-MR	Circular, entire, umbonate, dull, cream, opaque	<i>Bacillus safensis</i>	100	<i>B. safensis</i> MR7C
7	MS-MR	Circular, undulate, umbonate, dull, cream, opaque	<i>Bacillus safensis</i>	99	<i>B. safensis</i> MR7R
7	MS-SR	Circular, entire, convex, dull, white, opaque	<i>Bacillus megaterium</i>	100	<i>B. megaterium</i> SR7

Because enrichment passaging led to the isolation of several strains demonstrating spore-like morphologies and annotated as spore-forming taxa, isolated strains were prepared as spores for long-term storage. Previous work by Peet et al., (2015) demonstrated that spores loaded into replicate reactors under an scCO₂ headspace (e.g., *Bacillus sp.* OT1, *Bacillus sp.* MIT0214; FIG. 2) grew with frequencies dependent on incubation time and starting spore concentrations, while vegetative cells were unable to survive scCO₂ exposure. Spore preparations of *B. megaterium* SR7 and *B. licheniformis* MR4 maintained consistent viability over long periods (>2 years) in spore prep wash buffer at 4°C, though all *B. safensis* strains demonstrated markedly lower survival (decrease of CFUs/ml by at least four orders of magnitude in <6 months). Growth of *B. megaterium* SR7 and other strains was validated by triplicate incubation of spore stocks for 28-42 days using multiple media (Table 1). Growth was defined as demonstrating at least one order of magnitude increase in cell density relative to starting concentration (~10⁴ spores/ml; Table 6).

Table 6: Summary of results from strain isolate scCO₂ incubations in pure culture

Incubation	Duration	Strain	Media	Growth	Max cells/mL
P1	33 days	<i>B. megaterium</i> SR7	SR	1/2	1.0x10 ⁸
		<i>B. licheniformis</i> MR4	MR	3/3	1.2x10 ⁸
		<i>B. safensis</i> MR7C	MR	1/3	8.1x10 ⁷
		<i>B. safensis</i> MR7R	MR	1/3	4.9x10 ⁷
		<i>B. safensis</i> MR2	MR	1/2	8.8x10 ⁶
		<i>B. safensis</i> FM4	FM	2/3	3.5x10 ⁷
P2	28 days	<i>B. megaterium</i> SR7	SR	1/3	2.0x10 ⁷
			LB	3/3	6.8x10 ⁷
		<i>B. licheniformis</i> MR4	MR	1/3	1.8x10 ⁶
			LB	1/3	2.3x10 ⁷
		<i>B. safensis</i> MR7C	MR	1/3	1.5x10 ⁸
			LB	1/3	4.4x10 ⁶
P3	42 days	<i>B. megaterium</i> SR7	LB	3/3	3.5x10 ⁷
			MR	0/2	3.2x10 ⁴
		<i>B. safensis</i> MR2	LB	0/2	1.8x10 ⁴
			MR	1/3	1.9x10 ⁶
		<i>B. safensis</i> MR7R	LB	1/3	6.3x10 ⁶
			MR	3/3	6.1x10 ⁷
		<i>B. safensis</i> FM4	LB	3/3	1.2x10 ⁷
			MR	3/3	1.2x10 ⁷

Based on the results from the original four rounds of enrichment passaging (M1-M4) and subsequent pure culture scCO₂ incubation trials from spore stocks (P1-P3), strain *B. megaterium* SR7 generated the most consistently robust growth, especially in LB media (6/6 combined growth in P2-P3). Thus, strain *B. megaterium* SR7 was selected for physiological, metabolic and genomic investigation with the intent of optimizing growth under scCO₂. Isolates *B. licheniformis* MR4 and *B. safensis* FM4 also demonstrated strong growth during enrichment passaging and in pure culture.

Isolate SR7 genomics

The genome of *B. megaterium* SR7 was sequenced to determine its metabolic capacity and enable the development of genetic manipulation tools for bioproduct pathway engineering. PacBio sequencing (Pacific Biosciences; Menlo Park, CA) and assembly resulted in six contigs (DNA fragments) from *B. megaterium* SR7 (Table 7).

Table 7: SR7 Summary of PacBio genome sequencing/assembly and RAST annotation statistics

Contig	DNA Type	Length	% Bases Called	Coverage	ORFs	Plasmid-Associated	Sporulation/Germination
1	Chromosome	5449642	100.0	40.7	5,696	3	194
2	Plasmid p1	21958	99.9	57.0	35	11	6
3	Plasmid p2	17283	100.0	50.5	19	4	2
4	Plasmid p3	9202	79.2	20.8	13	3	1
5	Plasmid p4	7873	92.5	6.7	8	3	1
6	Plasmid p5	2921	52.2	0.5	4	2	1

The largest contig is 5,449,642 bp with 40.7X coverage and 39% GC content, while the other five contigs are between 2.9 kb and 22.0 kb (Table 7). Comparison of SR7 contigs with reference *B. megaterium* strain QM_B1551 showed nearly 1:1 synteny of the largest SR7 contig and the main chromosome of QM B1551, as well as similarity between the smaller SR7 contigs and QM B1551 plasmids. After synteny-based adjustments enabled the SR7 chromosome to be closed (FIG. 3), it was submitted to RAST for functional annotation along with the five smaller contigs. RAST chromosome analysis called 5,696 coding ORFs, with 13 complete rRNA operons with 5S, 16S and 23S rRNA genes and one extra 5S rRNA gene.

Genomic annotations of carbon metabolism in *B. megaterium* SR7 include genes associated with glycolysis, the Entner-Doudoroff Pathway, TCA Cycle, Pentose Phosphate Pathway, Glyoxylate Bypass, and acetogenesis from pyruvate. Annotation of the SR7 chromosome also reveals the genomic potential for broad fermentative reactions, including utilization of glucose, fructose, mannose, and xylose, and the production of butyrate, lactate, butanol, acetate, 2,3-butanediol, and ethanol.

No genes associated with direct carbon fixation pathways were detected in the genome (*e.g.*, Calvin Cycle, Wood-Ljungdahl Pathway, rTCA cycle, etc.). However, the annotation of carbonic anhydrase, which facilitates conversion of CO₂ to bicarbonate (Smith and Ferry, 2000), carbamoyl-phosphate synthase, which incorporates bicarbonate for pyrimidine and arginine biosynthesis (Arioli *et al.*, 2009), and phosphoenolpyruvate carboxylase, which catalyzes the irreversible addition of bicarbonate to phosphoenolpyruvate, indicates the capacity for SR7 to utilize and assimilate CO₂ species, potentially as a mechanism for aiding in high pCO₂ exposure survival (Santillan *et al.*, 2015, Arioli *et al.*, 2009). The presence of carboxylase may prove

useful for future engineering of CO₂-consuming metabolic pathways as a sustainable substrate in addition to solvent under scCO₂ conditions, especially in light of the previous demonstration of *B. megaterium* carboxylase activity under scCO₂ (Matsuda *et al.*, 2001).

5 Annotated inorganic redox metabolism-associated genes may ultimately prove useful by informing growth media amendments or elucidating the capacity for SR7 to grow on alternative substrates, including treated wastewater, *e.g.*, by denitrification (Yang *et al.*, 2012), reducing the need for expensive carbohydrate substrates. *B. megaterium* SR7 genes of this nature include assimilatory sulfite reductase (NAPDH-dependent), sulfite oxidase, assimilatory nitrate reductase, dissimilatory nitrite reductase (*nirBD*), nitric oxide reductase denitrification genes (10 *norQD*), and an arsenate reductase detoxification gene (*arsC*). Physiological annotations of the *B. megaterium* SR7 chromosome that hold potential utility as components of a microbial bioproduction system include a full suite of sporulation genes, siderophore assembly and uptake, flagellar motility, the twin-arginine translocation (TAT) system, and PHB metabolism, the last of which indicates a capacity for redirecting flux toward concentrated storage of excess carbon.

15 The endogenous TAT secretion system, may be useful for developing the ability to secrete specific products in the event that bioproduction focuses on the generation of proteins or enzymes.

Because the five smaller contigs failed to thoroughly annotate via RAST (*i.e.*, a majority of hypothetical genes), RAST-called ORFs were submitted to Blastx for amino acid level (20 annotation. All five contigs are annotated as containing plasmid replication, recombination, and mobility genes, as well as genes previously identified on other *Bacillus spp.* plasmids, and sporulation-related genes, content consistent with previously characterized *B. megaterium* plasmids (Eppinger *et al.*, 2011). As a result, the five putative plasmids native to *B. megaterium* SR7 are designated (in order of decreasing size) plasmids p1 through p5, the RAST statistics and (25 Blastx annotations for which are listed in Table 7. In comparison to the five putative plasmids in strain SR7 (59.2 kb total), seven (426 kb total) and three plasmids (91.3 kb total) were previously detected in strains QM B1551 and WSH-002, respectively, providing precedent for extra-chromosomal gene content in *B. megaterium*.

The *B. megaterium* SR7 genome size (5.51 Mbp) is slightly larger than several previously (30 sequenced *B. megaterium* strains, including QM B1551 (5.1 Mbp) and DSM319 (5.1 Mbp), and approximately 33% larger than strain WSH-002 (4.14 Mbp). *B. megaterium* isolate SR7 and

industrial strains QMB1551 and DSM319 share 96-97% average nucleotide identity (ANI). A comparison of shared gene content based on RAST annotations of *B. megaterium* SR7 and the three *B. megaterium* type strains reveal that approximately 12% of the SR7 genome consists of gene content not observed in three fully sequenced *B. megaterium* strains. However, the number of ORFs called by RAST appears to underestimate the number of gene calls in the original sequencing studies associated with each strain (*e.g.*, DSM319 RAST = 2,898 ORFs, Eppinger et al. (2011) = 5,272 ORFs; QM B1551 RAST = 2,915 ORFs, Eppinger et al., (2011) = 5,284 ORFs; WSH-002 RAST = 2,872 ORFs, Liu et al. (2011) = 5,269 ORFs). According to the RAST re-annotation of these submitted genomes, genes unique to SR7 include a gas vesicle structural protein (*gvpA*), genes associated with biotin synthesis/regulation (*bioHR*), a carboxysome structural gene (*ccmM*), a cell wall teichoic acid glycosylation gene (*gtcA*), several phage annotations, and chromosome/plasmid partitioning genes (*parAB*).

Physiological characterization of SR7 under ambient conditions

Strain *B. megaterium* SR7 was subjected to chemical and temperature characterization experiments under an ambient atmosphere to establish conditional growth ranges and optima of facultative aerobic growth. The results of these assays are presented in Table 8. pH growth experiments revealed the fastest growth between pH 6-7 with an extended lag phase of 76 hours for pH 4 and 10, and no growth after 123 hours at pH 2 and pH 12. LB and Biolog salinity assays revealed diminished growth of SR7 above 10 g/l NaCl. Increasing bicarbonate above 100 mM also led to decreased growth. SR7 growth is supported between 23°C and 45°C, with growth not observed after 73 hours at 9°C and 55°C. Sensitivity to all tested antibiotics (with intermediate sensitivity to spectinomycin; Table 9) may be exploited for aspects of biotechnology development methods, including selective markers for transformations. Biolog assays (Biolog, Inc; Hayward, CA) revealed SR7 growth was also inhibited by D-serine and Niaproof 4, which are known to inhibit cell wall synthesis and emulsify lipid membranes, respectively.

Table 8: Summary of viable SR7 growth in LB media over chemical and temperature ranges under aerobic conditions

Condition	Range	Optimal
pH ^a	4-10	6-7
[NaCl] (g/L) ^b	0-100	0-10
NaHCO ₃ (mM) ^c	0-300	0-100
Temperature (°C) ^d	23-45	37
Assayed ranges and durations: ^a pH: 2, 4, 6, 7, 8, 10, 12 over 123 h ^b Salinity: 0, 5, 10, 50, 80, 100 g/L over 36 h ^c Bicarbonate: 0, 100, 300, 500 mM over 36 h ^d Temperature: 9, 23, 27, 30, 37, 45, 55°C over 73 h		

5 Table 9: *B. megaterium* SR7 antibiotic sensitivity assay summary

Antibiotic	^a ug/mL	^b % Control	Assay	^c Sensitivity
Spectinomycin	100	47%	LB	S/R
Nalidixic Acid	-	14%	Biolog	S
Tetracycline	1.5	11%	LB	S
Minocycline	-	11%	Biolog	S
Lincomycin	-	11%	Biolog	S
Rifamycin SV	-	11%	Biolog	S
Aztreonam	-	10%	Biolog	S
Vancomycin	-	10%	Biolog	S
Streptomycin	10	10%	LB	S
^d D-Serine	-	10%	Biolog	S
Fusidic Acid	-	9%	Biolog	S
^d Niaproof 4	-	9%	Biolog	S
Chloramphenicol	35	9%	LB	S
Kanamycin	5	9%	LB	S
Troleandomycin	-	9%	Biolog	S
Ampicilin	50	8%	LB	S
^a Biolog does not publish antibiotic concentrations ^b (OD ₆₀₀ AB/OD ₆₀₀ Control)*100 in LB, average n = 2 (OD ₄₉₀ AB/OD ₄₉₀ Control)*100 for Biolog, average n = 2 ^c S = sensitive, R = resistant ^d Non-antibiotic treatment				

Biolog assays also established which potential sole carbon sources may be useful in future *B. megaterium* SR7 culturing and allowed comparison between *B. megaterium* SR7 and closely related *B. megaterium* strains DSM319 and QM B1551. While all three strains demonstrated robust growth on TCA Cycle intermediates citric acid and L-malic acid, DSM319 and QM B1551 both grew on L-lactic acid and L-glutamic acid, while SR7 did not (Table 10).

Table: 10: *B. megaterium* SR7 and alternative *B. megaterium* strains categorized by robust (+++), marginal (+) or no (-) growth on Biolog sole carbon sources (no metals added). Only carbon sources enabling at least one strain to demonstrate robust growth are listed

Carbon Substrate	SR7	DSM319	QM B1551
Citric Acid	+++	+++	+++
L-Malic Acid	+++	+++	+++
L-Lactic Acid	+	+++	+++
L-Glutamic Acid	+	+++	+++
α -D-Glucose	+	+++	+
Dextrin	+	+++	+
D-Mannitol	+	+++	+
D-Gluconic Acid	+	+++	+
L-Aspartic Acid	+	+++	+
N-Acetyl-D- Glucosamine	+	+++	+
L-Histidine	+	+++	+
Bromo-Succinic Acid	+	+++	+
D-Maltose	-	+++	+
Sucrose	-	+++	+
β -Hydroxy-D,L-Butyric Acid	+	+	+++
D-Saccharic Acid	-	-	+++

B. megaterium SR7 growth was markedly increased upon addition of trace metals solution to Biolog media (Table 11), including on substrates D-raffinose, α -D-glucose, γ -amino-butyric acid, myo-inositol, L-arginine, D-gluconic Acid, citric acid, N-acetyl-D-glucosamine, L-glutamic acid, D-turanose, and L-pyroglutamic acid. Malic acid appears to have facilitated robust growth only in the absence of metals. *B. megaterium* SR7 was able to grow on several carbon sources in the presence of metals that strains *B. megaterium* DSM319 and QM B1551 grew on without amendment (e.g., L-glutamic acid, α -D-glucose, sucrose, N-acetyl-D-

glucosamine, etc.), which suggests that metal-bearing co-factors specific to *B. megaterium* SR7 catabolism may require elevated metals concentrations to properly function. Initially, *B. megaterium* SR7 demonstrated robust growth on 2/71 Biolog substrates, improving to 12/71 upon addition of metals. These 12 substrates have thus been identified as potential sole carbon sources for metals-amended defined media.

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Table 11: *B. megaterium* SR7 robust (+++), marginal (+) and no (-) growth in unamended (I & II) and trace metals-amended carbon source Biolog plates. Maximum growth for each plate trial is noted by an asterisk. All substrates (and negative control) listed.

Carbon Substrate	I	II	+Metals
Citric Acid	+++	+++*	+++
α -D-Glucose	+	+	+++
L-Arginine	+	+	+++
D-Gluconic Acid	+	+	+++
L-Aspartic Acid	+	+	+++
N-Acetyl-D- Glucosamine	+	+	+++*
L-Glutamic Acid	+	+	+++
D-Turanose	+	+	+++
L-Pyrroglutamic Acid	+	+	+++
D-Raffinose	-	+	+++
γ -Amino-Butyric Acid	-	+	+++
myo-Inositol	-	+	+++
L-Malic Acid	+++*	+++	+
Gelatin	+	+	+
Pectin	+	+	+
Dextrin	+	+	+
α -D-Lactose	+	+	+
D-Mannitol	+	+	+
Methyl Pyruvate	+	+	+
D-Melibiose	+	+	+
D-Fructose	+	+	+
D-Arabitol	+	+	+
L-Alanine	+	+	+
D-Lactic Acid Methyl Ester	+	+	+
D-Galactose	+	+	+
L-Lactic Acid	+	+	+
β -Hydroxy-D,L- Butyric Acid	+	+	+
D-Cellobiose	+	+	+
D-Salicin	+	+	+
Glycerol	+	+	+
Gentiobiose	+	+	+
α -Keto-Glutaric Acid	+	+	+
L-Histidine	+	+	+
Stachyose	+	+	+
Benzo-Succinic Acid	+	+	+
D-Maltose	-	+	+
Carbon Substrate	I	II	+Metals
D-Trehalose	-	+	+
β -Methyl-D-Glucoside	-	+	+
Sucrose	-	+	+
Inosine	-	+	+
D-Sorbitol	+	-	+
β -Methyl Glucose	-	-	+
D-Gluconic Acid	+	+	-
Acetic Acid	+	+	-
L-Serine	+	+	-
Tween 40	-	+	-
D-Galacturonic Acid	-	+	-
L-Galactonic Acid Lactone	-	+	-
Acetoacetic Acid	-	+	-
Mucic Acid	-	+	-
Propionic Acid	-	+	-
Quinic Acid	-	+	-
D-Saccharic Acid	-	+	-
D-Fructose- 6-PO ₄	+	-	-
N-Acetyl-D- Galactosamine	+	-	-
Formic Acid	+	-	-
Negative Control	-	-	-
p-Hydroxy- Phenylacetic Acid	-	-	-
D-Mannose	-	-	-
Glycyl-L-Proline	-	-	-
α -Hydroxy- Butyric Acid	-	-	-
α -Keto-Butyric Acid	-	-	-
D-Fucose	-	-	-
D-Glucose- 6-PO ₄	-	-	-
Glucuronamide	-	-	-
N-Acetyl- β -D- Mannosamine	-	-	-
L-Fucose	-	-	-
D-Malic Acid	-	-	-
L-Rhamnose	-	-	-
D-Aspartic Acid	-	-	-
N-Acetyl Neuraminic Acid	-	-	-
D-Serine	-	-	-

***B. megaterium* SR7 activity under 1 atm CO₂**

As described in the Methods, culturing experiments under 1 atm CO₂ were used as a proxy for scCO₂ conditions. Modeling using the ideal gas law indicates that for rich media, predicted dissolved CO₂ concentrations for ambient air, 1 atm CO₂, and scCO₂ are 1.2x10⁻⁵ M, 2.6x10⁻² M and 2.7 M, respectively (Peet et al., 2015). Therefore, exposure of SR7 cultures to intermediate dissolved CO₂ content and pH conditions at 1 atm CO₂ may inform beneficial process improvements for enhanced growth under scCO₂.

Growth dynamics and process engineering

Assays conducted at 1 atm CO₂ showed that increased shake speed led to faster cell growth in spore-inoculated cultures (FIG. 4) and also facilitated more rapid growth of passaged vegetative cells (FIGs. 5A and 5B).

Increased shake speeds also enabled higher biomass accumulation, as the maximum OD600 reached by 150 and 250 rpm samples were 57% and 79% the OD600 maximum for 350 rpm, while maximum CFU counts reached by 150 rpm (1.5x10⁷ CFUs/ml) and 250 rpm (1.8x10⁷ CFUs/ml) samples were 43% and 51% of the maximum count for 350 rpm (3.5x10⁷ CFUs/ml), respectively (FIGs. 5A and 5B). However, it appears that cultures that reach maximum biomass accumulation due to increased mixing rates also may reach stationary phase and crash more quickly, a result often associated with end product toxicity in fermenting cultures (FIG. 5A). Therefore, due to the accelerated growth rate of *B. megaterium* SR7 at 250 RPM and the ability to sustain high biomass without experiencing a precipitous drop in CFU counts (as with 350 rpm), a shake speed of 250 RPM was utilized for all subsequent incubation experiments.

Minimal medium formulation (M9+)

Development of a minimal growth medium enables examination of microbial physiology, determination of nutritional growth requirements, and holds potential to reveal the metabolic pathways through which carbon flux occurs during growth under various conditions. Initial attempts to grow *B. megaterium* SR7 in M9 base medium under 1 atm CO₂ with 0.4% glucose or 0.4% xylose as sole carbon source in the presence and absence of a trace metals solution were unsuccessful (Table 12). Subsequent growth assays revealed that both a *de facto* vitamin/co-factor supplement (e.g., dilute LB/YE at concentrations insufficient to independently support

observable growth) and trace metals solution in glucose-amended media enabled robust growth (Table 12; FIGs. 6A and 6B). The use of NO₃⁻ as a potential alternative electron acceptor did not demonstrate any pronounced effects on growth rates or biomass accumulation, despite genomic evidence for potential nitrate/nitrite reduction capacity. Due to potential conflicts between xylose-induced biomass accumulation and heterologous gene expression, media development proceeded with glucose as sole carbon source. Since substituting out 0.01X LB for 0.01X YE (*e.g.*, 1X is the concentration of YE present in LB, 5 g/l; 0.01X YE = 50 mg/l) generated similar outcomes, media development proceeded with YE due to its more defined nature.

Table 12: M9 supplemented growth outcomes under 1 atm CO₂

M9 Amendments	Growth
0.4% Glucose	-
0.4% Xylose	-
0.4% Glucose + 1X Metals	-
0.4% Xylose + 1X Metals	-
0.001X LB	-
0.01X LB	-
0.001X YE	-
0.01X YE	-
0.001X LB + 0.4% Glucose	+
0.01X LB + 0.4% Glucose	+
0.01X YE + 0.4% Glucose	+
0.01X YE + 0.4% Glucose + 5 mM NaNO ₃	+
0.001X LB + 0.4% Glucose + 5 mM NaNO ₃	+
0.01X LB + 0.4% Glucose + 5 mM NaNO ₃	+
0.001X LB + 0.4% Glucose + 0.1X Metals	+++
0.01X LB + 0.4% Glucose + 0.1X Metals	+++
0.01X YE + 0.4% Glucose + 0.1X Metals	+++
0.01X YE + 0.4% Glucose + 0.25X Metals	+++
0.01X YE + 0.4% Glucose + 1X Metals	+++
0.01X LB + 0.4% Glucose + 5 mM NaNO ₃ + 0.1X Metals	+++
0.01X YE + 0.4% Glucose + 5 mM NaNO ₃ + 0.1X Metals	+++
LB/Yeast Extract (YE) Dilutions	
0.01X LB = 100 mg/L tryptone, 50 mg/L YE, 100 mg/L NaCl	
0.001X LB = 10 mg/L tryptone, 5 mg/L YE, 10 mg/L NaCl	
0.01X YE = 50 mg/L	
0.001X YE = 5 mg/L	

0.1X trace metals solution proved the most effective concentration for enabling rapid growth of passaged vegetative cultures. Although 1 atm CO₂ passaged cultures in M9 + 0.4% glucose + 0.01X YE amended with 0.25X and 1.0X trace metals achieved the same maximum OD₆₀₀ as 0.1X metals-amended cultures, lower OD₆₀₀ values at intermediate time points suggested diminished growth rates relative to 0.1X metals (FIG. 6A). Further investigation revealed that while cultures in the presence and absence of 0.1X trace metals reach intermediate OD₆₀₀ values at approximately the same rate, metals-amended cultures continue to grow while non-amended cultures appear to enter stationary phase (FIG. 6B). The effect of trace metals on accelerated anaerobic growth has previously been observed in (David et al., 2010), who suggested that bacteria require metal co-factors to improve growth outcomes.

The final combination of M9 + 0.01X YE + 0.1X metals + 0.4% glucose is designated “M9+” medium, and was used as the base semi-defined minimal medium for all subsequent sole carbon source experiments. After establishing M9+ medium components, 1 atm CO₂ growth curves conducted in both M9+ and LB media revealed SR7 anaerobic doubling times based on OD₆₀₀ of 1.93 ± 0.1 h and 2.07 ± 0.1 h, respectively (FIGs. 7A and 7B). OD₆₀₀ values and glucose consumption for M9+ media incubations appear to indicate log phase growth, then a brief stationary phase, followed by steady increases in OD and glucose consumption (though a decrease in CFUs).

Despite positive growth on glucose as a sole carbon source in M9+ defined medium, *B. megaterium* SR7 showed a reduced growth rate under the same culturing conditions in LB supplemented with 0.4% glucose, including both with and without metals solution, as demonstrated by an extended lag (FIG. 8A). Similar results were generated under aerobic conditions (data not shown). Incomplete glucose consumption after 24 hours (30-34% glucose remaining; FIG. 8B) further indicates that LB-amended glucose is not fully utilized either due to growth on an alternative substrate (*e.g.*, dilute YE or tryptone), or because glucose consumption during growth in LB medium is generating a toxic concentration of metabolites. Evidence from aerobic cultures demonstrate that after 2-3 hours, *B. megaterium* SR7 accumulates and maintains ~0.6 g/l acetate, while LB only cultures accumulate 0.4-0.5 g/l acetate after 5 hours, at which point it is consumed as a substrate (data not shown). These results suggest a potential mechanism for growth inhibition by glucose-associated end product toxicity as well as glucose

repression of acetate re-assimilation. Inspection by phase contrast light microscopy of *B. megaterium* SR7 grown in LB + 0.4% glucose under 1 atm CO₂ appears to show an increase in PHB granules (FIG. 9; polyhydroxybutyrates (PHBs). The membranes of many other cells appear to be nearly completely degraded, causing cells to look completely transparent. Because previous studies have shown high production of PHBs do not have a toxic effect on *B. megaterium* (Rodríguez-Contreras et al., 2013) it is considered unlikely that PHB accumulation itself is disrupting metabolism or cellular integrity.

SR7 cultures incubated under 1 atm CO₂ in M9+ and LB media generated a variety of fermentative products detected by HPLC (FIG. 10). After 6 hours, cultures in both media generated acetate (LB: 271 mg/L; M9+: 49 mg/L) while LB cultures also generated TCA intermediate succinate (300 mg/L), possibly due to availability of more complex proteinaceous substrates. After 48-hour incubations in M9+ medium, SR7 continued to generate acetate (352 mg/L), while also generating large amounts of lactate (1.54 g/L), and low concentrations of succinate (62 mg/L) and ethanol (17 mg/L). In contrast, 48-hour incubations under 1 atm CO₂ in LB medium generated a metabolite profile constrained only to succinate (1.11 g/L) and acetate (235 g/L), which indicates alternative carbon utilization absent lactic acid fermentation. No additional volatile products (*e.g.*, isobutanol, isopentanol, phenethyl alcohol) were detected by gas chromatography at any sub-sampled time points.

B. megaterium SR7 germination induction

Because germination and growth of spores under scCO₂ conditions has previously been shown to be a stochastic process (Peet et al., 2015), an effort was made to improve germination rates during scCO₂ incubations in order to be able to express heterologous enzymes more quickly and consistently. The literature has shown that a broad array of compounds, including several L-amino acids, and peptidoglycan are able to induce metabolically dormant endospores to germinate (Wei et al., 2010). These inducers have to be shown to activate germination in *Bacillus* through several independent pathways (Hyatt and Levinson, 1962). Two amino acids (L-alanine and L-leucine) previously shown to induce germination through different pathways were chosen for investigation with *B. megaterium* SR7 to increase the likelihood of success in an uncharacterized strain. Initial assays in LB under 1 atm CO₂ as a proxy for scCO₂ conditions

demonstrated that L-alanine-amended cultures germinated by 4.5 hours, while unamended cultures grew between 4.5 and 6 hours after inoculation (FIG. 11).

After germination, growth occurs at nearly identical doubling times by OD600 (M9A+: 0.86 h; M9+: 0.89 h), suggesting that the effect of alanine is specific to the germination process rather than improved growth rates. Heat treatment reduced *B. megaterium* SR7 germination (marginal growth at 6 hours) rates and increased doubling times (1.11 h), despite previously being shown to induce spore germination spores for certain *Bacillus* species (Hyatt and Levinson, 1962). It is possible though that in the case of *B. megaterium* SR7, rather than inducing germination, the heat treatment is lethal to a sub-population of spores, decreasing the number of viable spores available to germinate and grow. During the initial period of vegetative outgrowth spore concentrations remain nearly constant (FIG. 11). As a result, it appears that individual spores or sub-populations will germinate and commence vegetative growth while remaining spores stay dormant, at least initially. Therefore, adding an inducer such as L-alanine provides a consistent source of growth potential to a pool of dormant cells.

Because phosphate buffered saline (PBS) solution does not have an available carbon source, spore-inoculated PBS cultures with inducer amendments under 1 atm CO₂ headspace enabled investigation of the anaerobic germination process independent of growth. Results assayed by fluorescence microscopy, bulk fluorescence and OD600 demonstrate that germination is induced by 3 hours, including with 100 and 250 mM L-alanine, 25 mM L-leucine and heat-treated 100 mM L-alanine (FIGs. 1 and 12). All incubated cultures showed an approximate 2-fold increase (1.9-2.1-fold) in fluorescence magnitude after 3 hours, eventually reaching a maximum at the 8.5 hour endpoint of 2.4-fold the bulk fluorescence (100 mM L-alanine) of PBS only incubated spores. By 24 hours, every inducer-amended sample also had a lower OD600 than unamended PBS samples, indicative of the flooding of the spore interior after spore coat degradation, decreasing the cell's index of refraction.

A similarly pronounced inducer effect was observed by fluorescence microscopy direct filter counts based on spore staining patterns indicative of dormancy and germination. All treatments increased the percentage of germinated cells by 3 hours relative to PBS incubated spores (FIG. 12). According to filter counts, unamended PBS incubated spores maintained a constant, low-level abundance of germinated cells, increasing from 14.7% germinated at t₀, to 19.8% at 8.5 hours. Inducer-treated cultures increase more substantially, from 16.7-18.9% at t₀

to 63.8-75.1% at 8.5 hours. Since all cells that showed stain membrane penetration, including whole cell and center-localized (FIG. 1), were considered “germinated,” it is possible that “percentage germination” values in FIG. 12A may be overestimates. Microscopic inspection of inducer-amended PBS incubations did not reveal any vegetative cell morphologies, suggesting that L-alanine and L-leucine are not being utilized as a carbon source for growth in PBS.

The effect of heat treatment on PBS cultured spores (also amended with 100 mM L-alanine) generated mixed results. After initially increasing in bulk fluorescence, heat-treated cultures steadily decreased in bulk fluorescence, while microscopy indicated that heat treatment increased germination to the highest observed frequencies (FIG. 12A). However, this result may be due to spore coat damage during heat treatment at 70°C that caused more cells to become susceptible to membrane penetration by Syto9 cell stain. Therefore, without further physical evidence it is difficult to conclude whether the apparent germination inducing effect by heat treatment is a genuine result or false positive.

Delayed germination induction experiments in which spores incubated under 1 atm CO₂ for 14.5 hours in PBS were amended with L-alanine or L-leucine and assayed for germination after 9.5 hours demonstrated the capacity to actively germinate in the presence of inducers mid-culture. Higher concentrations of L-alanine and L-leucine did not appear to improve the extent of germination relative to lower concentrations, suggesting that the capacity for SR7 spores to be germinated saturates at or below 100 mM L-alanine and 10 mM L-leucine. The observed effects caused by both amino acid inducers were comparable in magnitude (Table 13). Follow-up investigation of a physiological state change in endospores caused by alanine amendment to carbon-free PBS cultures was ultimately verified by use of FCM, as explained below in section “Physiological signatures of induced germination.”

Table 13: Germination assays 9.5 hours after delayed induction in PBS under 1 atm CO₂ (OD decrease indicative of germination)

Inducer	mM	Fluorescence Fold Increase	OD ₆₀₀ Fold Decrease
L-alanine	250	1.4	1.2
	100	1.4	1.2
	25	2.1	1.5
L-leucine	25	2.0	1.2
	10	2.0	1.2

5 Physiological signatures of induced spore germination

Additional investigation using flow cytometry (FCM) sought to build upon preliminary LB-based evidence (FIGs. 11, 12A and 12B; Table 13) to verify a physiological effect of alanine on spores during the transition from dormant to germinated cell. FCM data collected on Syto16-stained SR7 cells from unamended and L-alanine-amended PBS cultures revealed two populations capable of gating on side and forward scatter (FIG. 14): 1) PBS only incubated cells (Population 1) and 2) L-alanine-amended PBS cells (Population 2). Based on Syto16 and propidium iodide (PI) fluorescence intensities, three additional populations could subsequently be gated based on unique Syto16 and propidium iodide (PI) fluorescence signatures. These individual populations appear well correlated with visual evidence by fluorescence microscopy of three staining patterns of varying intensity (whole cell, center localized, edge localized; FIG. 1). These staining patterns and germination stage categories can be thus be mapped onto each other schematically (FIG. 13).

Population 1 (PBS) and 2 (PBS + L-alanine) display marked differences in terms of fluorescence magnitudes and distributions (FIG. 14). A wide majority of spores incubated in PBS are gated as dormant cells (74.3%), with 22.0% and 3.7% gated as activated and germinated, respectively (FIGs. 14 and 15). When L-alanine is amended to PBS cultures, fluorescence distributions shift towards activated (44-69%) and germinated (1.38-31.8%) fluorescence signatures (FIGs. 14 and 15). These results reinforce the implication that L-alanine acts to induce physiological changes involved in the progression from dormant endospore to germinated cell.

***B. megaterium* SR7 growth and activity under supercritical CO₂**

Results generated under aerobic and 1 atm CO₂ conditions investigating the physiology, growth dynamics and germination induction of *B. megaterium* SR7 were integrated in an effort to generate robust growth and production of natural products under scCO₂. Chemical induction experiments in P-LB medium spore-loaded scCO₂ incubations (Table 14) revealed that L-alanine confers a statistically significant improvement in germination rates and growth outcomes relative to inducer-free cultures (FIG. 16), while L-leucine reduced growth frequency under scCO₂ conditions relative to controls. Growth was defined as at least one order of magnitude increase in biomass according to epifluorescence cell counts:

- 1) high growth: ≥ 40 -fold increase in direct cell counts relative to t_0 cell density
- 2) low growth: >10 -fold increase in direct cell counts relative to t_0 cell density
- 3) germinated: <10 -fold increase, mixture of vegetative cells and spores
- 4) dormant: <10 -fold increase, only spore morphologies observed

Table 14: Growth outcomes for unamended and induced scCO₂ cultures

Incubation	Duration	Media	Growth
A	18 days	P-LB	3/7
		P-LBA	5/7
		P-LBA (+Heat)	3/6
		P-LBL	1/7
		P-LBAL	2/7
		Neg Ctrl	0/4
B	20 days	P-LB	1/7
		P-LBA	5/7
		P-LBL	0/6
		P-LBAL	2/6
		Neg Ctrl	0/4
C	18 days	P-LB	1/6
		P-LBA	3/5
		Neg Ctrl	0/4

Overall, growth was observed in 63% of all cultures amended with L-alanine, while only 36% of unamended reactors showed growth (Table 14). Median fold increase in cell concentration for P-LBA cultures was 37.5 and for unamended phosphate-buffered LB (P-LB;

Table 1) was 22.8. Using growth frequency and fold change as inputs for non-parametric modeling of scCO₂ growth outcomes established that L-alanine conferred a statistically significant improvement on growth ($p = 0.0036$) relative to P-LB cultures by a Wilcoxon/Kruskal-Wallis Test. L-leucine (P-LBL media) only generated growth in 7.7% of reactors, while the combined treatment of L-alanine and L-leucine (P-LBAL) resulted in 31% growth frequency. Diminished growth in L-leucine reactors suggests a neutral to inhibitory effect on *B. megaterium* SR7 scCO₂ germination and growth, which is unexpected based on 1 atm CO₂ results (Table 14). As the L-alanine + heat treatment reactors (50%) also did not grow as well as non-heated L-alanine reactors, L-leucine and heat treatment were discarded as potential growth enhancing components of the microbial bioproduction system.

After verifying the positive growth effect of L-alanine on spore-loaded P-LBA scCO₂ cultures, two rounds of 18-20 day scCO₂ incubations of SR7 spores in M9A+ displayed growth in 11/18 reactors and 5/7 reactors, respectively. The total frequency of growth in M9A+ (64%) is thus comparable to P-LBA (63%), though M9A+ appears to increase the frequency of high level (>40 fold) growth (56% in M9A+ vs. 26% in P-LBA; FIG. 16). Despite similar overall frequencies, median biomass accumulation was improved for cultures grown in M9A+ (64.3 fold increase) relative to P-LBA (37.5 fold).

As statistical tests did not establish significance ($p = 0.381$) in differential growth outcomes for P-LB and M9+, subsequent system development proceeded with semi-defined M9A+ minimal media, which simplifies pathway engineering architecture and metabolic flux analysis due to growth on a single carbon source. In order to more fully understand the relationship between starting *B. megaterium* SR7 spore concentration and likelihood of growth in M9A+ media, a logistic regression model for growth frequency was generated in part using data from an 18 day scCO₂ time course experiment (sampled at 6, 12, and 18 days) with starting spore concentrations varied over six orders of magnitude. The results of the incubation are summarized in Table 15, using a 10-fold increase in filter cell counts as the threshold for growth. After merging the time course growth data (Table 15) with previously generated results from M9A+ incubated spores (Table 14) a total of 91 experimental samples and 24 negative controls subjected to logistic regression analysis demonstrated that both loaded spore density ($p = 0.0057$) and incubation time ($p = 0.003$) have statistically significant impacts on growth frequency, while the interaction of their effects was not significant ($p = 0.89$). The overall regression model

generated the following equation (plotted in FIG. 17) to describe growth frequency (Z) as a function of incubation time (X) and starting inocula concentration (Y).

$$Z = \frac{1}{1 + e^{4.7849 - 0.228 * X - (3.8207 * 10^{-6}) * Y}}$$

5

***B. megaterium* SR7 fermentation products under scCO₂**

Cultures from 6, 12, and 18-day SR7 time course incubations in M9A+ media demonstrating growth (>10-fold increase in cell counts) under scCO₂ were analyzed for natural fermentation products by HPLC. Cultures generated several detectable metabolites, including for succinate (up to 2.4 mg/l), lactate (up to 17.8 mg/l), and acetate (up to 9.5 mg/l) (FIGs. 18A and 18B). All metabolites were also detected in culture under 1 atm CO₂ conditions grown in similar M9+ media (FIG. 10), suggesting shared features of active fermentative pathways under both conditions. Normalization of product concentrations by total cell counts enables calculation of metabolite productivities on per cell basis. Maximum per cell productivity values (mg product cell⁻¹) are 1.1x10⁻¹⁰, 5.0x10⁻¹⁰, and 1.3x10⁻⁹ for succinate, lactate and acetate, respectively. These productivities are comparable to results observed under 1 atm CO₂ (assuming OD600 = 1.0 corresponds to 10⁸ cells/ml, based on filter counts), which displayed maximum productivities (mg product cell⁻¹) in M9+ media of 5.5x10⁻¹⁰, 3.4x10⁻¹⁰, and 5.8x10⁻¹⁰ for succinate, lactate, and acetate, respectively. Therefore, a relationship within roughly an order of magnitude appears to exist between concentration of per cell metabolite production and total cell numbers per culture.

The work described herein allowed for the identification and isolation a scCO₂-compatible strain, *B. megaterium* SR7. This strain was used to develop optimal process engineering and culturing modifications in order demonstrate the capacity for enhanced growth and natural product generation under scCO₂.

EXAMPLE 2: Metabolic Engineering of *Bacillus megaterium* SR7 for Heterologous Gene Expression and Advanced Biofuel Synthesis and Recovery under Biphasic Aqueous-Supercritical CO₂ Conditions

The scCO₂ harvesting systems described herein were evaluated for production of
5 biofuels, which due to the semi-hydrophobic chemistry of alcohols like isobutanol and butanol, readily causes compound partitioning from the aqueous phase into scCO₂ (e.g., Kow >4; Timko et al., 2004). Although short-to-medium chain alcohols (e.g., isobutanol, isopentanol) were produced in the methods described herein, scCO₂ culturing methods holds may be useful for extraction of a broad range of high-value bioproducts.

10 Isobutanol production requires modification of the amino acid valine biosynthesis pathway by directing flux of the intermediate α -ketoisovalerate (α -KIV) away from L-valine production and instead towards isobutyraldehyde and finally isobutanol (Atsumi et al., 2008; FIG. 19). α -KIV itself is generated from the condensation of two pyruvates (via pyruvate kinase, Pyk), which is decarboxylated (via acetolactate synthase, IlvIH) to form 2-acetolactate, then
15 reduced (via acetohydroxy acid isomeroreductase, IlvC) and dehydrated (via dihydroxy acid dehydratase, IlvD) to α -KIV. Insertion of exogenous pathway genes for keto-acid decarboxylase (kivD) and alcohol dehydrogenase (adh) then facilitates isobutanol production from α -KIV.

The results described herein demonstrate a two-phase harvesting method for stripping of microbially produced chemicals using in situ scCO₂ extraction. These methods involve the
20 development of a genetic system for expression of single and multi-gene pathways under scCO₂ and the first demonstration of in situ bioproduct recovery by partitioning into scCO₂, thereby establishing a new branch of microbial bioproduction by enabling access of an engineered bacterial strain to the unique properties of sustainable solvent supercritical carbon dioxide.

25 **Methods**

Strain, media and culture conditions

As described herein, environmental strain *Bacillus megaterium* SR7 was isolated through enrichment culture and serial passaging of fluids sourced from the deep subsurface McElmo Dome supercritical CO₂ formation (Example 1). Previous *B. megaterium* SR7 development
30 under 1 atm CO₂ included the formulation of semi-defined minimal medium M9+, which consists of M9 base medium amended with 0.4% D-glucose, 50 mM yeast extract, 0.1X trace

metals solution (Boone *et al.*, 1989). The addition of 100 mM L-alanine to M9+ (resulting in medium “M9A+”) was previously shown to increase rates of SR7 spore germination and growth rate under scCO₂ conditions. Therefore, all culturing experiments conducted under 1 atm CO₂ occur in M9+ medium, and under scCO₂ in M9A+ medium. All cultures were incubated at 37°C and 250 rpm based on previous results showing enhanced growth rates and population longevity under these conditions. All 1 atm CO₂ and scCO₂ experiments were prepared within an anaerobic chamber (Coy Products) containing an atmosphere of 95% CO₂ and 5% H₂. Experiments conducted under 1 atm CO₂ used 10 ml of CO₂-degassed culture media in 100 ml serum vials with clamped rubber stoppers. Incubations under scCO₂ used ¾ inch 316 stainless steel tubing fitted with quarter turn plug valves (Swagelok (Solon, OH) or Hylok (Houston, TX)) for 10 ml total capacity. As described in Example 1, reactors were filled to ½ capacity (5 ml) with inocula and degassed media, after which the headspace was pressurized with extraction grade CO₂ gas at a rate of 2-3 atm min⁻¹ until reaching a final pressure of 100 atm. After pressurization, reactors were incubated in a 37°C warm room and mixed at 250 rpm until unloading.

Development for SR7 genetic manipulation and expression

Vector construction

All primers used in plasmid construction, final vector constructs, transformed strains and associated references are presented in Tables 15A and 15B. The *lacZ* gene was PCR amplified from plasmid pKVS45 LacZ_LVA with primers LacZ_F and LacZ_R. Shuttle vector pRBBm34 (Amp^R (*E. coli*), Tet^R (*B. megaterium*); pBR322 Ori (*E. coli*), RepU (*B. megaterium*)) was used as a scaffold for pathway genes. PCR products and pRBBm34 were digested with SpeI and SphI prior to ligation to create the pJBxL plasmid (FIG. 20, left panel). The xylose repressor and promoter of pRBBm34 were replaced with a hyper-spank promoter (P_{Hyper-spank}) and *lacI* using circular polymerase extension cloning (CPEC). The pRBBm34 plasmid was PCR linearized with two sets of primers to remove *xyIR* and P_{xyI}: pRBBm34_F / Bla_R and Bla_F / pMM1520R. P_{Hyper-spank} and *lacI* were PCR amplified from pDR111 using pMM1520-P_{Hysp}_F and LacI-pRBBm34_R. Standard CPEC cloning was used to assemble the three PCR products into the P_{Hyper-spank} plasmid. The *lacZ* gene with a ribosome-binding site was PCR amplified from

the plasmid pKVS45 LacZ_LVA using: RBS-LacZ_F and LacZ_R. PCR products and the P_{Hyper-spank} plasmid were digested with SalI and SphI prior to ligation to create the pJBhL plasmid.

Table 15A: Primers used for vector construction

5

Name	Sequence (5' > 3')	Target	Reference
LacZ_F	GTCCAAACTAGTACCATGATTACGG ATTCAGTGGC (SEQ ID NO: 3)	pKVS45 LacZ_LVA	Solomon et al., 2012
LacZ_R	CCGCCGGCATGCTCATTATTTTGA CACCAGACCAACTGG (SEQ ID NO: 4)		
pRBBm34_F	CGGCCGGCACCTCGCTAAC (SEQ ID NO: 5)	pRBBm34	Biedendieck et al., 2007
Bla_R	GGTGCCTCACTGATTAAGCATTGG (SEQ ID NO: 6)		
Bla_F	CCAATGCTTAATCAGTGAGGCACC (SEQ ID NO: 7)	pMM1520	Malten et al., 2005
pMM1520_R	AGATCCACAGGACGGGTGTG (SEQ ID NO: 8)	pDRIII	Guerout-Fleury et al., 1996
pMM1520- P _{Hysp} _F	CACACCCGTCCTGTGGATCTGACTC TCTAGCTTGAGGCATC(SEQ ID NO: 9)		
LacI- pRBBM34_R	GTTAGCGAGGTGCCGCCGGGATCCT AACTCACATTAATTGCG (SEQ ID NO: 10)	pKVS45 LacZ_LVA	Solomon et al., 2012
RBS-LacZ_F	AGCTTAGTCGACAGGGGGAAATGTA CAATGACCATGATTACGGATTCACT GGC (SEQ ID NO: 11)		
KivD_F	GTCCAAACTAGTATGTATACAGTAG GAGATTACCTATTAGACCG (SEQ ID NO: 12)	pCOLA KivD, Fjoh_2967	Sheppard et al., 2014
KivD_R	GAGGAGCATGCGAGCTCGGATCCT CATTATGATTTATTTGTTCAGCAA ATAGTITACCC (SEQ ID NO: 13)		
RBS-ADII6_F	GAGGAGGGATCCTCGACAGGGGGAA ATGTACAATGAGCTACCCGGAAG TTCG (SEQ ID NO: 14)	pACYC (car,sfp)	
ADH6_R	CCGCCGGCATGCAATGCGGCCGCTC ATTAGTCGCTGAATTCTTTATCGTA ACCAACC (SEQ ID NO: 15)		
RBS-YqhD_F	TAATGAGGATCCTCGACAGGGGGAA ATGTACAAATGAACAACCTTAATCT GCACACCC (SEQ ID NO: 16)	E. coli MG1655 gDNA	Common lab strain
YqhD_R	GCATGCAATGCGGCCGCTCATTAG CGGGCGGCTTCGTATATAC (SEQ ID NO: 17)		

10

Table 15B: Vector constructs and strains used in the work described herein

Strain	Plasmid	Description / Genotype	Reference
<i>B. megaterium</i> SR7	Endogenous only	Wild-type isolate from scCO ₂ subsurface formation	Thesis Ch. 3
SR7JR1	pJR1	CmR; mob, oriT, rep (<i>E. coli</i>), pUCTV2 ori ^{ts} (<i>Bacillus</i>), sacB (<i>B. subtilis</i>)	Richhardt et al., 2010
SR7x	*pJBx	P _{Xyl} -empty construct	This study
SR7xL	*pJBxL	P _{Xyl} lacZ; Tet ^R	This study
SR7h	*pJBh	P _{Hyper-spank} -empty construct; Tet ^R	This study
SR7hL	*pJBhL	P _{Hyper-spank} lacZ; Tet ^R	This study
SR7xK	*pJBxK	P _{Xyl} kivD _{LI} ; Tet ^R	This study
SR7xKA6	*pJBxKA6	P _{Xyl} kivD _{LI} , adh6 _{Sc} ; Tet ^R	This study
SR7xKY	*pJBxKY	P _{Xyl} kivD _{LI} , yqhD _{Ec} ; Tet ^R	This study
SR7xKAB	*pJBxKAB	P _{Xyl} kivD _{LI} , adhA _{Bm} ; Tet ^R	This study
SR7xKAL	*pJBxKAL	P _{Xyl} kivD _{LI} , adhA _{LI} ; Tet ^R	This study
SR7xKAP	*pJBxKAP	P _{Xyl} kivD _{LI} , adhA _{Ec} ; Tet ^R	This study
SR7xGFP	*pJBxGFP	P _{Xyl} sfGFP; Tet ^R	This study
SR7hGFP	*pJBhGFP	P _{Hyper-spank} sfGFP; Tet ^R	This study
*pRBBm34 derivative (Biedendieck et al., 2007)			

- 5 For solventogenesis strain engineering, *kivDLI* sourced from *Lactococcus lactis* and *adh6Sc* from *Saccharomyces cerevisiae* were placed downstream of xylose-inducible promoter PXyl on pRBBm34. Vector construction began by PCR amplifying *kivDLI* from pCOLA *KivD*, *Fjoh_2967* using primers KivD_F and KivD_R. PCR products and the pRBBm34 plasmid were digested with SpeI and SphI prior to ligation to create the pJBxK plasmid. *Adh6Sc* from *S.*
- 10 *cerevisiae* was PCR amplified from pACYC (*car*, *sfp*; *adh6*) with the same ribosome binding site as was used for *kivDLI* using primers RBS-ADH6_F and ADH6_R. *Adh6Sc* was added between the BamHI and SphI restriction sites in PXyl *KivDLI* to create pJBxKA6. *YqhDEc* from *E. coli* was PCR amplified from *E. coli* MG1655 genomic DNA with the same ribosome binding site as was used for *kivDLI* using primers RBS-YqhD_F and YqhD_R. *YqhDEc* was added between the
- 15 BamHI and SphI restriction sites in PXyl *KivDLI* to create pJBxKY (FIG. 20, right panel). All constructs were verified by DNA sequencing.

Transformation methods

Initial attempts to genetically transform strain SR7 with shuttle vector pRBBm34 (Addgene; Cambridge, MA) used an established *Bacillus* electroporation protocol (Zhang et al. (2011; *Analytical Biochemistry*). Modifications to the method included the addition of cell wall
5 weakeners (3.9% glycine, 80 mM DL-threonine) one hour prior to electroporation, and testing a wide range of plasmid concentrations (10-200 ng/μl) and cell densities (OD₆₀₀ 0.6-1.2). Conjugation-based transformation was attempted with SR7 using mating strain *E. coli* S-17 and plasmid pJR1 (provided courtesy of the Meinhardt Lab, University of Muenster, Germany; Table 1B), following the protocol of Richhardt et al. (2010). To optimize the protocol, a range of
10 donor to recipient strain volumes were tested (*e.g.*, 10:1 to 1:1000) after reaching protocol-prescribed OD₆₀₀ values. Post-transformation counter-selection included pasteurization and the *sacB* suicide system. The final transformation method attempted was protoplast fusion based on von Tersch and Robbins (1990) and Biedendieck et al. (2011) using shuttle vector pRBBm34. The cell wall removal step was optimized to increase viable protoplasts by modifying lysozyme
15 concentrations and transformed protoplast incubation times. Counter-selection occurred by plating protoplasts on a soft agar overlay above LB agar containing 5 μg/ml tetracycline.

Plasmid maintenance

Several assays were utilized to verify exogenous plasmid stability in SR7 during growth
20 under 1 atm CO₂. To assay for maintenance of pRBBM34 in SR7 under 1 atm CO₂, singleton incubations of SR7 empty vector control strain (SR7x), which constitutively expresses tetracycline resistance, were inoculated at a concentration of 10⁵ spores/ml and passaged three times in LB for 24 hours with and without supplementation of 0.5 μg/ml tetracycline. After each
25 passage, cultures were plated on LB agar with or without 0.5 μg/ml tetracycline to determine if cultures grown without antibiotics maintained the transformed vector over multiple growth cycles in the absence of a selective pressure. SR7 wild-type and SR7x strains were also assayed to determine minimum required tetracycline concentration to select for transformed strains containing the vector. Cultures inoculated with 10⁵ spores/ml were incubated in LB amended with a range of tetracycline concentrations under both aerobic (Tet 0.05-10.0 μg/ml) and 1 atm
30 CO₂ conditions (Tet 0.1-10.0 μg/ml) and scored for growth by OD₆₀₀ relative to cultures that were not amended with Tet.

Heterologous single gene expression under 1 atm CO₂ and scCO₂

B. megaterium SR7 strains SR7xL and SR7hL (bearing genetic constructs pJBxL and pJBhL, respectively) and empty vector control strains SR7x and SR7h were assayed for protein expression in *B. megaterium* SR7 under 1 atm CO₂ and scCO₂ conditions. 1 atm CO₂ cultures grown overnight were diluted in fresh media to OD₆₀₀ 0.01, cultured for 2 hours, then amended with 0.4% D-xylose (P_{Xyl}; SR7x, SR7xL) or 5 mM IPTG (P_{Hyper-spank}; SR7h, SR7hL) to induce expression. After 24 hours, 1 ml of culture volume was spun down for 5 min x 21,000g and the remaining pellet was stored at -20°C until analysis.

Supercritical CO₂ cultures were loaded with 3x10⁵ spores/ml (as described in Example 1) of strain SR7xL. A subset of reactors was amended with 0.5% xylose inducer. Reactor cultures were incubated for 21 days then depressurized and prepared for fluorescence microscopy as previously described. 2 ml of culture volume was spun down for 5 min x 21,000g, after which the supernatant and pellet were separately stored at -20°C until analysis. Supernatant was prepared for GC-MS analysis by methods described below and for HPLC analysis by methods described in Example 1.

Pellets from 1 atm CO₂ and scCO₂ cultures were lysed by addition of 100 µl of Bacterial Protein Extraction Reagent (B-PER; Thermo Scientific; Waltham, MA) and vortexed for 30 minutes. After lysed pellets were centrifuged for 20 min x 18,500g at 4°C, supernatants were prepared for total protein analysis using the Pierce™ BCA Protein Assay Kit (Thermo Scientific; Waltham, MA) according to manufacturer's instructions. Colorimetric signatures proportional to total sample protein were measured by OD₅₆₂, including for cell-free B-PER negative controls. Total protein standard curves were generated using 0.05-1.0 mg/ml of bovine serum albumin (BSA) according to the same protocol. Samples and B-PER negative control were prepared for LacZ activity assays by adding 70 µl of lysed culture supernatant to 730 µl of assay buffer (0.1 M sodium phosphate buffer, 10 mM KCl, 1 mM MgSO₄) and 200 µl of β-galactosidase substrate (4 mg/ml o-nitrophenyl-β-D-galactoside, ONPG). LacZ activity was quantified as the rate of OD₄₂₀ absorbance per minute, as the product of ONPG cleavage by B-galactosidase absorbs at 420 nm. Absorbance rate was normalized by total protein per culture using BSA standard curves. Protein-normalized rates were converted to specific activity using the assay extinction coefficient and volume to generate units of µmol min⁻¹ mg⁻¹.

Heterologous biofuel production under 1 atm CO₂

Vegetative cultures of SR7x and SR7xKA6 were prepared by growing 10⁵ spores/ml of each strain in CO₂-degassed LB tet_{0.5} for overnight growth. Stationary phase cultures were then
 5 diluted in 10 ml of fresh LB + tet_{0.5} to OD₆₀₀ 0.01. After 2 hours, passaged cultures were amended with 5 mM α -KIV substrate and 0.4% D-xylose to induce gene expression. Passaged cultures were grown for 24 hours post-induction, with sub-sampling at 4 and 24 hours by aseptic needle extraction. After 1 ml samples were centrifuged for 5 minutes x 21,000g, 500 μ l supernatant was pipetted into separate tubes with 500 μ l of ethyl acetate solvent ($\geq$ 99.9% pure
 10 GC-grade, Sigma Aldrich) and vortexed for 5 minutes. The ethyl acetate fraction was pipetted into analysis vials (Agilent) and loaded on the Agilent Technologies 7890B GC system (using Agilent J&W VF-WAXms GC Column) (Agilent Technologies; Santa Clara, CA) and 5977A MSD for gas chromatography-mass spectrometry (GC-MS) analysis using MassHunter Qualitative Analysis (Agilent; Santa Clara, CA) software to measure compound concentrations.
 15 Peaks in the resulting total ion current (TIC) chromatogram were input into the NIST MS Search 2.2 database for compound prediction. Prior to running incubated samples, standard curves were generated using a range of concentrations (0.2-5.0 g/l) of expected products (isobutyraldehyde, isobutanol, isopentanol, phenethyl alcohol, acetate) using flame ionization detector (FID) spectra. Integrated total ion current (TIC) chromatogram peaks for differentially produced
 20 compounds were measured and converted to g/l concentrations according to standard curve conversion factors.

Alcohol dehydrogenase screening

To assay for differential alcohol dehydrogenase activity under aerobic and 1 atm CO₂
 25 conditions, pRBBm34 vectors were constructed using previously described methods with xylose-inducible promoter P_{Xyl} upstream of *kivD*_{LI} and one of five alcohol dehydrogenase variants: *adh6* / pJBxA6 (*S. cerevisiae*), *adhA*_{Bm} / pJBxKAB (*B. megaterium* SR7), *adhA*_{LL} / pJBxKAL (*L. lactis*), *adhP*_{Ec} / pJBxKAP (*E. coli*), and *yqhD* / pJBxKY (*E. coli*) (constructs and strains summarized in Tables 15A and 15B).

30 For aerobic screens, freezer stocks of each strain were streaked onto LB agar plates supplemented with tetracycline (5 μ g/ml) and grown at 37°C overnight. For each alcohol

dehydrogenase, three colonies were added separately to 5 ml of LB with tetracycline and grown at 37°C overnight. Each sample was sub-cultured to an OD₆₀₀ of 0.05 in 3 ml of LB with tetracycline in a 50 ml screw-capped glass tube. Cultures were grown at 37°C and 250 RPM until an OD₆₀₀ of ~1.0 was reached, at which point 5mM α -ketoisovalerate (α -KIV) precursor was added and protein production induced by supplementing with 0.5% D-xylose. Cultures were grown at 37°C and 250 RPM. Time points were taken at 4 hours, 24 hours and 48 hours by removing 1 ml of culture volume. Samples were centrifuged at 20,000xg for 5 minutes and the supernatant removed. Alcohols were extracted from the supernatant using a 1:1 ratio of supernatant to ethyl acetate and vortexed at maximum speed for 5 minutes. The ethyl acetate fraction was recovered by centrifugation at 20,000xg for 5 minutes and removal of the upper solvent fraction. Sample analysis by GC-FID and concentrations of produced alcohols by standard curve calculations used previously described methods.

1 atm CO₂ cultures inoculated with 10⁵ spores/ml of each strain were grown overnight and passaged by syringe needle into fresh CO₂-degassed LB + tet_{0.5}. Two hours after passaging, cultures were amended with 5 mM α -KIV substrate and 0.5% D-xylose for gene induction. Sampling of strain variant cultures took place at 24 and 48 hours by syringe needle. Samples were then prepared for GC-MS analysis and post-run data processing as previously described.

Full isobutanol pathway

Although production of isobutanol through a two-step conversion of α -KIV using ketoisovalerate decarboxylase and various alcohol dehydrogenases has been demonstrated, α -KIV is an expensive substrate for isobutanol production. It may be desired to use an inexpensive, six-carbon substrate such as glucose for isobutanol production. Isobutanol production from glucose utilizes two endogenous cellular pathways, glycolysis to convert glucose to pyruvate and valine synthesis to convert pyruvate to α -KIV (FIG. 19). The *ilvCD* and *ilvBN* operons from *E. coli* were added to pJBxKA6 or pJBxKY vectors to generate the full 6 enzyme pathway for the conversion of pyruvate to isobutanol. After transforming the plasmids into SR7, the functionality of the pathway was assessed in the presence of glucose and xylose (as inducer and carbon source) for SR7 grown in M9+ media (FIG. 26C).

After initial biofuel screens, the pathway was regenerated such that each gene contained a ribosome-binding site that has previously shown high production in SR7. Instead of using the

ilvBN operon from *E. coli*, the acetolactate synthase gene, *alsS*, was selected from *B. subtilis* to be paired with *ilvC* and *ilvD* from *E. coli*. The new plasmid contained five genes, including the alcohol dehydrogenase ADH6_{sc} that was previously tested in SR7. After confirming the transformation of the new plasmid in SR7, cells were grown in M9+ with 10-25 g/L glucose and 5 g/L xylose to determine if the full isobutanol pathway would lead to titers greater than 125 mg/L isobutanol that had been observed for the partial pathway.

Assay for quantification of isobutanol production under scCO₂

A headspace extraction setup was constructed to collect the scCO₂ phase with dissolved species from high-pressure growth reactors. 316 stainless steel lines and valves connected to the reactor pressurization manifold enabled direct depressurization of the scCO₂ headspace into ethyl acetate solvent for subsequent GC-MS analysis. To generate standard curves for isobutanol scCO₂ phase extraction, duplicate 10 ml incubation reactors were filled with cell-free LB medium amended with 5% isobutanol, 0.5% isobutanol and unamended LB. Reactors were pressurized with CO₂ to 100 atm, heated to 37°C and shaken for 3 days to allow equilibration. Individual reactors were then slowly depressurized into 10 ml of chilled ethyl acetate at a rate of ~1 atm/min. This process was repeated a second time with several modifications, including submerging reactors in a heat bath at 55°C, increased depressurization rates (1.5-2 atm/min) and extraction into larger solvent volume (20 ml). A standard curve generated by GC-MS analysis of the initial extraction run enabled conversion of FID isobutanol peak areas to mg/l concentrations.

To determine whether SR7xKY produces isobutanol during growth under scCO₂ headspace, high-pressure reactors were loaded with 3x10⁵ spores/ml of SR7xKY, control strain SR7xL, or cell-free media controls. A subset of reactors was amended with 0.5% xylose to induce gene expression. Reactors were pressurized to 100 atm CO₂, heated to 37°C and incubated under scCO₂ for 21-22 days while shaking at 250 rpm. Reactors with identical inocula/media conditions (*e.g.*, strain ± xylose) or cell-free controls were simultaneously depressurized into chilled ethyl acetate at a rate of ~1 atm/min. Between each round of unloading, manifold lines and valves were flushed with ethyl acetate. Samples were prepared for GC-MS analysis as previously described. Quadruplicate technical replicates were run for all scCO₂ bulk phase-collected samples. Culture supernatant glucose concentrations were measured using the YSI 2900 with YSI 2814 glucose starter kit after spinning down 1 ml culture volume

for 5 minutes x 21,000g. Depressurized cultures were prepared for epifluorescence microscopy by methods described in Example 1. Cultures were considered to have grown when demonstrating at least 10-fold increase in cell counts relative to loaded spore concentrations. The limit of detection was considered to be one half of a cell per 15 grids, which corresponds to 1.15x10³ cells/ml.

Results

Development of a genetic system for *B. megaterium* SR7

Three methods for transforming strain *B. megaterium* SR7 were tested: electroporation, conjugation and protoplast fusion. Genetic transformation of SR7 by electroporation using plasmid pRBBm34 was unsuccessful despite multiple attempts to modify protocol parameters based on published studies in other *B. megaterium* strains (Moro et al., 1995). Transformation by conjugation using the *E. coli* S-17 mating strain and vector pJR1 (Richhardt et al., 2010; Table 15B) gave mixed results with conferral of chloramphenicol resistance up to 10 µg/ml and positive PCR amplification of the plasmid-specific marker (*sacB*) in the resistant strains confirming transformation, albeit at a low frequency (*e.g.*, 1 transformant per 10⁷ SR7 cells). However, subsequent attempts to transform SR7 by the described conjugation protocol were not successful and thus prevented its further use in this specific study.

Protoplast fusion transformation, previously demonstrated in several *B. megaterium* strains (Bunk et al., 2010) proved successful at introducing all constructs used in this study via shuttle vector pRBBm34. Despite protocol modifications that increased viable protoplasts by fifty-fold, transformation efficiencies remained low (~1 transformed cell/10⁷ viable protoplasts), frequently generating 1-3 successfully transformed colonies per µg of plasmid DNA. Protoplast transformation first enabled conferral of constitutive tetracycline resistance (10 µg/ml aerobic; 1.0 µg/ml under 1 atm CO₂). Maintenance of tetracycline resistance under 1 atm CO₂ was verified by nearly identical growth of cultures passaged three times in either LB or tetracycline-amended LB on unamended and tetracycline-amended plates. All subsequent genetic manipulation of strain SR7 was conducted using the modified protocol for protoplast fusion transformation.

Inducible heterologous enzyme production under 1 atm CO₂ and scCO₂

After demonstrating constitutive antibiotic expression, two promoters (PXyl and PHyper-spank) were investigated for inducible protein expression in SR7. The D-xylose-inducible PXyl promoter (FIG. 21) is endogenous to all sequenced *B. megaterium* strains, including SR7:

Rather than using the xylose promoter native to SR7, a previously optimized PXyl system (Biedendieck et al., 2007) was used to avoid uncharacterized endogenous promoter regulation specific to SR7. The IPTG-inducible hyper-spank promoter (PHyper-spank) had previously been transformed into and expressed in *B. subtilis* (van Ooij and Losick, 2003), but had never been utilized in *B. megaterium*.

Plasmids pJBxGFP and pJBhGFP, where reporter superfolder GFP was placed under the control of the PXyl and PHyper-spank promoters, respectively, demonstrated induced expression in SR7 at nearly equal strengths under aerobic conditions assayed by GFP fluorescence intensity (data not shown). Low-level fluorescence in uninduced cultures appeared to show minor leakiness by PXyl. However, since fluorescent protein reporters including GFP are typically active only under aerobic conditions, an anaerobically functional reporter was necessary to verify promoter activity under 1 atm CO₂. Therefore, both promoters were placed upstream of the lacZ reporter, which is O₂-independent. Cultures of transformed strains SR7xL and SR7hL passaged under 1 atm CO₂ and induced with D-xylose and IPTG, respectively, were analyzed for LacZ production 24 hours after induction. Total protein-normalized LacZ specific activities (*e.g.*, activity of enzyme per mg total protein; $\mu\text{mol min}^{-1} \text{mg}^{-1}$; U/min) for duplicate lacZ strain cultures, empty vector controls, and a LacZ assay reagent (B-PER; Thermo Scientific; Waltham, MA) control are displayed in FIG. 22.

LacZ specific activity values from duplicate incubations of xylose-amended cultures of SR7xL (1.26-4.41 U/min) and SR7x (0.06-0.11 U/min) demonstrate that LacZ activity is increased by induction relative to empty vector controls. Relative to empty vector samples and the B-PER assay control (≤ 0.14 U/min), LacZ activity increased 9-32 fold. Differential expression of LacZ by IPTG induction of P_{Hyper-spank} was also observed, but at lower total protein-normalized specific activity levels than by xylose-induction (0.31-1.36 U/min). LacZ production under aerobic and anaerobic 1 atm CO₂ conditions represents the first successful use of IPTG-inducible P_{Hyper-spank} in *B. megaterium*. This development, and verification of strong

P_{Xyl} activity under 1 atm CO₂ expands the list of genetic tools available for SR7 engineering, as well as alternative *B. megaterium* strains used for biotechnological applications.

After exhibiting superior total protein-normalized LacZ activity under 1 atm CO₂, the SR7xL strain was investigated for expression under scCO₂. Duplicate cultures with and without 0.4% xylose inducer demonstrated robust germination and growth after 21 days under scCO₂ conditions, with appearance of vegetative cell morphologies and at least 15-fold increase in cell counts relative to starting cultures. Duplicate cultures of induced and uninduced reactors showing vegetative cell morphologies, but not robust outgrowth (<10-fold increase in cell counts) were utilized for comparison of LacZ activity in germinated/low-level growth cultures.

Both cultures that grew under scCO₂ in the presence of xylose showed elevated total protein-normalized LacZ specific activity (0.66-0.90 U/min) relative to uninduced cultures that grew (0.06-0.23 U/min) (FIG. 23). Uninduced cultures may display low-level LacZ activity due to minor leakiness of the xylose promoter, as also demonstrated under aerobic conditions.

Duplicate induced cultures that did not grow but appeared to have germinated (by microscopy) displayed activity values (0.06-0.17 U/min) on par with the negative control (0.14 U/min), indicating that active growth is required for heterologous enzyme expression under scCO₂. Successful LacZ production by SR7 under scCO₂ was the first demonstration that exogenous gene expression in a scCO₂ headspace bioreactor is possible.

Engineering and expression of a heterologous pathway for biofuel synthesis in scCO₂-tolerant strain SR7 under aerobic, 1 atm CO₂ and scCO₂ conditions

The final two steps in the production of isobutanol using the valine biosynthesis pathway requires catalytic conversion of α -KIV to isobutyraldehyde by α -ketoisovalerate decarboxylase (KivD), followed by conversion to isobutanol by alcohol dehydrogenase (FIG. 19). Since the *kivD* gene is not present in the SR7 genome, it required exogenous introduction in order to be expressed. The well-described *Lactococcus lactis* version of keto-isovalerate decarboxylase commonly used for isobutyraldehyde production (de la Plaza *et al.*, 2004) was utilized in this study. Though the SR7 genome indicates that the gene then required for conversion of isobutyraldehyde to isobutanol, alcohol dehydrogenase, is present in the cell, its production is likely lower than if transformed on a plasmid with a strong promoter. As a result, the *E. coli* version, *adh6*_{Ec}, was initially used in SR7 due to laboratory availability.

While upstream optimization may enable efficient conversion of glucose to α -KIV, initial pathway engineering relied on an exogenous supply of α -KIV to constrain heterologous enzyme activity assays to the final two steps of the pathway. Because the isobutanol pathway genes should be functional under both anaerobic and aerobic conditions, induction of the final two steps was first characterized under aerobic and 1 atm CO₂ conditions to validate expression under both conditions. After demonstrating initial activity, subsequent screening for highly active alcohol dehydrogenase enzymes in SR7 under aerobic and 1 atm CO₂ ultimately enabled the use of an optimized construct under scCO₂. 1 atm CO₂ passaged cultures of strains SR7xKA6 and SR7x (empty vector control) in LB media grew similarly well 24 hours after gene expression was induced. Based on averaged OD₆₀₀ values, heterologous pathway expression appeared to impose a metabolic burden that results in a 24% decrease in biomass yield relative to the empty vector control (FIG. 24). GC-MS analysis verified production of several biofuel products in the 1 atm CO₂ cultures grown in LB after 4 and 24 hours, including expected compounds isobutyraldehyde and isobutanol (Table 16). In a somewhat surprising result, isopentanol and phenethyl alcohol were also produced, indicating that pJBxKA6 genes *kivD_{LI}* and *adh6_{Sc}* appear to redirect flux of alternative amino acid biosynthesis pathways, including those of leucine (to isopentanol) and phenylalanine (to phenethyl alcohol; FIG. 19). No biofuel peaks were detected in either of the P_{Xyl} Empty replicate cultures.

The biofuel strain replicates (A & B) showed accumulation of the intermediate product isobutyraldehyde at the 4-hour time point (A: 1.34, B: 1.66 mM) with trace level accumulation of isobutanol (A & B: 0.01 mM) and isopentanol (A & B: 0.01 mM) and no detectable phenethyl alcohol (Table 16).

Table 16: Summary of bioproducts (mM, mg/l) generated by SR7xKA6 under 1 atm CO₂

[Biofuel] (mM)						
Time	Replicate	Isobutyraldehyde	Isobutanol	Isopentanol	Phenethyl Alcohol	Sum
4	A	1.66	0.01	0.01	0	1.69
4	B	1.34	0.01	0.01	0	1.35
24	A	1.7	4.00	1.95	0.22	7.87
24	B	1.85	4.08	1.98	0.26	8.17
[Biofuel] (mg/L)						
Time	Replicate	Isobutyraldehyde	Isobutanol	Isopentanol	Phenethyl Alcohol	Sum
4	A	23.0	0.1	0.1	0.0	23.3
4	B	18.6	0.1	0.1	0.0	18.8
24	A	23.6	54.0	22.1	1.8	101.5
24	B	25.7	55.0	22.5	2.1	105.3

Culture Conditions	
Supplemented: α -KIV: 5 mM	
Strain: SR7 P _{xyg} hivD ₁₂ Adh6 _{sc}	
Media: LB + tet 0.1 ug/mL	
Induced: 0.5% xylose	

Compounds	Std Curve Conversion y = counts; x = mM
Isobutyraldehyde	y = 27623x; R = 0.81
Isobutanol	y = 169971x; R = 1.00
Isopentanol	y = 184505x; R = 0.97
Phenethyl Alcohol	y = 434871x; R = 1.00

A marked shift in production was observed in duplicate cultures at the 24-hour time point, with significant accumulation of isobutanol (A: 4.00, B: 4.08 mM), isopentanol (A: 1.95, B: 1.98 mM) and small amounts of phenethyl alcohol (A: 0.22, B: 0.26 mM), while maintaining comparable aldehyde accumulation (A: 1.70, B: 1.85 mM). It therefore appears that while a certain concentration of aldehyde will build up due to the limits of Adh6_{sc} activity in SR7, by 24 hours the majority of α -KIV substrate has been converted to final biofuel products isobutanol and isopentanol.

1 atm CO₂ cultures of SR7xKA6 generated bioproducts at a slower rate than under aerobic conditions, but final 24-hour titers were similar under both conditions (FIG. 25), indicating reduced catalytic efficiency but comparable total substrate conversion. At 4 hours, the sum of 1 atm CO₂ bioproduct concentrations was 28% of the summed concentrations under aerobic conditions, increasing to 88% of the aerobic sum by 24 hours. Specifically with regard to isobutanol, by 24 hours the 1 atm CO₂ incubations generated 93% of the concentration detected in aerobic cultures. Overall, these results suggest that reduced production rates under 1 atm CO₂ relative to aerobic conditions may be due to slower microbial growth/metabolism or diminished enzyme activity.

While nearly identical amounts of isobutanol were produced under both aerobic and 1 atm CO₂ conditions, 1 atm CO₂ titers of isopentanol and phenethyl alcohol were about half as concentrated as under aerobic conditions. Therefore, it appears that alternative amino acid

pathway enzymes (FIG. 19) may be operating at reduced efficiency in siphoning off α -KIV substrate, possibly due to dependence on O₂-dependent co-factor NAD(P)H.

Alcohol dehydrogenase screening

The accumulation of isobutyraldehyde in initial SR7xKA6 cultures under 1 atm CO₂ (Table 16, FIG. 25) prompted additional screening of alcohol dehydrogenase gene variants in order to improve the rate and completeness of isobutyraldehyde conversion to isobutanol. This enzymatic reaction is of particular importance in the proposed biofuel production system because isobutyraldehyde is soluble in scCO₂ and thus premature partitioning of accumulated isobutyraldehyde into the scCO₂ headspace would reduce overall yields, titers and purity of the desired isobutanol end product. Vectors constructed with PXyl kivDLI alone and with one of five alcohol dehydrogenase variants (*adh6Sc*, *adhABm*, *adhALL*, *adhPEc*, and *yqhDEc*) were thus assayed for biofuel production under aerobic and 1 atm CO₂ conditions. Aerobic results for GC-MS detected compounds after 4 and 24 hours are presented in FIGs. 26A and 26B.

Results from subsequent alcohol dehydrogenase variant screens (including raw and OD-normalized values) under 1 atm CO₂ are presented in FIGs. 27A-27D.

Under aerobic conditions concentrations of aldehyde and alcohol products demonstrate that *yqhDEc* variant cultures (SR7xKY) outperformed all other alcohol dehydrogenases according to several metrics. By 4 hours, while all other variants generated isobutyraldehyde above 1.5 mM, SR7xKY cultures prevented intermediate accumulation by converting nearly all α -KIV substrate to isobutanol and isopentanol (FIG. 26A). By 24 hours, while all alcohol dehydrogenase variants had converted isobutyraldehyde to alcohol products, SR7xKY cultures generated the highest titers for both isobutanol (4.6 mM) and isopentanol (3.4 mM). Overall, *YqhDEc* results in >90% conversion of α -KIV substrate to biofuel products (FIG. 26B).

Under 1 atm CO₂ isobutyraldehyde, isobutanol, and isopentanol concentrations were nearly identical for all strains at both 24 and 48 hours based on raw values (FIGs. 27A and 27C), suggesting that effectively all α -KIV substrate had been converted by 24 hours. The fact that low levels of isobutyraldehyde persist at both 24 and 48 hours also suggests that alcohol dehydrogenase activity may become limited once the aldehyde concentration drops below a threshold level, as all aldehyde concentrations from both time points fell within a narrow range, (0.193-0.457 mM; 0.014-0.033 g/l). The best performing enzyme variants after 24 and 48 hours

as determined by maximum alcohol and minimum aldehyde concentrations are listed in Table 17.

Table 17: 1 atm CO₂ alcohol dehydrogenase variant performance summary based

Time (h)	Enzyme	[Isobutanol] _{Max}		Enzyme	[Isopentanol] _{Max}		Enzyme	[Isobutyraldehyde] _{Min}	
		mM	g/L		mM	g/L		mM	g/L
24	YqhD	4.43	0.342	Adh6 _{sc}	1.893	0.167	YqhD	0.193	0.014
48	YqhD	4.448	0.343	Adh6 _{sc}	2.025	0.178	AhdA _{LI}	0.203	0.015

OD-normalized product concentrations (FIGs. 27B and 27D) suggest that AdhA_{LI} may be especially efficient at product generation on a per-cell basis, which in addition to displaying the lowest aldehyde concentration at 48 hours indicates it may be one of the better performing variants. In addition to yqhD_{Ec} strain SR7xKY demonstrating the fastest aldehyde conversion rates and highest final titers under aerobic conditions (FIG. 26), results from 1 atm CO₂ cultures also displayed the highest final titers (FIG. 27C), although performance differences under 1 atm CO₂ were marginal relative to aerobic results. With available data especially encouraging for variant YqhD_{Ec}, subsequent incubation experiments under scCO₂ proceeded with the pJBxKY construct-bearing strain SR7xKY.

Bench scale abiotic isobutanol scCO₂ and aqueous phase extractions

In situ extraction via scCO₂ relies on the partitioning of a compound from the aqueous phase to the scCO₂ phase followed by product recovery. After manifold modifications, batch reactors used for culturing under scCO₂ in this study were utilized for bench scale *in situ* extraction, as described in the Methods. Standard curves generated for partitioning of isobutanol from aqueous media into the scCO₂ phase demonstrated that isobutanol at concentrations from 0.5-5.0 mM (37-371 mg/l) could be quantitatively stripped from the media phase and recovered in the scCO₂ phase (FIGs. 28A and 28B).

Process modifications including continuous reactor heating, increased depressurization rates and increased ethyl acetate solvent volume appeared to significantly improve supercritical CO₂ phase recovery efficiencies during a second round of abiotic isobutanol extractions, increasing the percent of total isobutanol recovered from the scCO₂ phase by an order of magnitude from 2% to 20% between the initial and second runs. Overall mass balance

calculations of the second run demonstrated that between 75-90% of loaded isobutanol concentration was recovered by the sum of aqueous and scCO₂ phase products after three-day scCO₂ incubations. Since the batch bioreactor set up used in this work is not optimized for solvent stripping using scCO₂, 2-20% product recovery in scCO₂ is satisfactory in the context of this study. However, further work to optimize the reactor and stripping configuration may enable more efficient *in situ* extraction.

Biosynthesis and *in situ* extraction of natural products and biofuels under scCO₂

Having established alcohol dehydrogenase variant YqhD_{Ec} as the best performing enzyme for isobutanol production, cultures loaded with spores of SR7xKY in the presence of xylose inducer were anticipated to generate biofuel products. Conversely, metabolically inactive cultures and LacZ-generating SR7xL control cultures were not expected to show signatures of alcohol production. Uninduced biofuel strain cultures showing growth were anticipated to generate low-level biofuel concentrations due to the mildly leaky nature of P_{Xyl}. A summary of growth outcomes from scCO₂-incubated cultures of genetically modified strains and wild-type SR7 is presented in Table 18.

Table 18: Summary of growth outcomes for cultures of SR7 wild-type and modified strains in M9A+ media incubated under scCO₂

Inocula	± Xylose	Starting spores/mL	M9A+ Growth Frequency	Max Biomass (cells/mL)
SR7xKY	+	3x10 ⁵	33% (5/15)	5.96x10 ⁷
	-	1x10 ⁵	17% (1/6)	2.88x10 ⁷
SR7xL	+	5x10 ⁵	13% (2/15)	1.34x10 ⁷
	-	3x10 ⁵	33% (2/6)	9.69x10 ⁶
Wild-type SR7	-	3x10 ⁴	64% (16/25)	1.63x10 ⁷
Media Control	+	b.d.	b.d.	b.d.

Decreased growth frequencies observed in transformed strains relative to wild-type SR7 may indicate a metabolic burden associated with carrying and expressing the pRBBm34 vector, as observed under 1 atm CO₂ (FIG. 24), that reduces germination frequency and/or vegetative outgrowth, though these hypotheses will require additional investigation.

Natural fermentation products were detected by HPLC in the media phase of all reactors demonstrating growth (>10-fold increase in cell counts) over 21-22 day scCO₂ incubations, including induced and uninduced cultures of both SR7xL and SR7xKY. Detected compounds were consistent with those generated by wild-type SR7 scCO₂ cultures (Example 1), including succinate (up to 73.2 mg/l), lactate (up to 2.8 g/l), and acetate (up to 1.3 g/l) (FIGs. 29A and 29B), which reinforces the suggestion of growth via the TCA Cycle and mixed acid fermentation. Total cell-normalized metabolite concentrations demonstrate maximum per cell productivities of 7.6×10^{-9} , 5.3×10^{-8} , and 2.5×10^{-8} mg product cell⁻¹ for succinate, lactate and acetate, respectively, which are also similar to maximum per cell productivities of the wild-type strain under 1 atm CO₂ and scCO₂ (Example 1). The quantification of natural metabolites thus has potential for use as an indicator of active growth under scCO₂.

Biofuels were detected by GC-MS in the media phase of all five reactors loaded with SR7xKY that showed growth and were induced with xylose (FIGs. 30A, 30B, and 31; Table 5). Of the two SR7xKY cultures showing growth in the absence of xylose, one showed low level biofuel production (0.3 mg/l isobutanol, 0.1 mg/l isopentanol), putatively as the result of the mildly leaky xylose promoter. No biofuel was generated in any of the reactors that did not show vegetative growth, verifying that metabolic activity (*e.g.*, growth) under scCO₂ is required for heterologous compound production (*e.g.*, FIG. 23). No biofuels were detected in SR7xL cultures or media only negative controls.

Measured isobutanol concentrations in the aqueous phase of induced cultures ranged from 1.6 to 93.5 mg/l, while isopentanol concentrations varied from 0.5 to 29.7 mg/l. Observed maximum titers of 0.094 g/l isobutanol and 0.030 g/l isopentanol in scCO₂ incubations are approximately two orders of magnitude lower than under 1 atm CO₂, possibly due to reduced growth rates and biomass accumulation, or potential redirection of carbon flux under scCO₂ conditions.

In order to maximize direct scCO₂ phase biofuel compound recovery, all reactors loaded with strain SR7xKY that were induced were depressurized into a single collection solvent to maximize the likelihood of biofuel recovery. Similarly, all reactors loaded with SR7xKY that were uninduced were pooled via a single collection solvent. A pronounced GC-MS peak for isobutanol was observed only for the pooled samples from induced reactors while no isobutanol peak was observed from non-induced samples, indicating inducible gene expression led to

biofuel generation under scCO₂ (FIGs. 30B and 31). Based on the abiotic scCO₂-extracted isobutanol standard curve (FIG. 28), the total scCO₂ phase isobutanol concentration was 10.2 mg per liter of media (0.138 mM; Table 19), which represents 5.2% of the total recovered isobutanol from both the media and scCO₂ phases. The detection of biogenic isobutanol in the scCO₂ phase represents the first biofuel production under scCO₂ conditions, as well as the first harvesting of biofuels from microbial cultures incubated under scCO₂.

Table 19: Summary of scCO₂-incubation outcomes for SR7xKY-loaded columns that showed increased biomass relative to starting concentrations

Culture Sample	Induced (+Xyl)	Replicate	Filter Count (cells/mL)	Filter Std Dev	Fold Growth [t22]/[t0]	[Glucose] (g/L)	Isobutanol (IBuOH)			Isopentanol (IPnOH)			Sum [Biofuel] mg/L
							GC Area	mM	mg/L	GC Area	mM	mg/L	
Cell-Free M9A+ Control	Yes	A	0.00E+00	0.00E+00	0	3.21	0	0	0	0	0	0	0
		C	0.00E+00	0.00E+00	0	3.14	0	0	0	0	0	0	0
		D	0.00E+00	0.00E+00	0	3.10	0	0	0	0	0	0	0
		Bulk (scCO ₂)					0	0	0	0	0	0	0
SR7xKY	No	C	2.88E+07	8.13E+06	195.3	3.05	765	0.004	0.316	239	0.001	0.094	0.411
		E	4.36E+05	3.68E+05	3.0	3.14	0	0.000	0.000	0	0.000	0.000	0.000
		Bulk (scCO ₂)					0	0	0	0	0	0	0
	Yes	A	5.96E+07	1.66E+07	190.0	2.45	8229	0.046	3.403	1698	0.008	0.669	4.072
		B	5.37E+07	1.01E+07	171.3	0.01	204775	1.144	84.690	75485	0.338	29.745	114.435
		C	3.48E+07	6.73E+06	111.0	1.91	225969	1.263	93.455	72353	0.324	28.511	121.966
		G	3.88E+07	9.54E+06	123.5	2.70	4146	0.023	1.715	1181	0.005	0.465	2.180
		P	3.18E+07	1.91E+05	101.5	2.60	3922	0.022	1.622	2126	0.010	0.838	2.460
		Bulk (scCO ₂)					563	0.138	10.212	0	0	0	10.212

In addition to endogenous metabolites and heterologous biofuels, differentially extracted compounds present in the bulk scCO₂ phase of grown cultures that are absent in the aqueous phase may hold promise as SR7 natural products able to be extracted by the scCO₂ phase. If these products can be identified, it is possible that optimization of product-generating pathways may enable future production and extraction of these unknown compounds. Peaks differentially present in the scCO₂ bulk phase include compounds with retention times of 19.75, 25.34, 25.4, and 26.97 minutes, however these compounds were not identified.

Full isobutanol pathway

Due to the endogenous ability of SR7 to produce α -KIV, conversion to isobutanol and isopentanol was observed for just the keto-isovalerate decarboxylase and alcohol dehydrogenase

containing cells (pXyl KivD, ADH6 and pXyl KivD, YqhD), with titers of approximately 125 mg/L for isobutanol and 175 mg/L for isopentanol when feeding 10 g/L glucose. The addition of the valine synthesis operons from *E. coli* did not show any increased production suggesting that the operons were not correctly transcribed in SR7 or at least one of the pathway enzymes was not functional in SR7.

After regenerating the full isobutanol pathway to include ribosome binding sites for each individual gene of the pathway, observed production titers included approximately 275 mg/L for isobutanol and 200 mg/L for isopentanol (FIG. 26D). Further, after additional glucose was supplied to 25 g/L, isobutanol titers reached almost 400 mg/L (FIG. 26D). Inclusion of this pathway in SR7 shows the potential for this organism to make biofuel from low-cost, common substrates such as glucose as well as begins the metabolic engineering of this host to make a variety of products.

EXAMPLE 3: Continuous Butanol Extraction using Supercritical CO₂

Butanol is an example of a potentially valuable bioproduct that may be produced using the methods and cells described herein. Butanol can be used as a drop-in biofuel or as a fuel additive that can be blended with gasoline at much higher proportions than ethanol. To date, economical production of butanol has been hampered largely by its cytotoxicity, which becomes limiting at levels as low as several weight percent. Compared to other butanol recovery approaches, scCO₂-extraction has sterilization and potential energy balance advantages. Because scCO₂ selectively extracts butanol instead of water, a highly concentrated butanol stream can be recovered which requires minimal post-processing purification.

Specifically, butanol extraction performance was evaluated, initially using a batch-wise extraction to study the effects of initial butanol concentration, extraction vessel pressure, and scCO₂ volumetric flow rate on butanol extraction rate. Additionally, the data was modeled using a standard liquid-liquid mass transfer model to determine the values for the mass transfer coefficient, κ_a . In all cases tested, the mass transfer model adequately described the experimental data. Best-fit values of κ_a did not vary within our estimated limits of uncertainty for variation in extraction pressure (from 10.3 to 13.7 MPa – the range over which the scCO₂-tolerant bacterial strain has exhibited growth); therefore it was determined that operation at lower

pressures should be favored to achieve better process economy. Similar analysis was performed to interpret the mass transfer coefficient from correlations developed for gas-liquid and liquid-liquid extraction and compared to interfacial area results obtained from scCO₂-droplet size analysis.

A diagram of an example continuously CO₂-stripped multiphase reactor that may be used in the methods described herein is presented in FIG. 32. Briefly, use of scCO₂ provides a number of benefits to the system and allows for the continuous production and stripping of the bioproduct when the bioproduct is present in the solvent (scCO₂) phase of the reactor. Stripping the bioproduct may maintain a low concentration of the bioproduct in the reactor and alleviate end product toxicity.

Specifically, FIG 38A shows compression of CO₂ to liquid, near-critical or supercritical state (here, 100 bar and 40 °C), feeding it to a fermenter producing butanol for extraction, then recovery by de-pressurization. Table 20 provides performance metrics for the extraction process, including energy requirement and butanol purity. The data in Table 20 were calculated using thermodynamic efficiencies of 90% and mechanical efficiency of 30%, as is typical. The purity can be increased by additional steps. FIG 38B provides energy requirements for the operations in the overall process. CO₂ compression represents the main energy requirement (C301, C302, and C303). Heat exchange, especially to cool CO₂ during stage-wise compression, is the next most significant energy requirement (E301, E302, and E303, as well as V301 and V302).

Turbine expansion (T-301) partially offsets the compression energy.

Table 20: Performance metrics for extraction process

Mass % Butanol in Product	96.0%
Butanol Energy Requirement (MJ/kg)	3.2
Butanol recovery	97.8%

Such an example reactor was used to study higher alcohol (*e.g.*, 1-butanol, n-butanol, pentanol, hexanol) extraction using scCO₂ and provided a simple mass transfer model of extraction. Extraction of the bioproducts using multiphase reactors, such as the reactor presented
5 in FIG. 32, allows for dynamic extraction by flowing the scCO₂ phase and a non-flowing aqueous (media) phase. The conditions used in the experiments were focused on 100 bar and 40°C, conditions in which *B. megaterium* SR7 is capable of growing. Both the alcohol bioproduct and the rate of extraction were varied between experiments.

The results of the extraction experiments for n-butanol, isobutanol, n-pentanol, n-
10 hexanol, are presented in FIGs. 33, 34, 36, and 37, respectively. Briefly, for n-butanol and isobutanol, a slow rate of extraction was found to be most efficient (FIGs. 33 and 34). The fast flow rate (12.5 mL/min) resulted in extraction of the most butanol, whereas the 5.4 mL/min rate extracted the most isobutanol. Overlaying the extraction profiles for n-butanol and isobutanol shows that scCO₂ extraction of the two bioproducts occurs at the same rate at 5.4 mL/min scCO₂
15 (FIG. 35).

For n-pentanol, the 3.2 mL/min scCO₂ flow rate resulted in the superior extraction, whereas the extraction trends declined with increasing flow rates above 3.2 mL/min (FIG. 36). For n-hexanol, it was observed that increasing the scCO₂ flow rate above 5.4 mL/min did not increase the extraction rate (FIG. 37).

20 EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

25 All references, including patent documents, disclosed herein are incorporated by reference in their entirety.

What is claimed is:

CLAIMS

1. A method of producing a bioproduct, comprising culturing a cell in a multiphase reactor
5 comprising an aqueous phase and a solvent phase, wherein the solvent phase comprises
supercritical CO₂ (scCO₂), near critical CO₂, or liquid CO₂.
2. The method of claim 1, wherein the solvent phase comprises scCO₂.
- 10 3. The method of claim 1, wherein the solvent phase comprises near critical CO₂,
4. The method of claim 1, wherein the solvent phase comprises liquid CO₂.
5. The method of any one of claims 1-4, wherein the cell is a viable cell.
15
6. The method of any one of claims 1-5, wherein the cell is a bacterial cell.
7. The method of claim 6, wherein the bacterial cell is resistant to scCO₂.
- 20 8. The method of claim 6 or 7, wherein the bacterial cell is a spore-forming bacterium
belonging to the genus *Firmicutes*.
9. The method of claim 8, wherein the bacterial cell is a *Bacillus* spp.
- 25 10. The method of claim 9, wherein the bacterial cell is selected from the group consisting of
Bacillus cereus, *Bacillus subterraneus*, *Bacillus amyloliquefaciens*, *Bacillus safensis*, and
Bacillus megaterium.
11. The method of claim 10, wherein the bacterial cell is *Bacillus megaterium* SR7.

30

12. The method of any one of claims 1-11, wherein the cell is in spore-form or has been acclimated to scCO₂.

13. The method of claim 12, wherein the cell has been acclimated to scCO₂ by previous exposure to scCO₂.

14. The method of any one of claims 1-13, wherein the bioproduct is selected from the group consisting of isobutanol, butanol, isopentanol, phenyl-ethyl-alcohol and 4-methyl-pentanol.

15. The method of any one of claims 1-14, wherein the cell is engineered to recombinantly express one or more genes.

16. The method of any one of claims 1-15, wherein the bioproduct is partitioned into the scCO₂, near critical CO₂, or liquid CO₂.

17. The method of any one of claims 1-16, wherein the bioproduct is a hydrocarbon, oxygenated hydrocarbon, aldehyde, alcohol, fatty acid, or ketone.

18. The method of claim 17, wherein the bioproduct is isobutanol and the cell is engineered to recombinantly express an isoketovalerate decarboxylase and an alcohol dehydrogenase.

19. The method of claim 18, wherein the isoketovalerate decarboxylase is a *Lactococcus lactis* gene.

20. The method of claim 18 or 19, wherein the alcohol dehydrogenase is an *E. coli* gene.

21. The method of claim 17, wherein the bioproduct is 4-methyl-pentanol and the cell is engineered to recombinantly express one or more enzymes selected from the group consisting of an acetolactate synthase, a ketol-acid reductoisomerase, a dihydroxy-acid dehydratase, an alpha-ketoisovalerate decarboxylase, an aldehyde dehydrogenase, a propionyl-CoA transferase, a beta-keto-thiolase, a beta-keto-acyl-CoA reductase, an enoyl-CoA hydratase, a trans-enoyl-CoA

reductase, a carboxylic acid reductase, a 4'-phosphopantetheinyl transferase, and an alcohol dehydrogenase.

22. The method of any one of claims 1-21, wherein the bioproduct is isolated from the solvent phase.

23. The method of any one of claims 1-22, wherein the bioproduct is isolated from the aqueous phase.

24. The method of any one of claims 1-23, wherein the solvent phase further comprises less than or equal to about 3% inert helium.

25. The method of any one of claims 1-24, wherein the aqueous phase comprises a growth media.

26. The method of claim 25, wherein the growth media comprises a spore germination inducer.

27. The method of claim 26, wherein the spore germination inducer is D-alanine.

28. The method of any one of claims 1-27, wherein the culturing comprises incubating the cell in the multiphase reactor for at least 5 hours.

29. The method of anyone of claims 1-28, wherein the culturing comprises incubating the cell in the multiphase reaction at a temperature between 35°C – 40°C.

30. A *Bacillus megaterium* cell that recombinantly expresses an isoketovalerate decarboxylase and an alcohol dehydrogenase.

31. A reactor comprising
- (a) (i) supercritical CO₂ or a mixture of supercritical CO₂ and one or more other solvents,
(ii) near critical CO₂ or a mixture of near critical CO₂ and one or more other solvents,
or (iii) liquid CO₂ or a mixture of liquid CO₂ and one or more other solvents; and
- 5 (b) at least one viable cell.

32. The reactor of claim 31, wherein the viable cell is a bacterial cell.

33. The reactor of claim 32, wherein the bacterial cell is resistant to scCO₂.

10 34. The reactor of claim 32 or 33, wherein the bacterial cell is a spore-forming bacterium belonging to the genus *Firmicutes*.

35. The reactor of claim 34, wherein the bacterial cell is a *Bacillus* spp.

15 36. The reactor of claim 35, wherein the bacterial cell is selected from the group consisting of *Bacillus cereus*, *Bacillus subterraneus*, *Bacillus amyloliquefaciens*, *Bacillus safensis*, *Bacillus licheniformis*, and *Bacillus megaterium*.

20 37. The reactor of claim 36, wherein the bacterial cell is *Bacillus megaterium* SR7.

38. The reactor of any one of claims 31-37, wherein the viable cell is in spore-form or has been acclimated to scCO₂.

25 39. The reactor of claim 38, wherein the viable cell has been acclimated to scCO₂ by previous exposure to scCO₂.

40. The reactor of any one of claims 31-39, further comprising a bioproduct.

30 41. The reactor of claim 40, wherein the bioproduct is partitioned into the scCO₂, near critical CO₂, or liquid CO₂.

42. The method of claim 41, wherein the bioproduct is a hydrocarbon oxygenated hydrocarbon, aldehyde, alcohol, fatty acid, or ketone.

5 43. The method of claim 42, wherein the bioproduct is selected from the group consisting of isobutanol, butanol, isopentanol, phenyl-ethyl-alcohol and 4-methyl-pentanol.

44. The reactor of any one of claims 31-43, wherein the viable cell is engineered to recombinantly express one or more genes.

10

45. The reactor of claim 43 or 44, wherein the bioproduct is isobutanol and the viable cell is engineered to recombinantly express an isoketovalerate decarboxylase and an alcohol dehydrogenase.

15 46. The reactor of claim 45, wherein the isoketovalerate decarboxylase is a *Lactococcus lactis* gene.

47. The reactor of claim 45 or 46, wherein the alcohol dehydrogenase is an *E. coli* gene.

20 48. The reactor of claim 43 or 44, wherein the bioproduct is 4-methyl-pentanol and the cell is engineered to recombinantly express.

49. The reactor of any one of claims 40-48, wherein the bioproduct is isolated from the scCO₂ phase.

25

50. The reactor of any one of claims 31-49, wherein the one or more other solvent is inert helium.

51. The reactor of any one of claims 31-50, further comprising a growth media.

30

52. The reactor of claim 51, wherein the growth media comprises a spore germination inducer.

53. The reactor of claim 52, wherein the spore germination inducer is D-alanine.

5

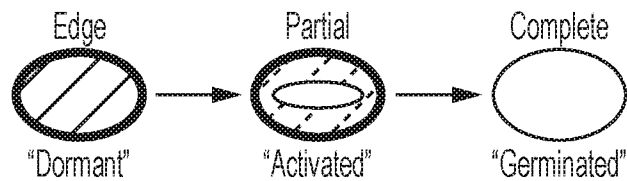


FIG. 1

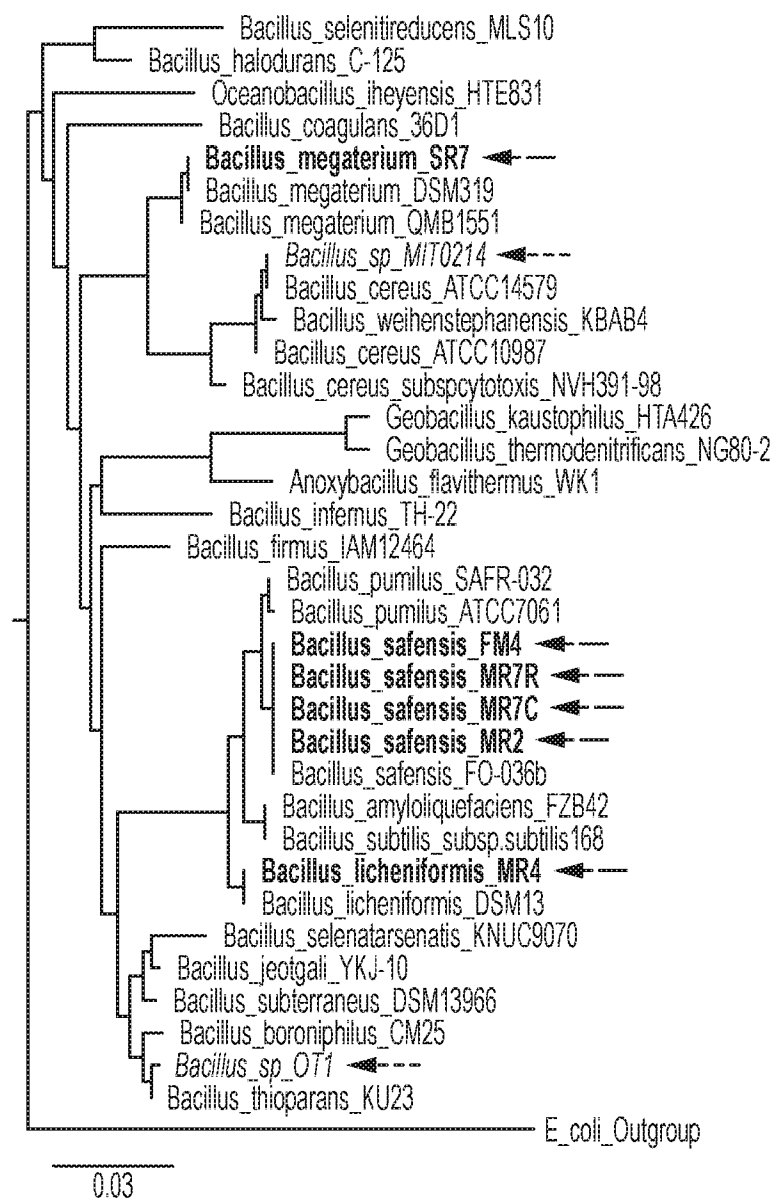


FIG. 2

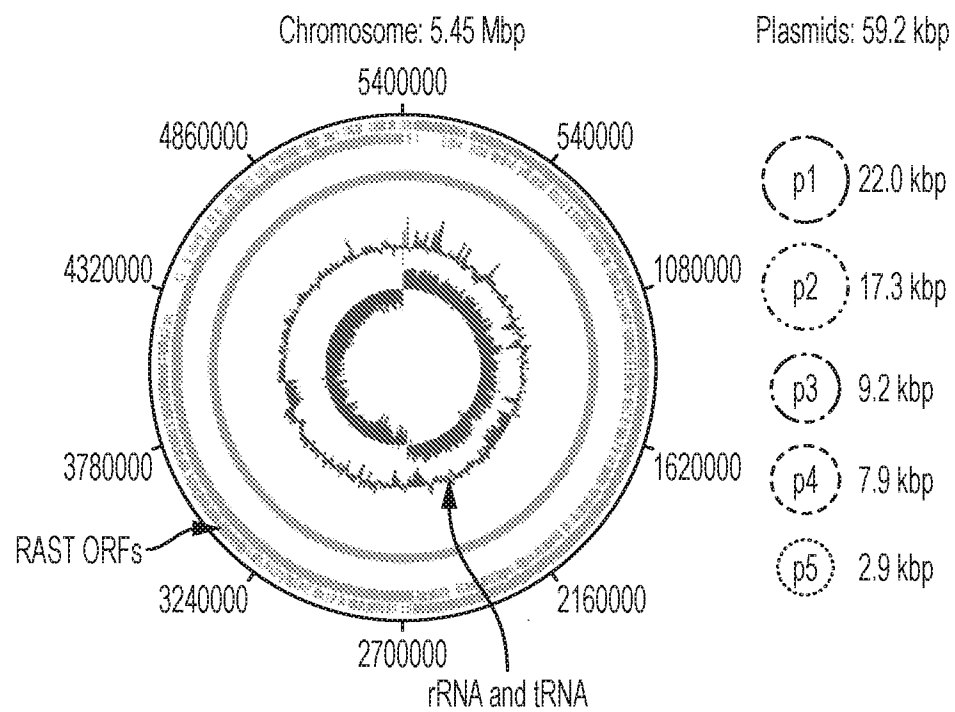


FIG. 3

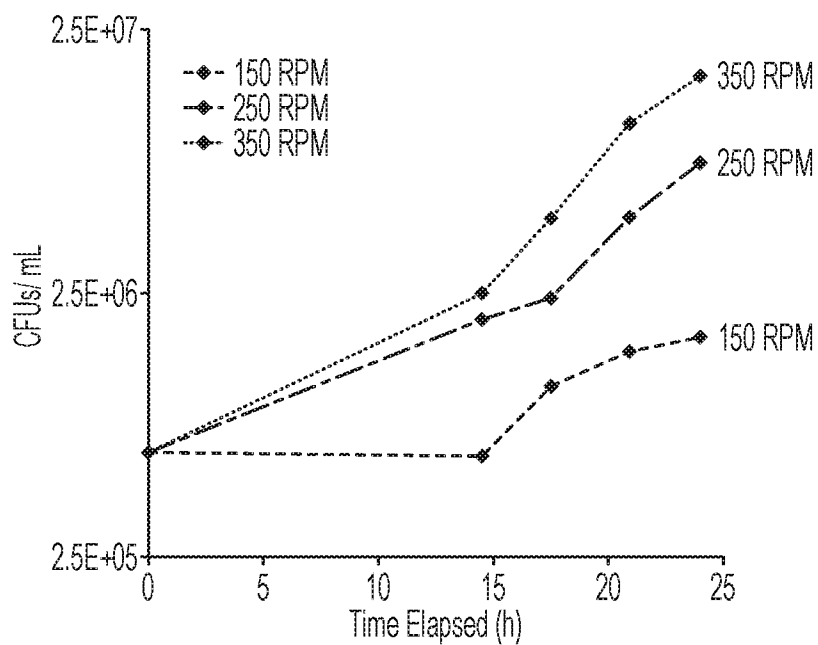


FIG. 4A

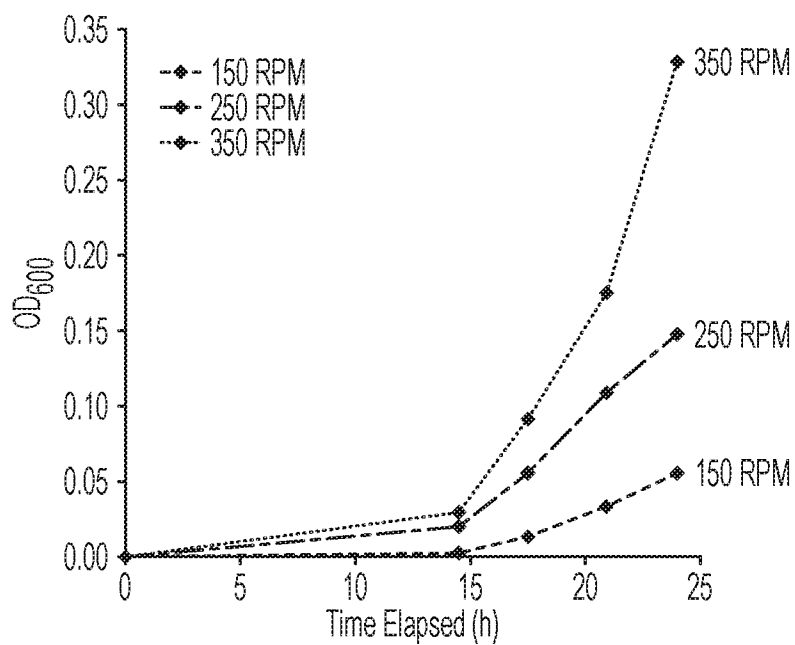


FIG. 4B

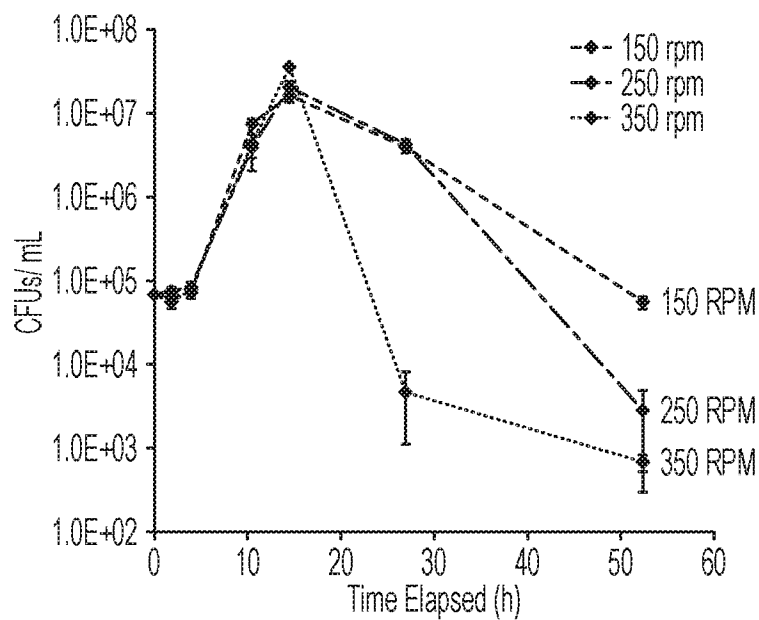


FIG. 5A

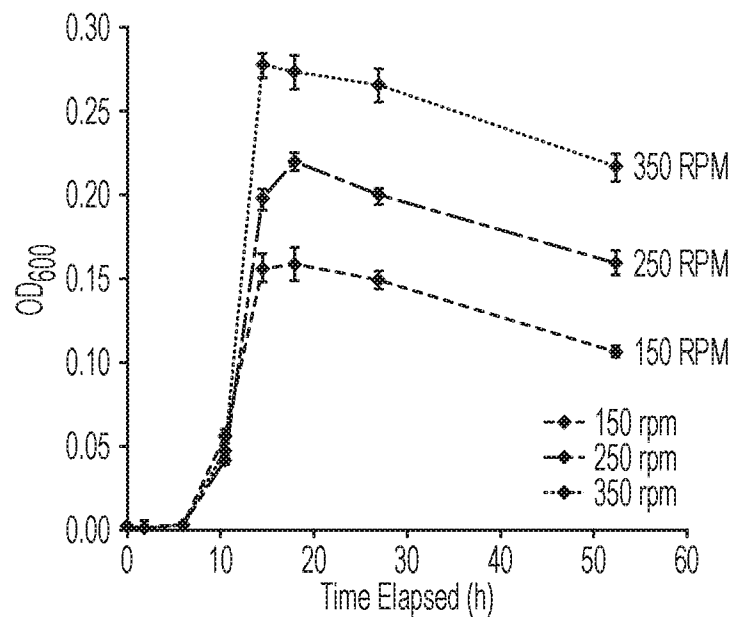


FIG. 5B

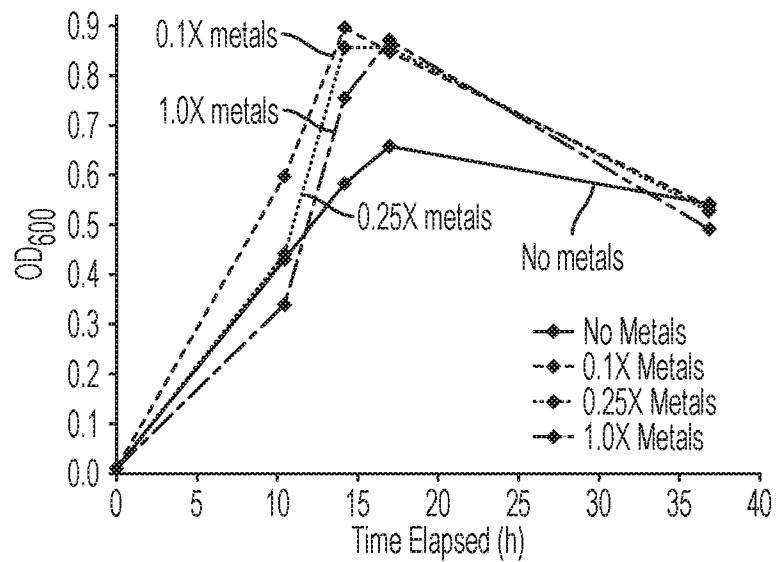


FIG. 6A

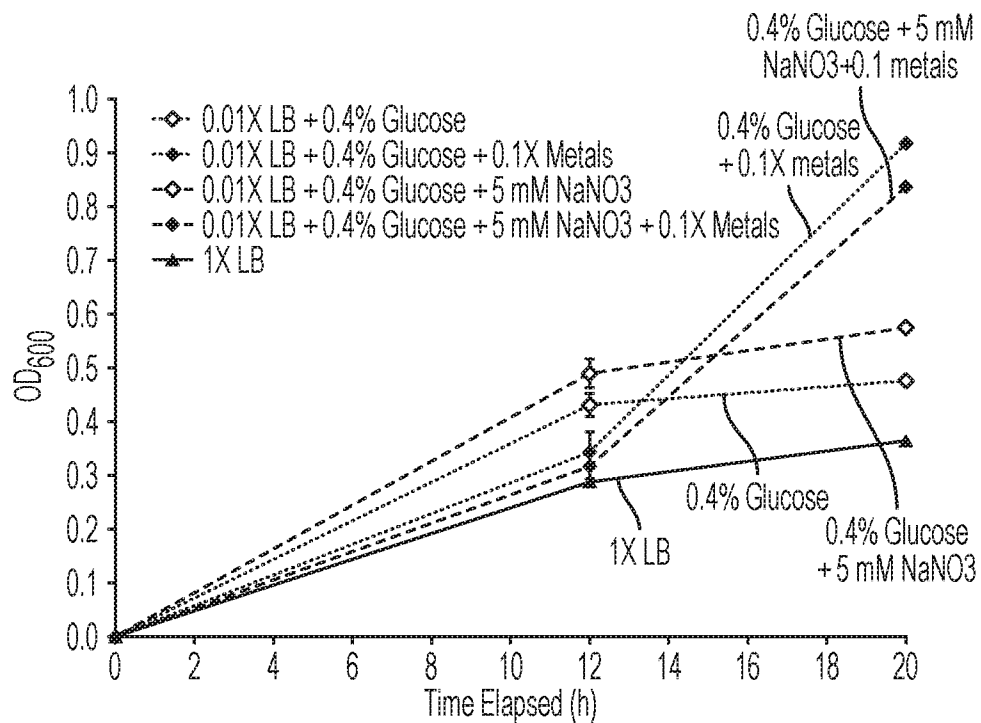


FIG. 6B

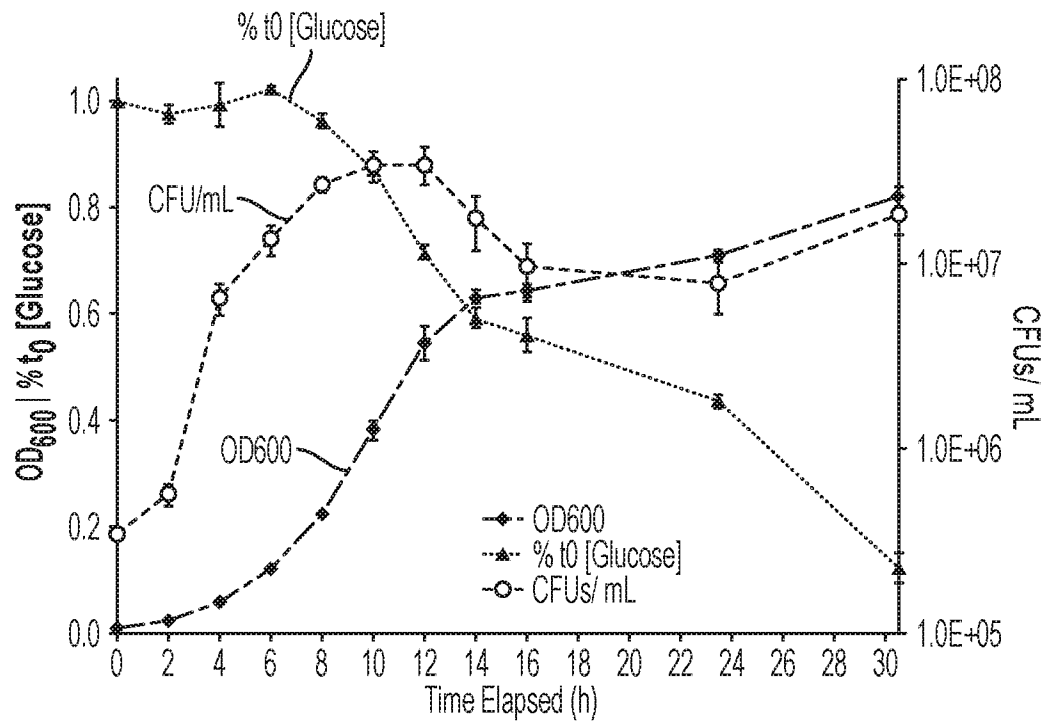


FIG. 7A

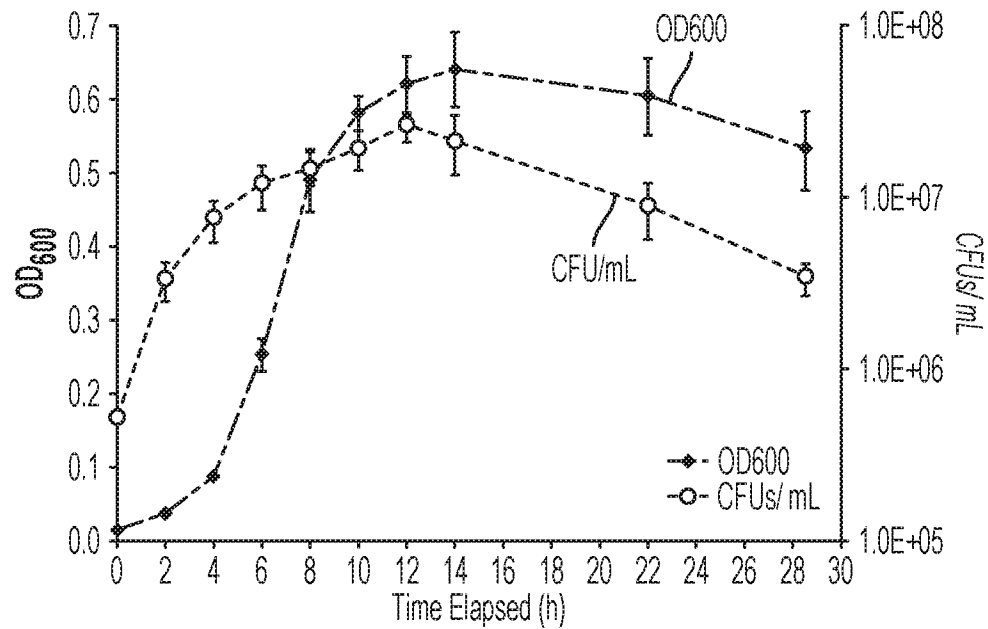


FIG. 7B

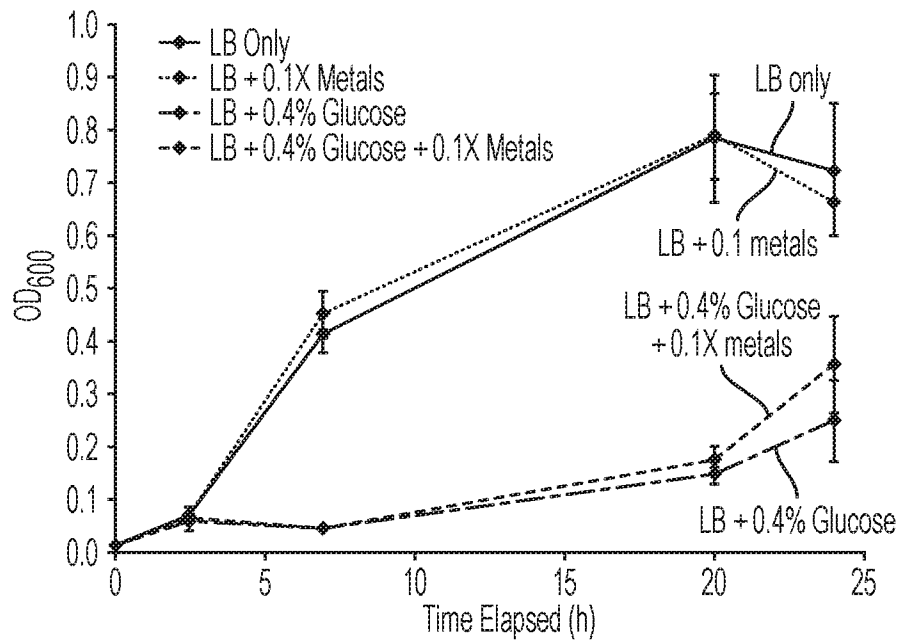


FIG. 8A

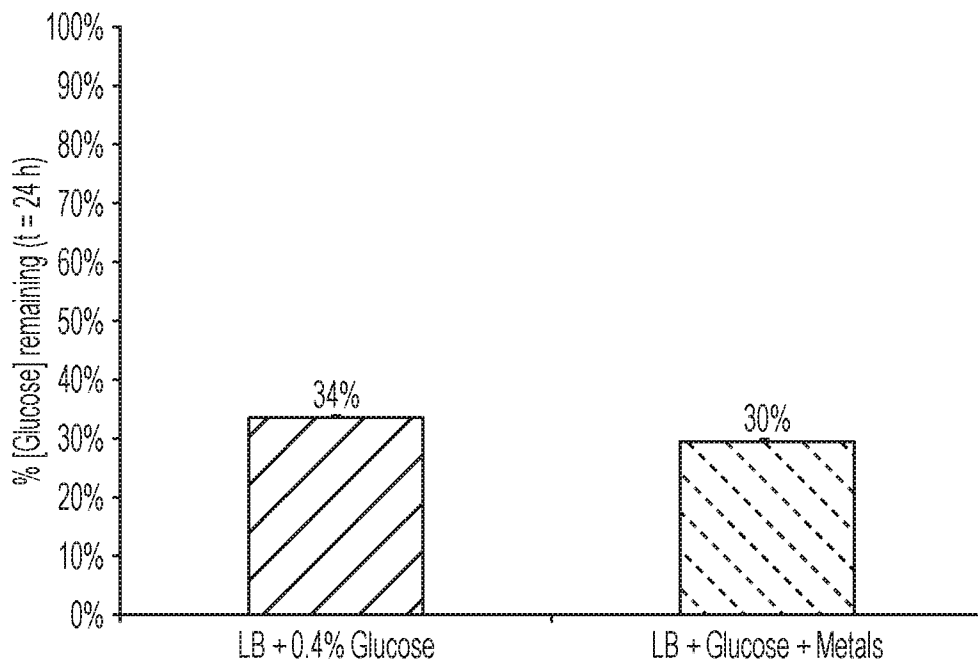


FIG. 8B

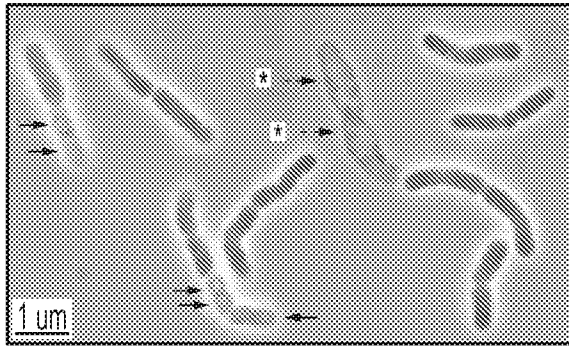


FIG. 9A

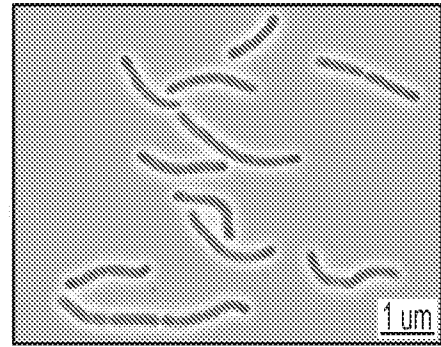


FIG. 9B

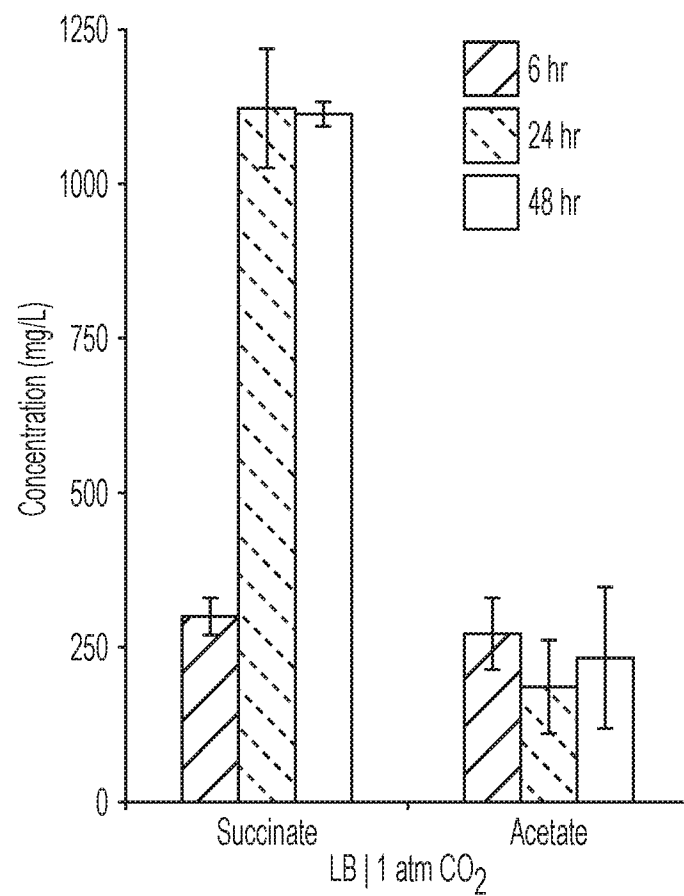


FIG. 10A

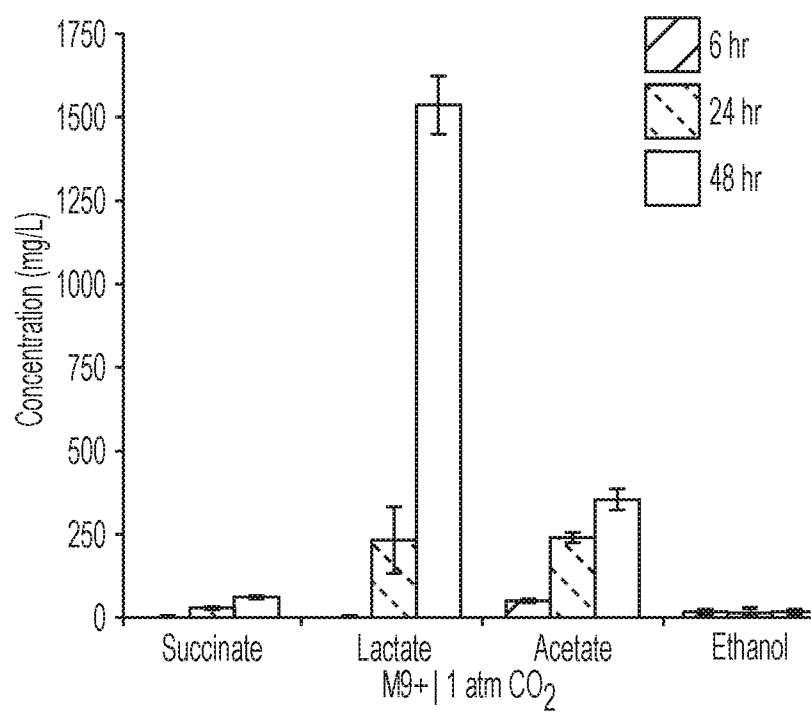


FIG. 10B

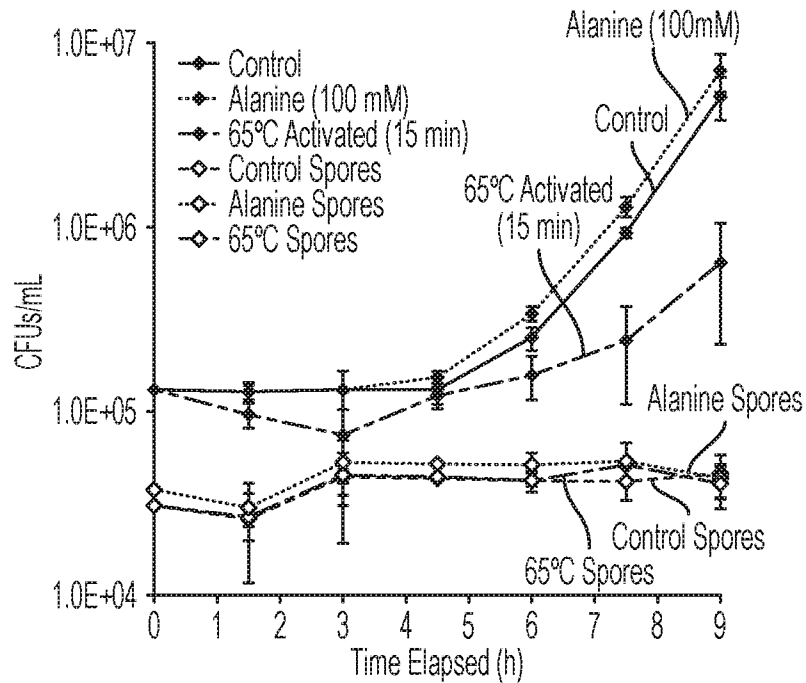


FIG. 11

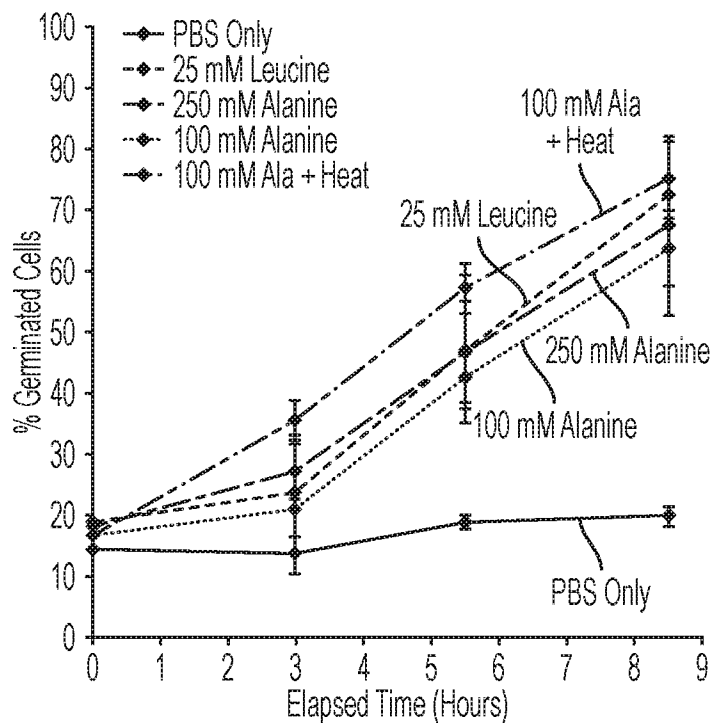


FIG. 12A

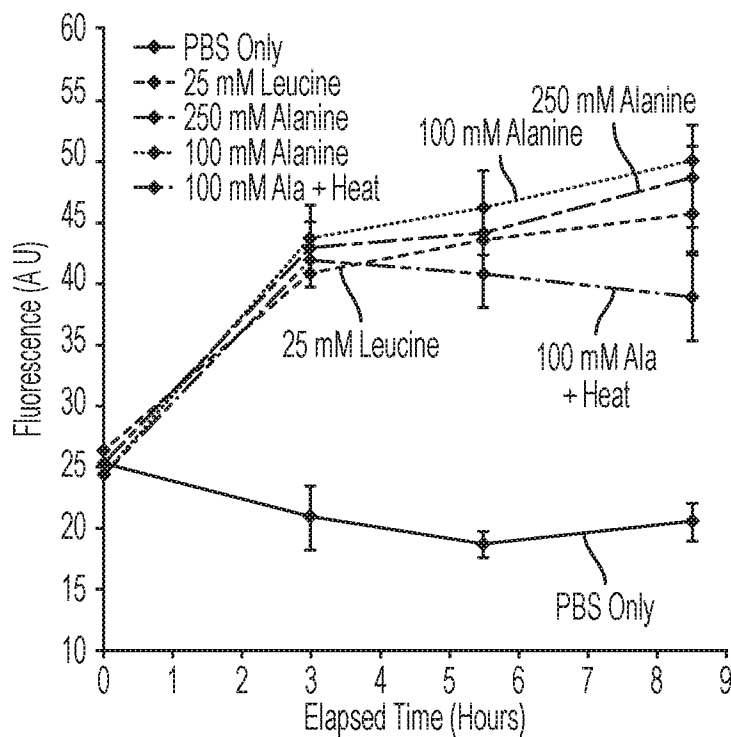


FIG. 12B

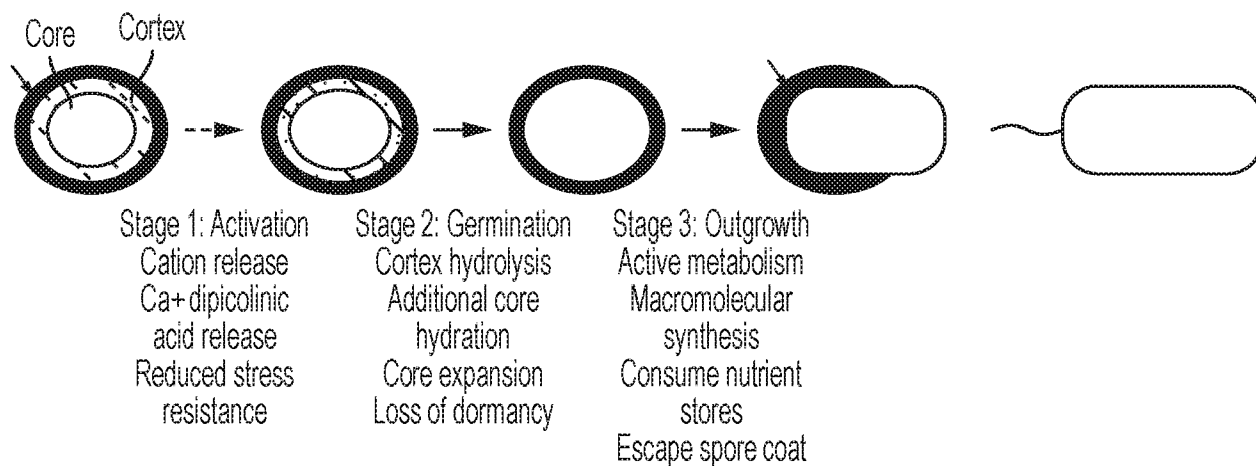


FIG. 13A

Classification	FCM Fluorescence	DNA Stain Distribution	Physiological State
"Dormant"	Low	Edge-localized	Intact Spore
"Activated"	Medium	Center-localized	Stage 1-2
"Germinated"	High	Whole cell	Stage 2-3

FIG. 13B

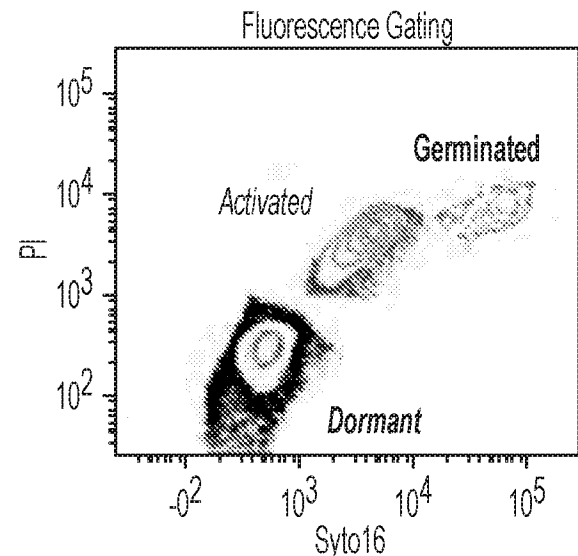
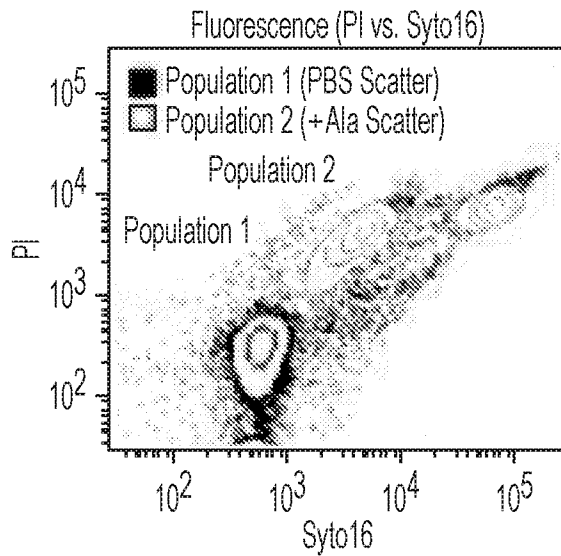
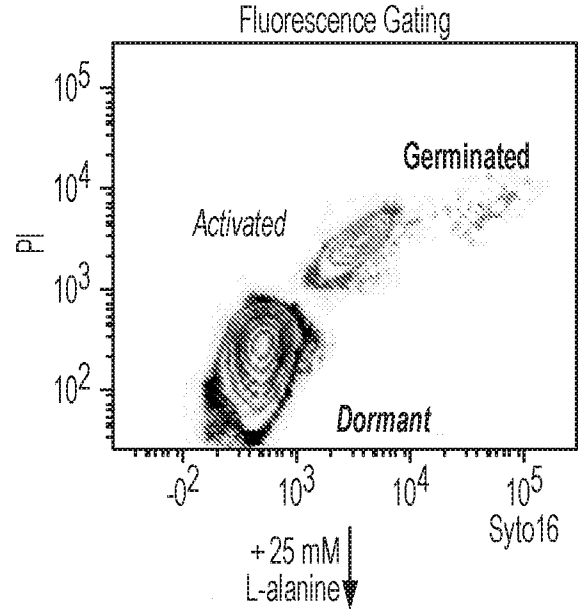
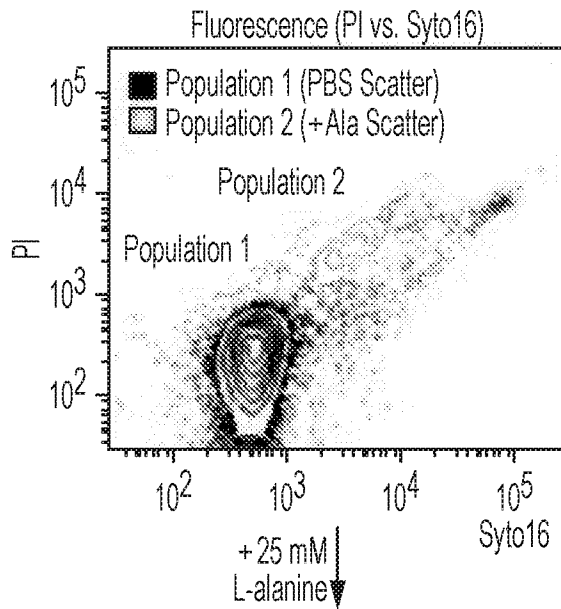


FIG. 14A

FIG. 14B

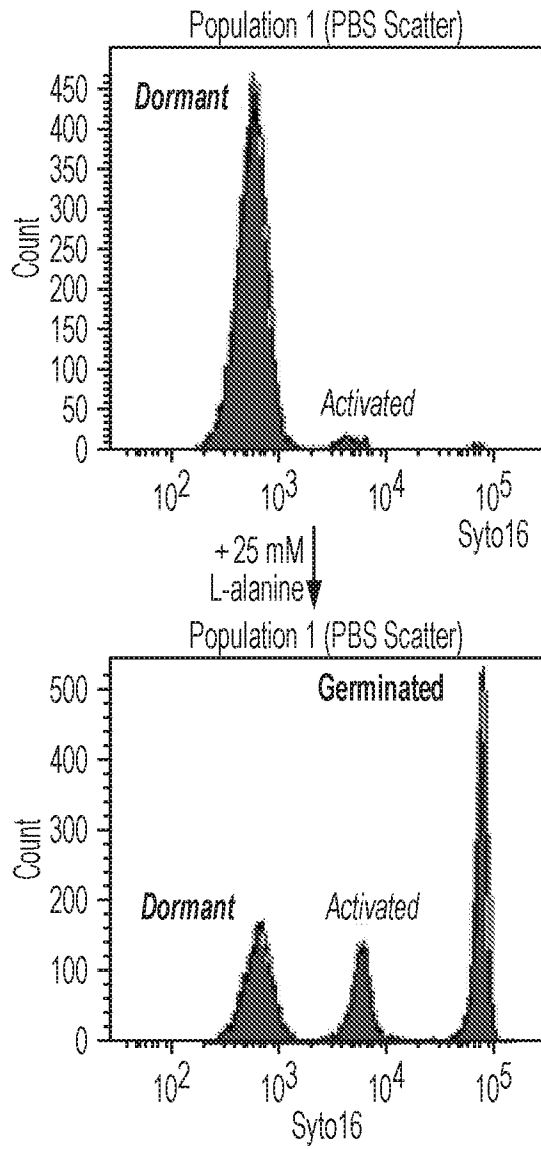


FIG. 14C

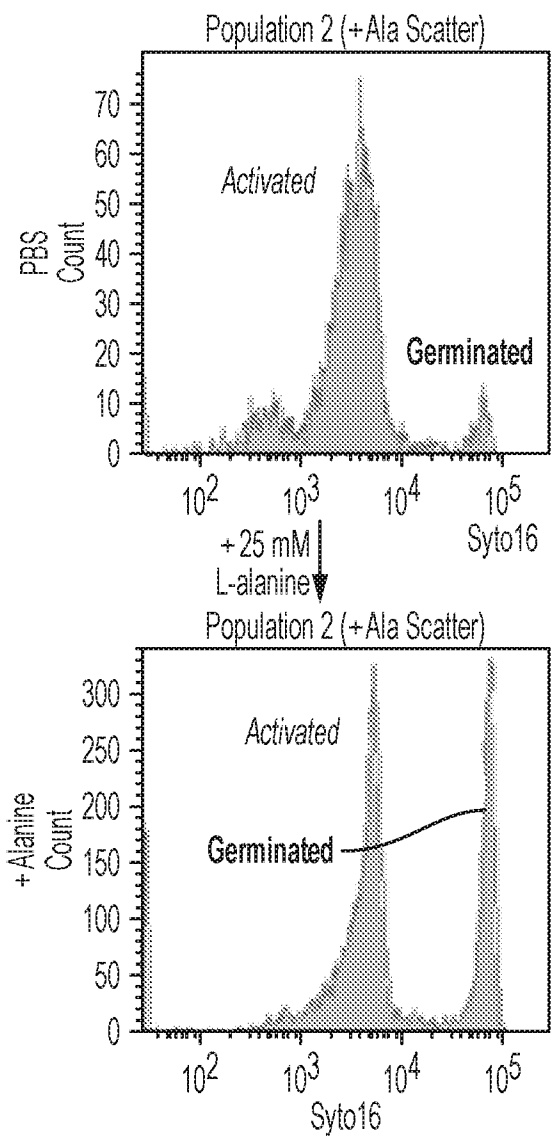
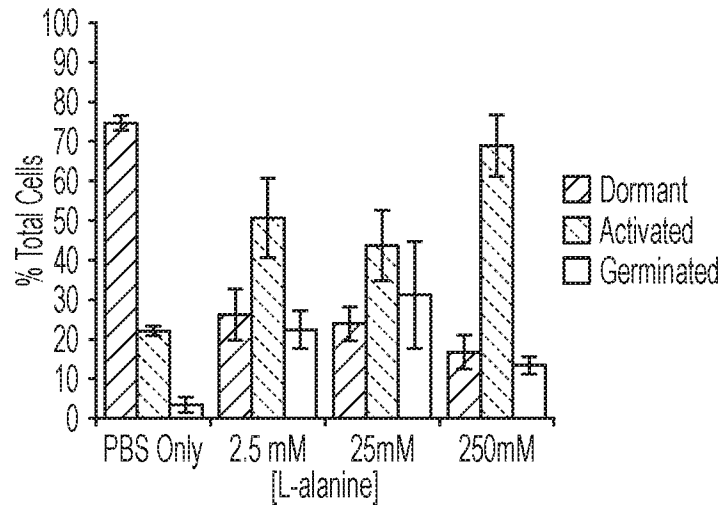


FIG. 14D



	% Dormant	% Activated	% Germinated
Spore Stock	82.9 ± 3.2	1.0 ± 0.2	16.1 ± 2.1
PBS Only	74.3 ± 2.2	22 ± 1.2	3.7 ± 1.7
2.5 mM	26.5 ± 6.4	50.5 ± 9.9	23.1 ± 4.1
25 mM	24.2 ± 4.1	44 ± 8.9	31.8 ± 13
250 mM	17.2 ± 4.5	69 ± 7.0	13.8 ± 3.2

FIG. 15

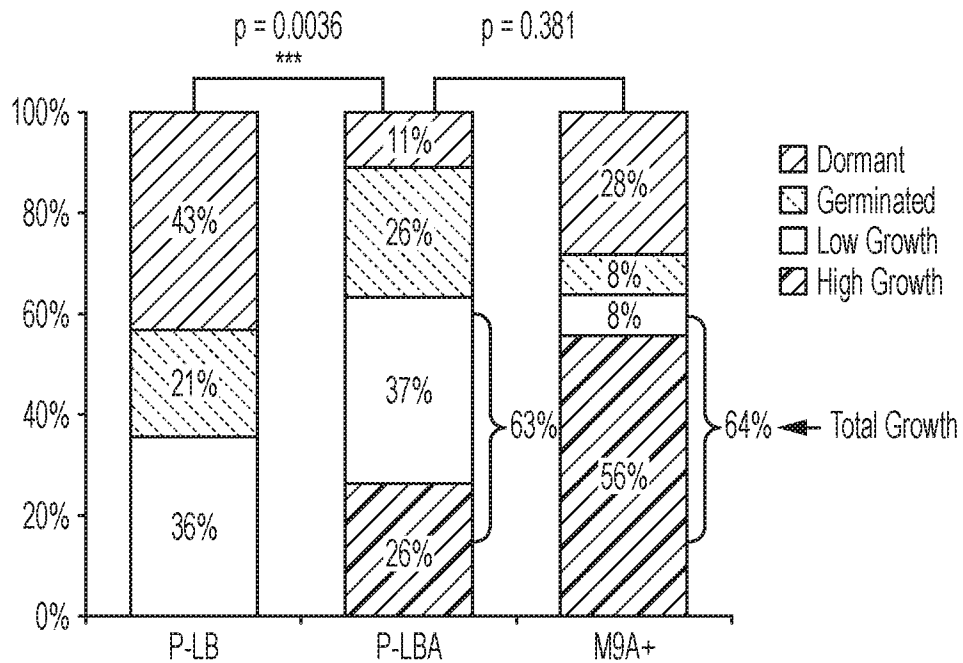


FIG. 16

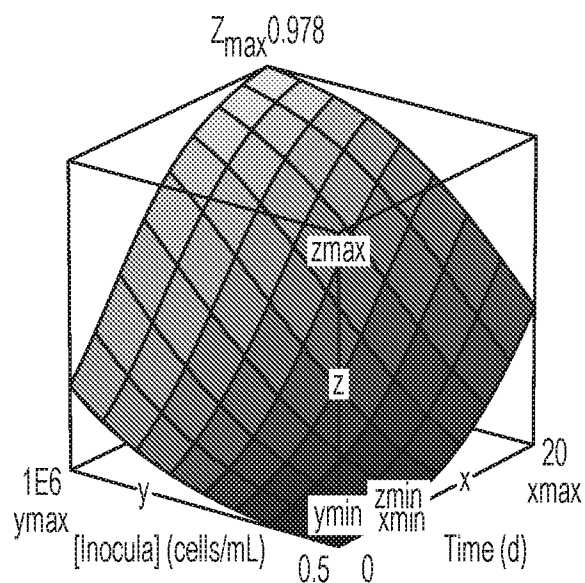


FIG. 17

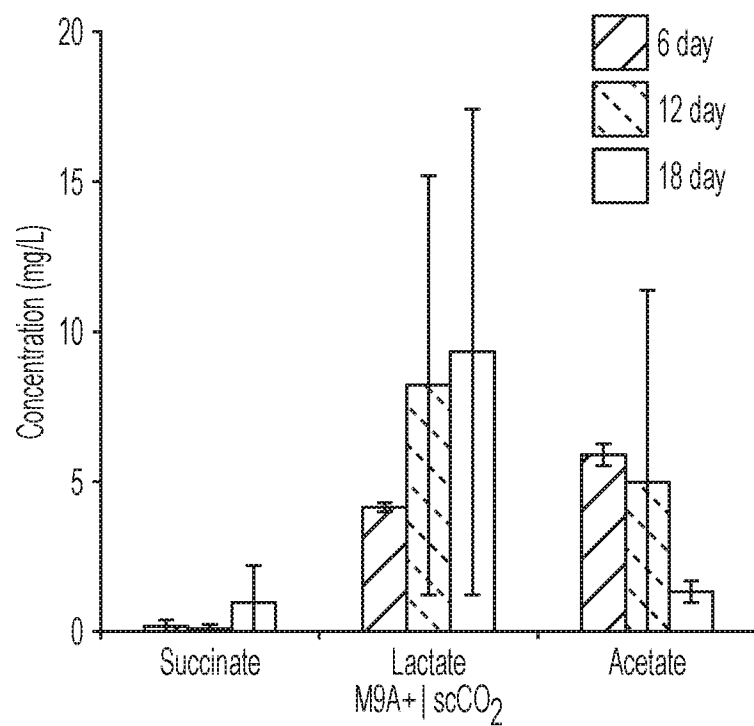


FIG. 18

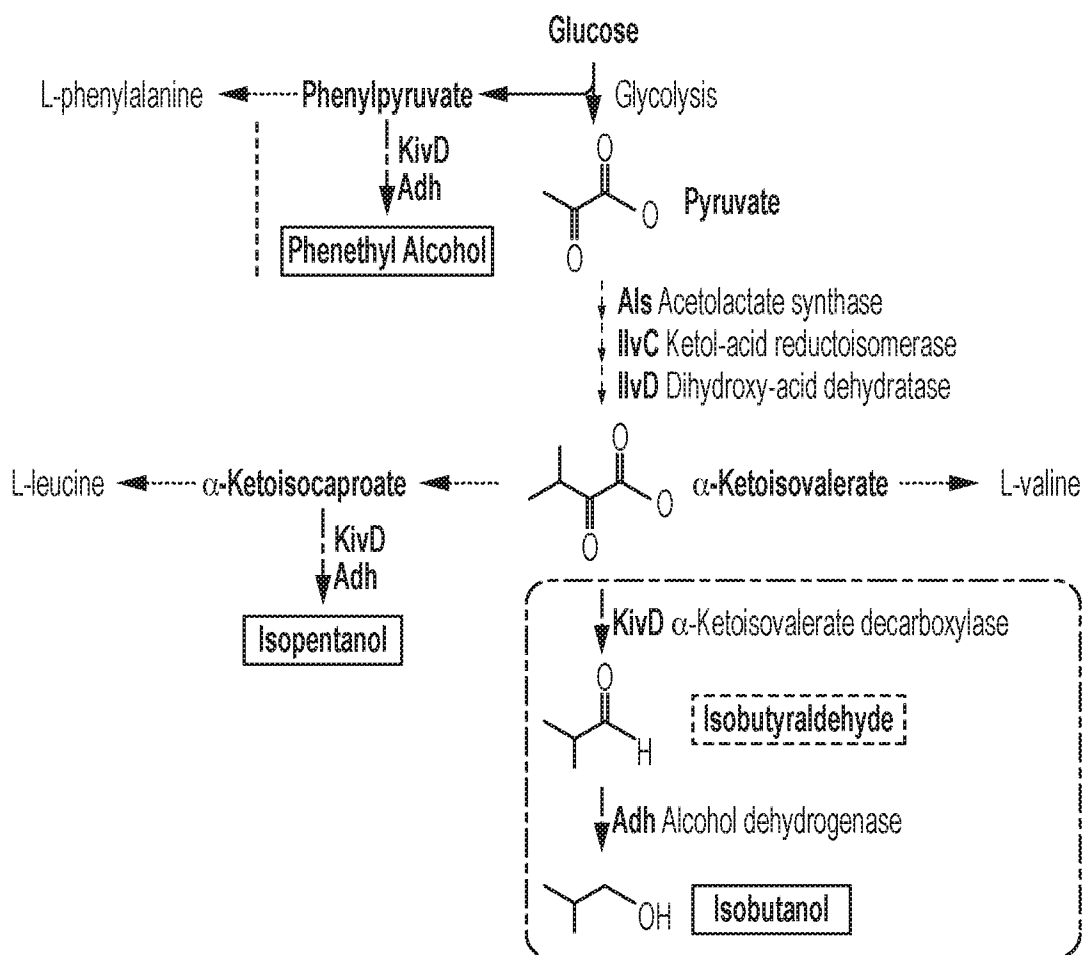


FIG. 19

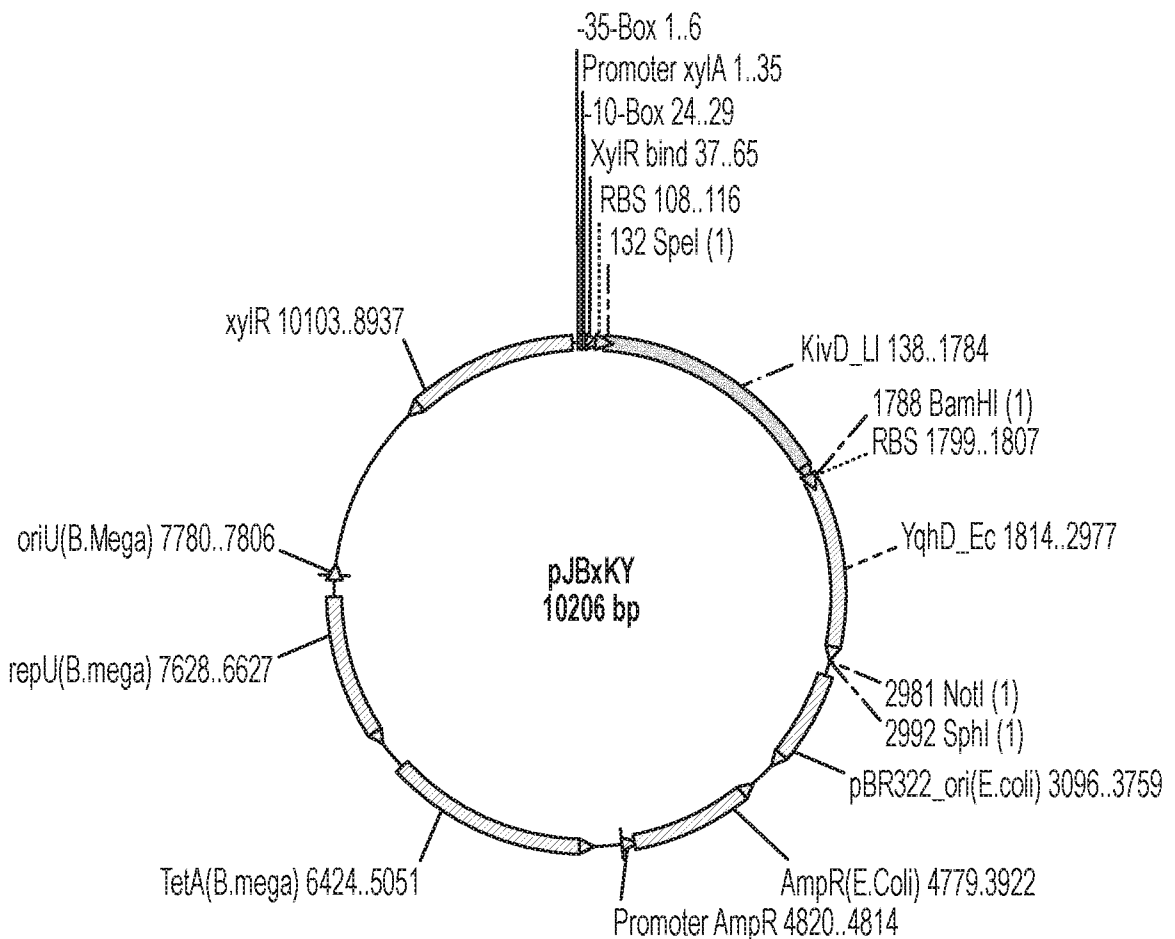
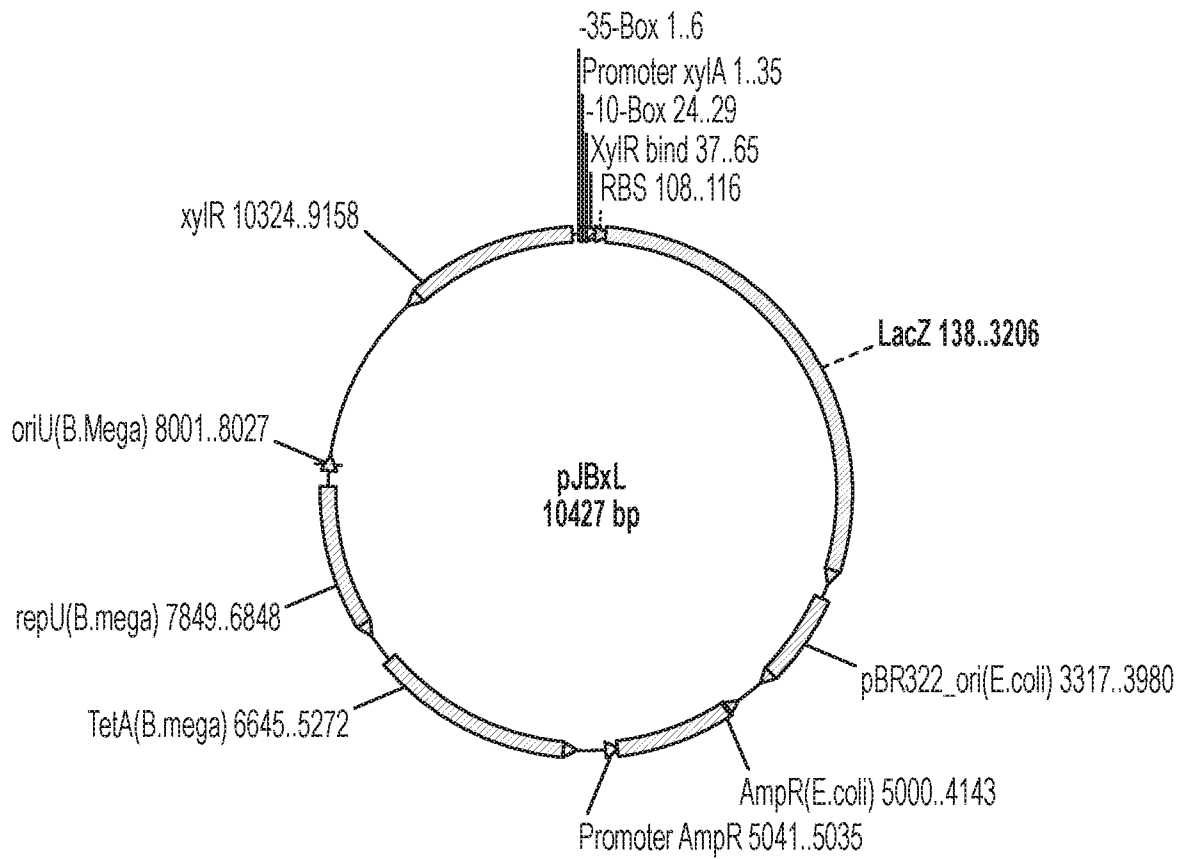


FIG. 20

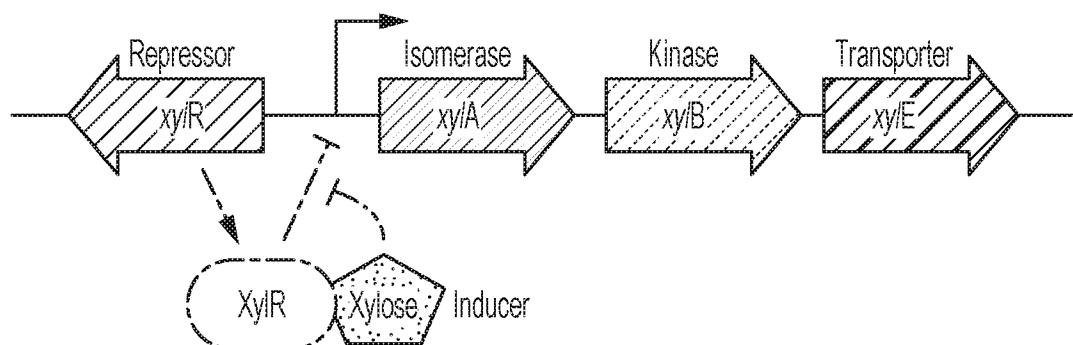


FIG. 21

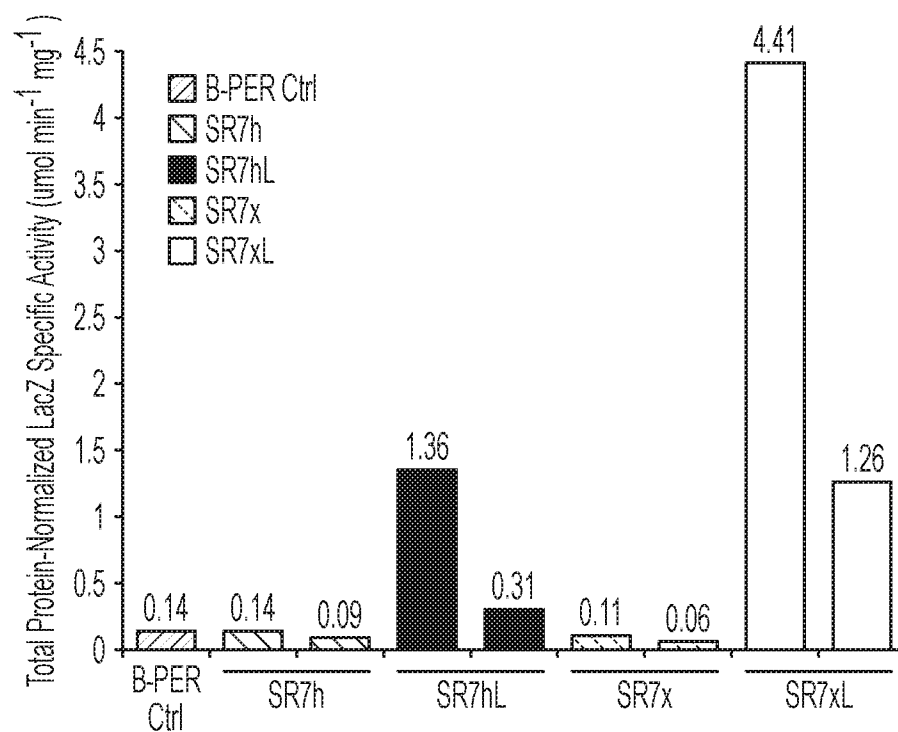


FIG. 22

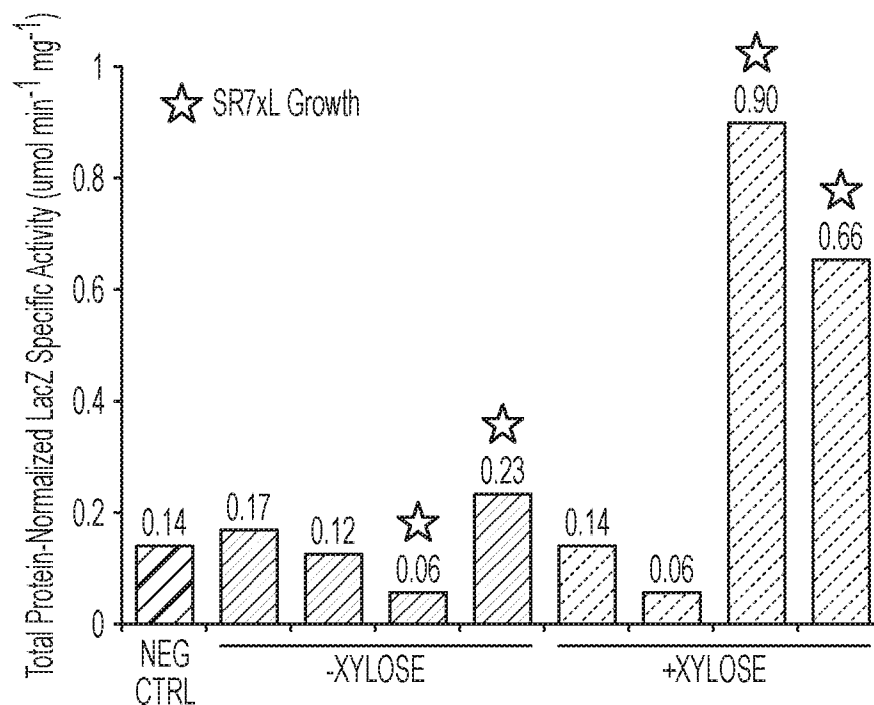


FIG. 23

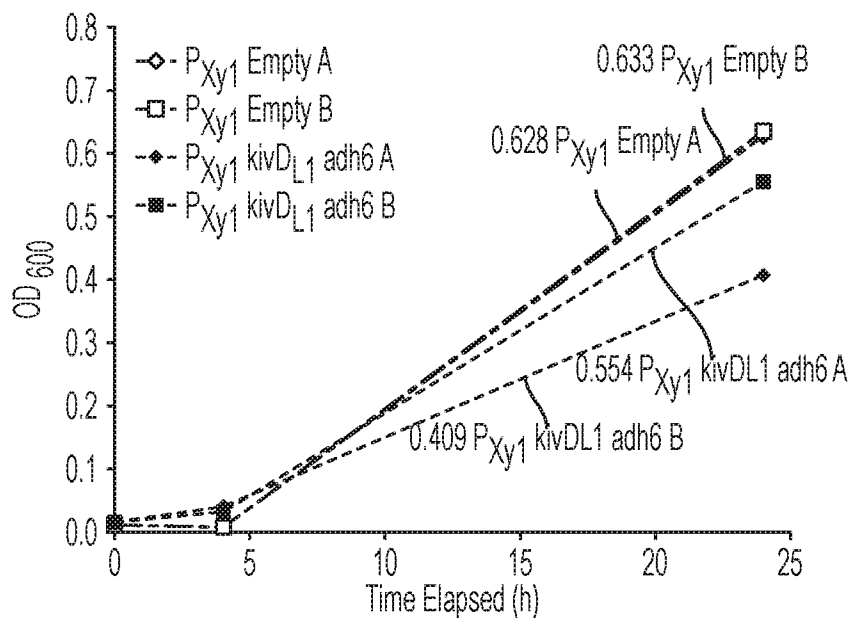


FIG. 24

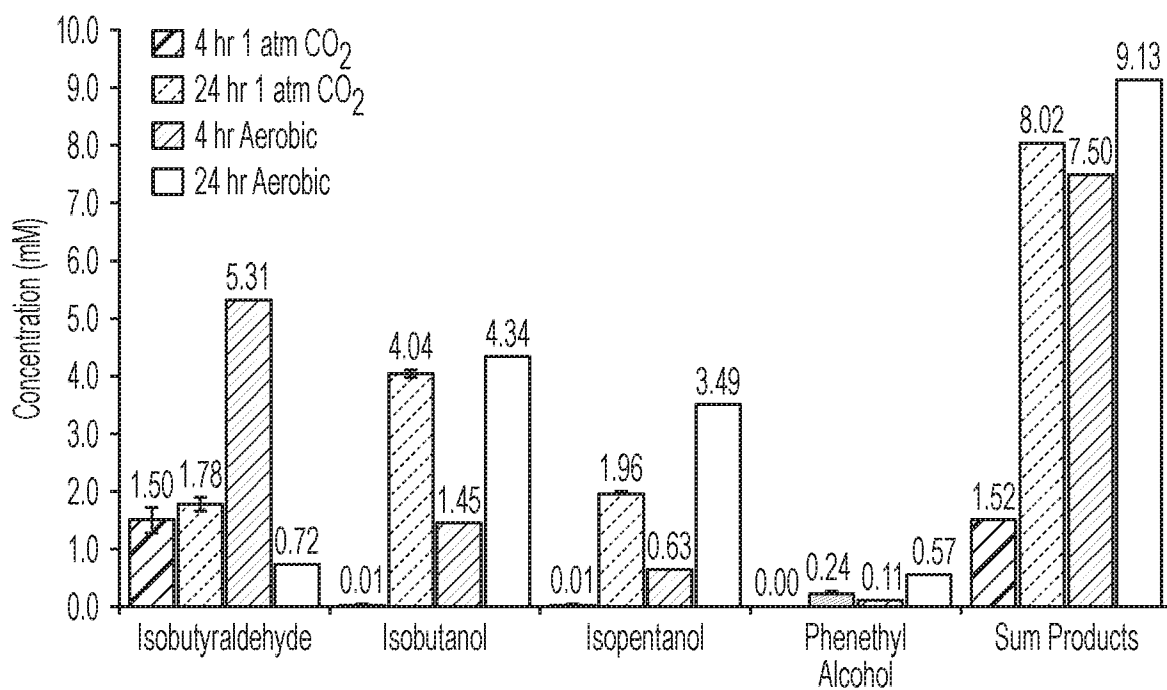


FIG. 25

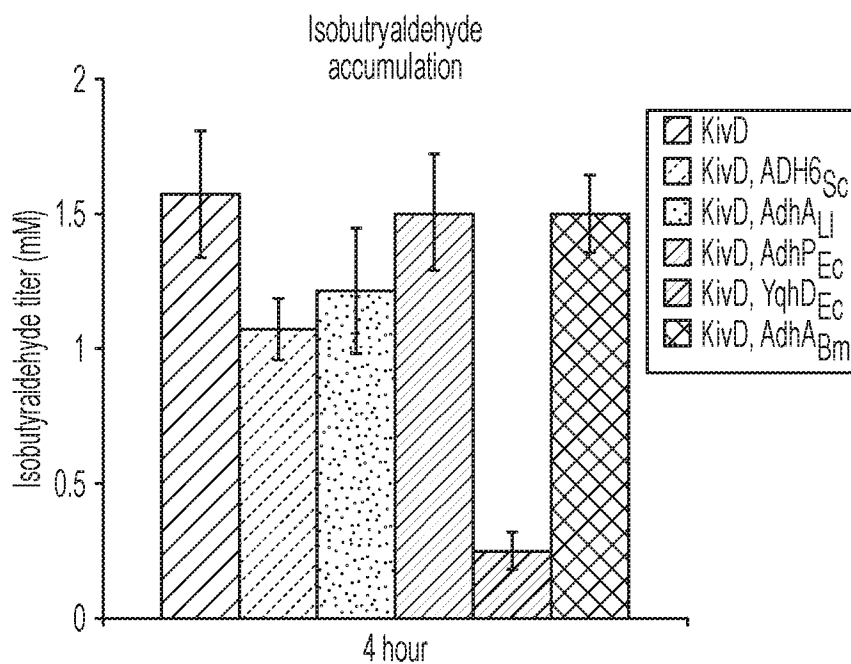


FIG. 26A

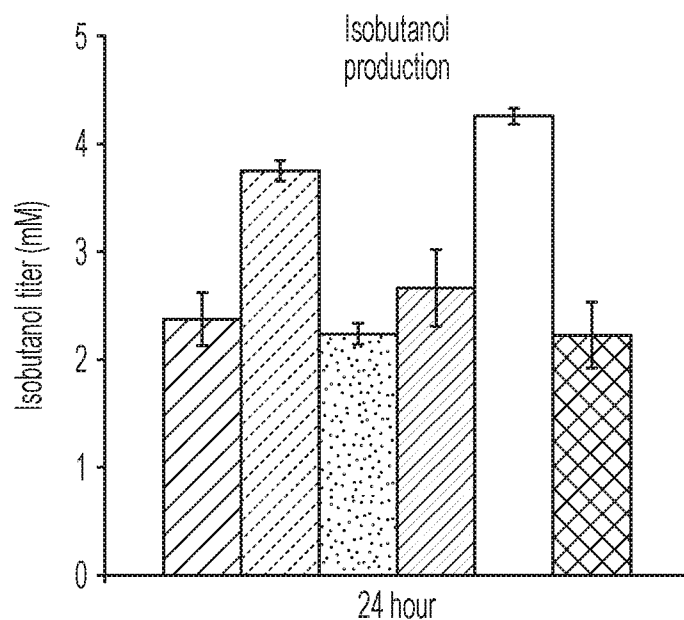


FIG. 26B

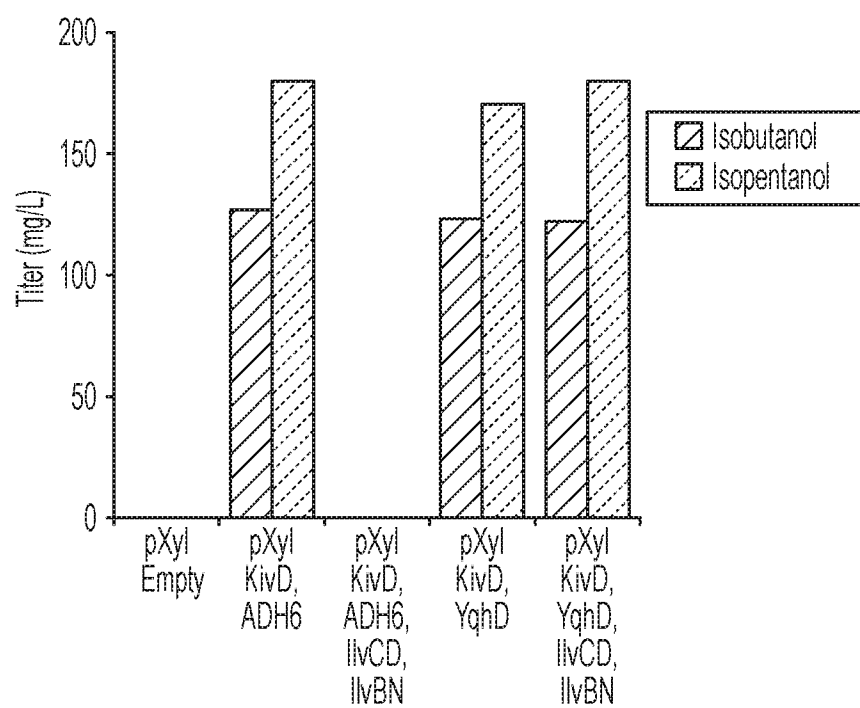


FIG. 26C

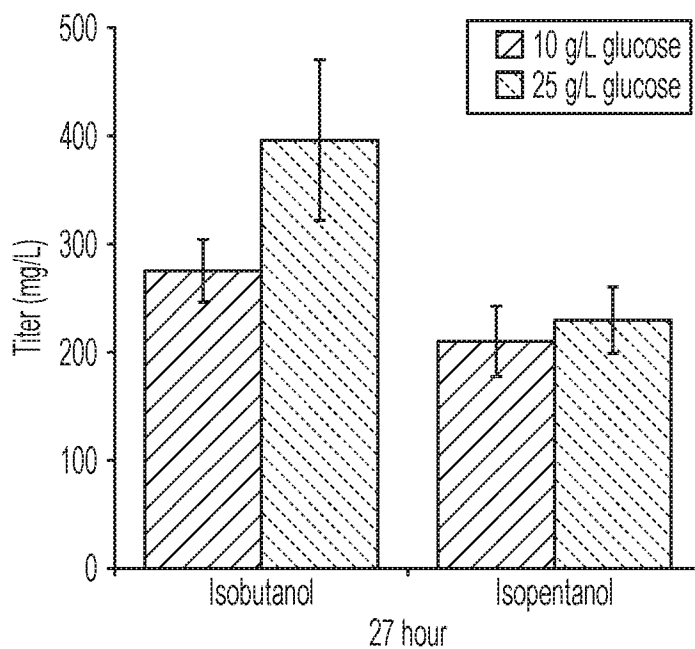


FIG. 26D

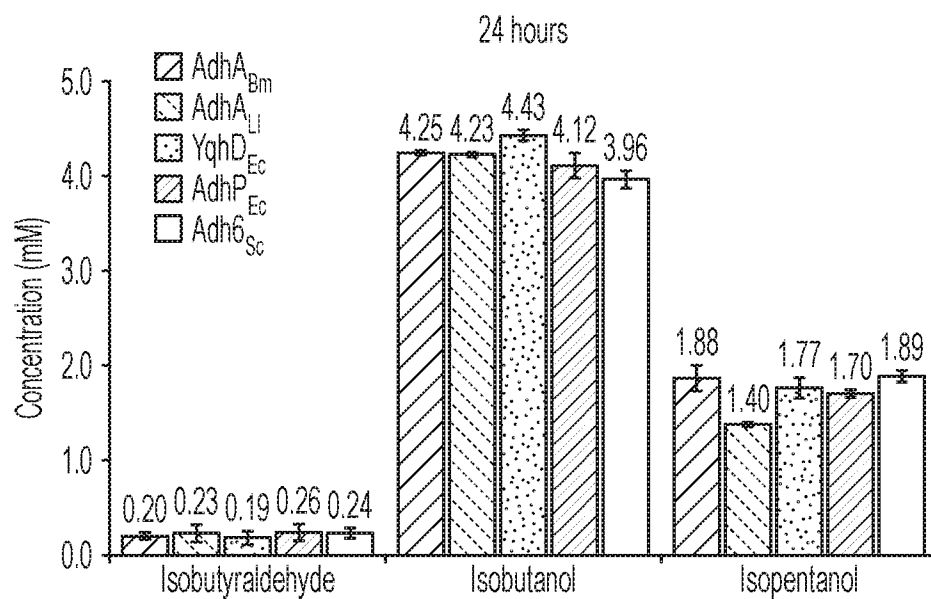


FIG. 27A

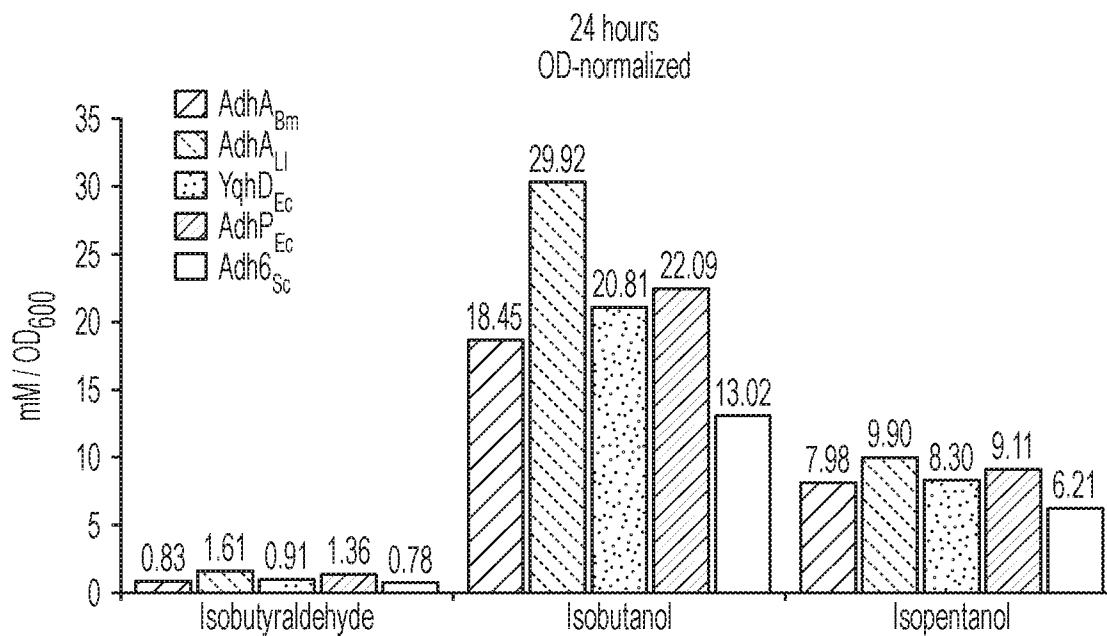


FIG. 27B

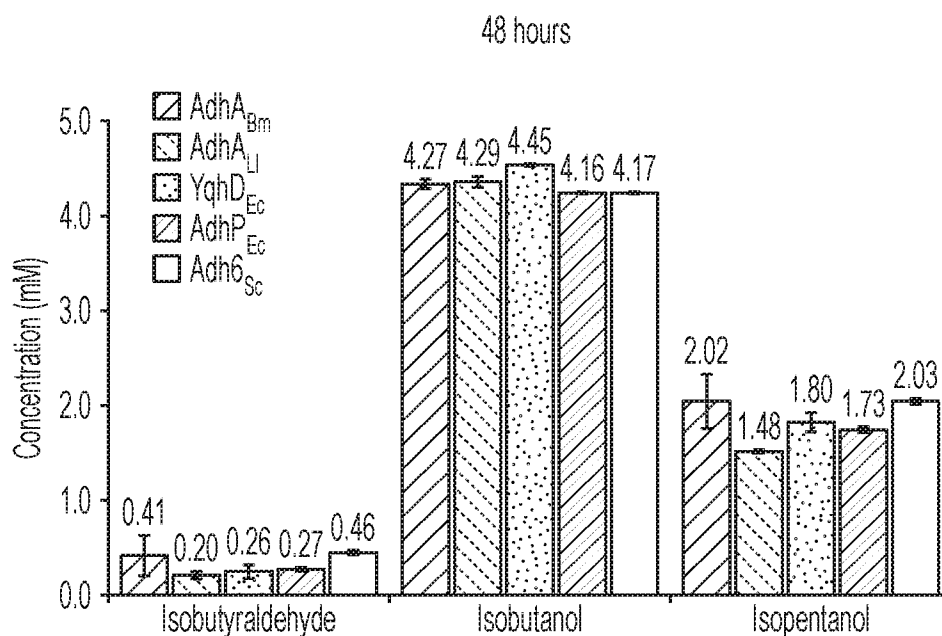


FIG. 27C

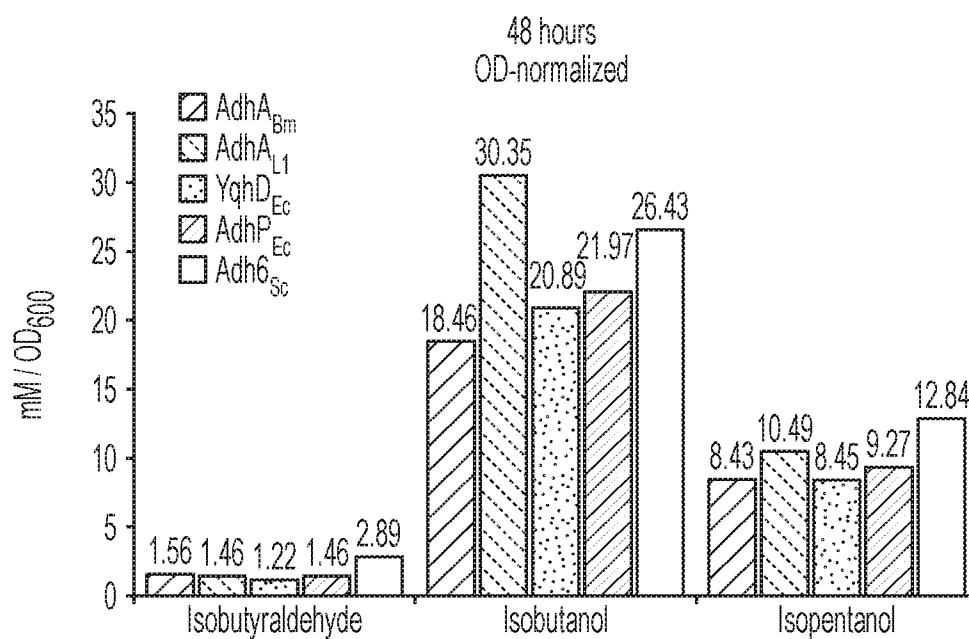


FIG. 27D

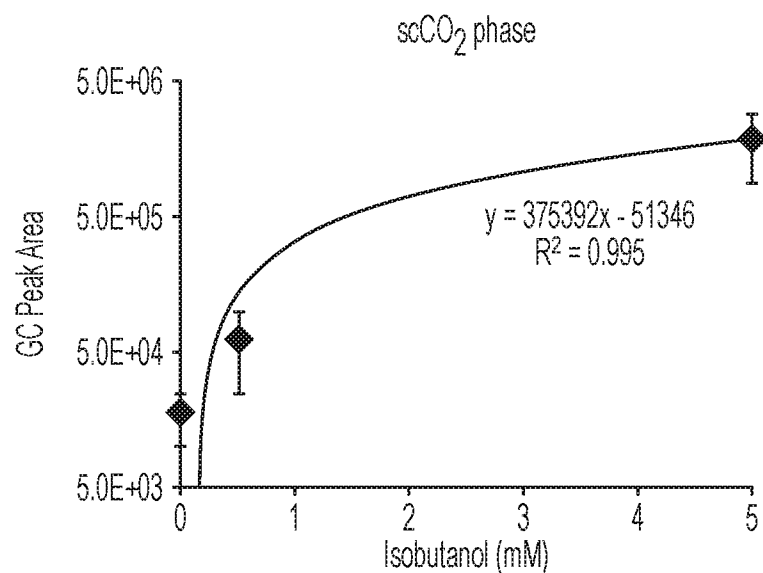


FIG. 28A

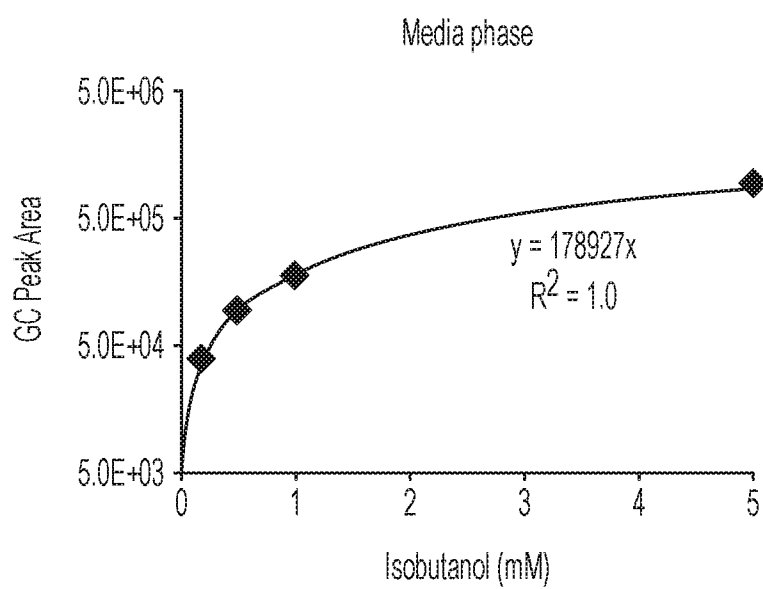


FIG. 28B

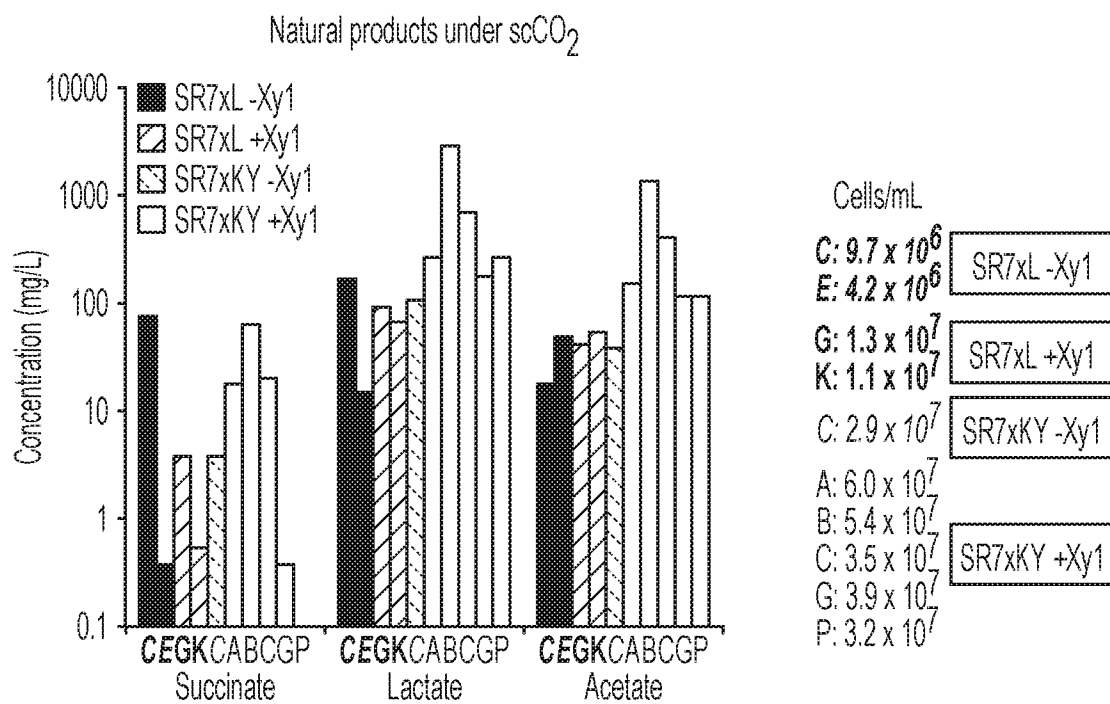


FIG. 29A

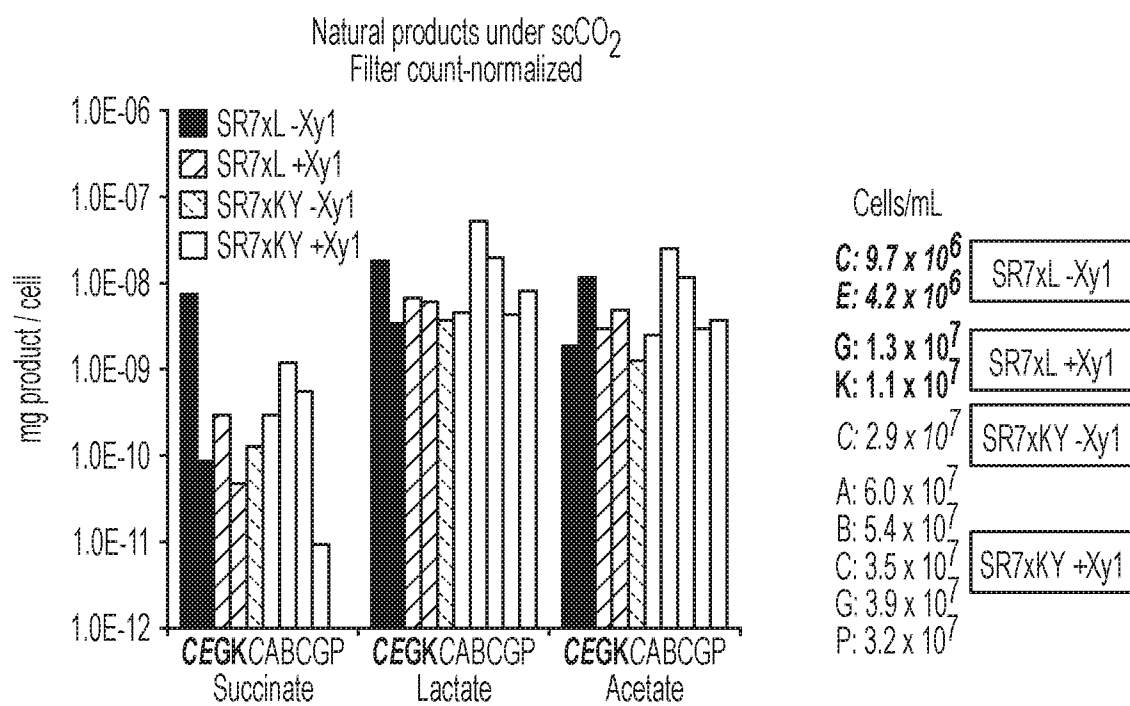


FIG. 29B

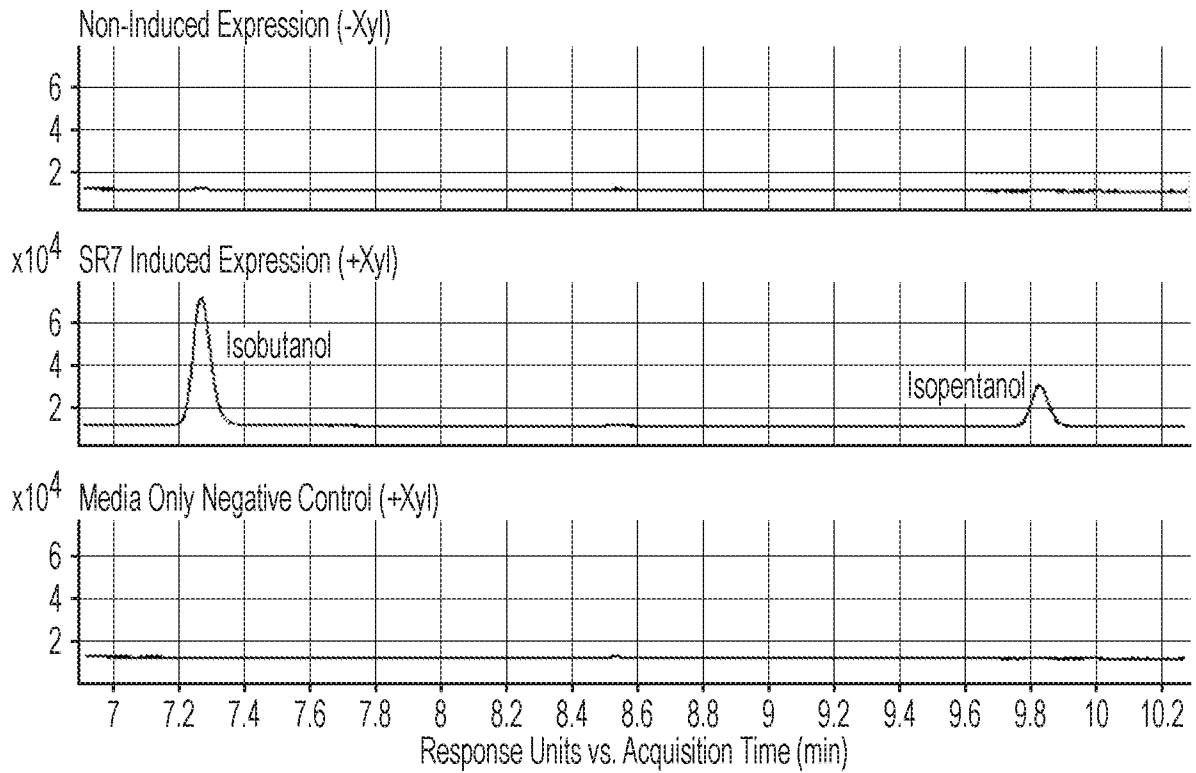


FIG. 30A

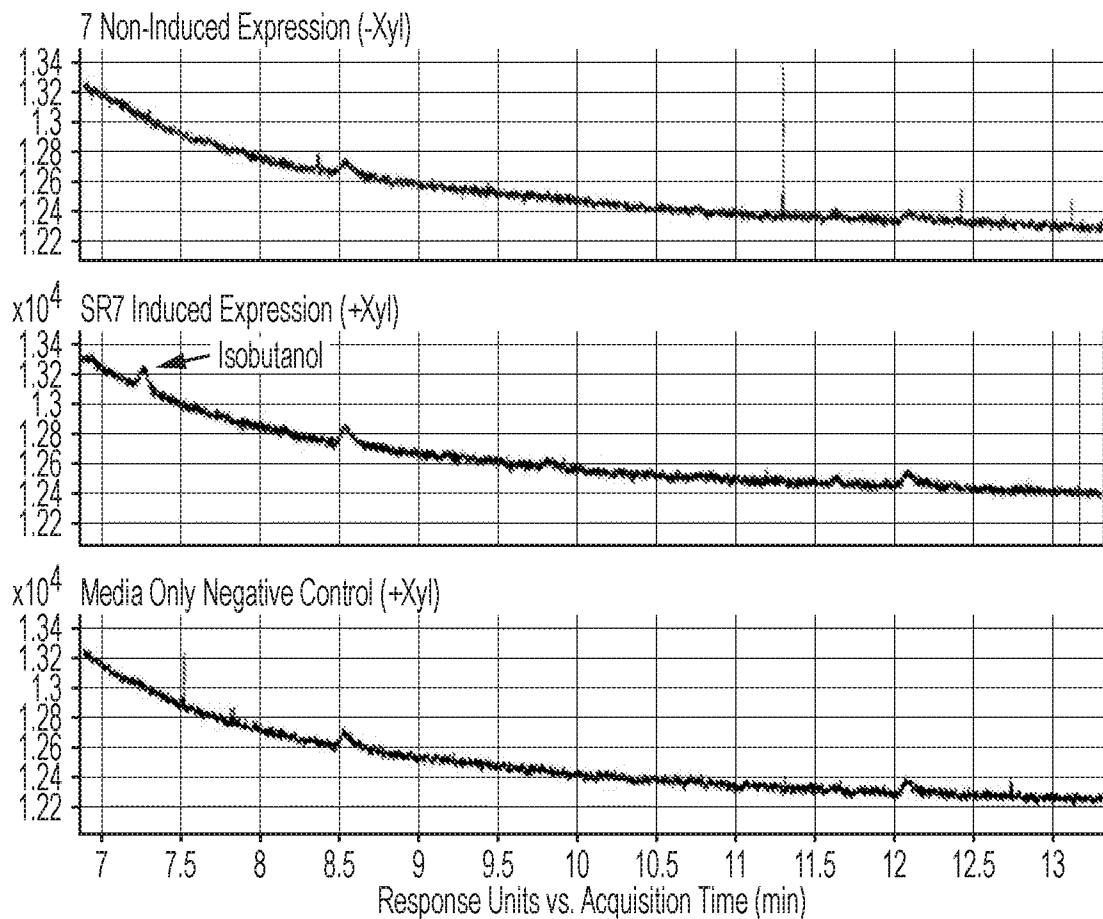


FIG. 30B

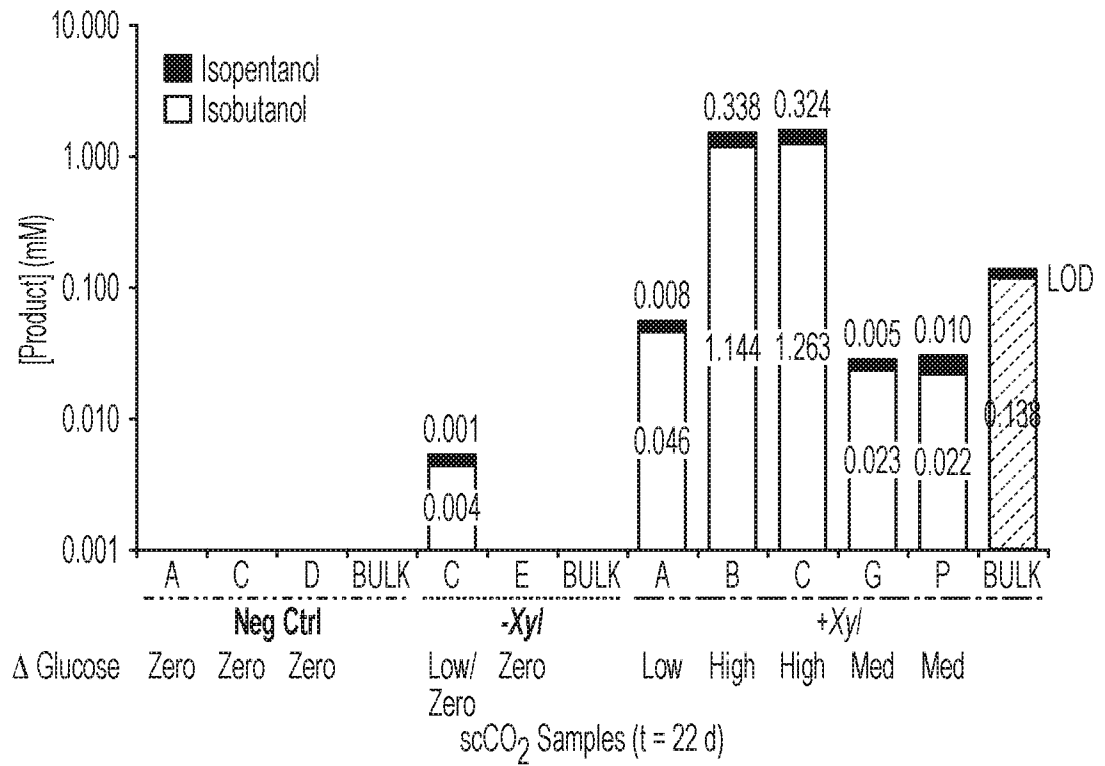


FIG. 31

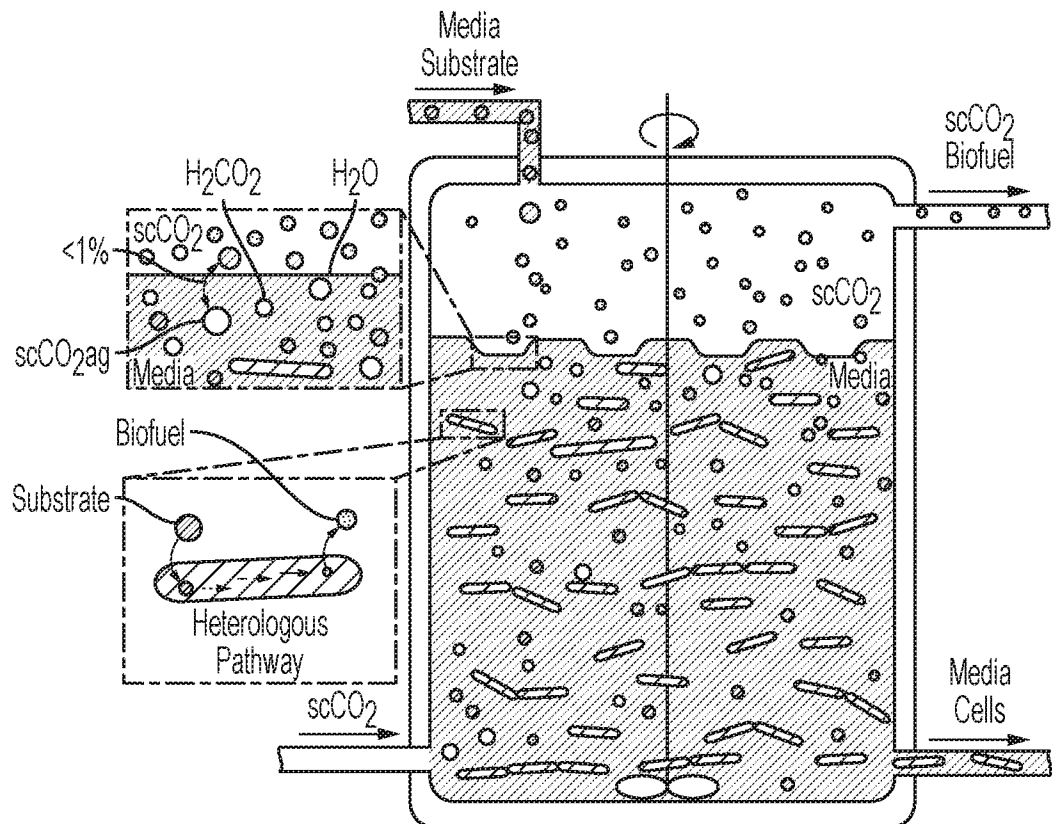


FIG. 32

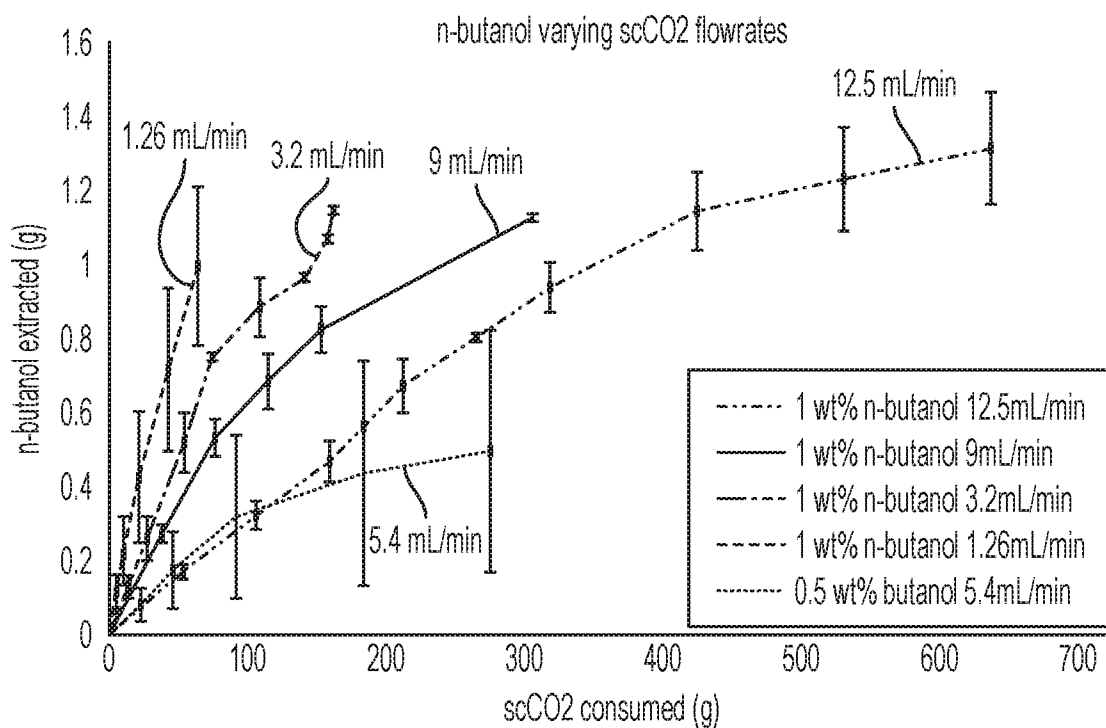


FIG. 33

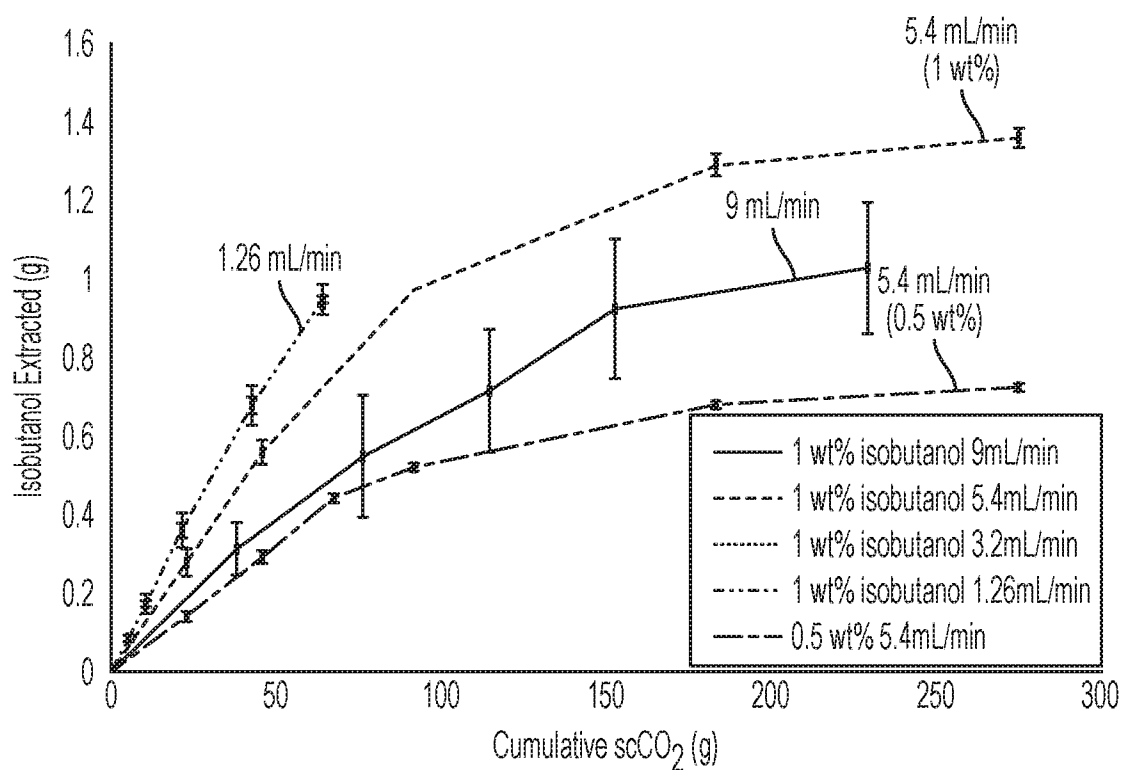


FIG. 34

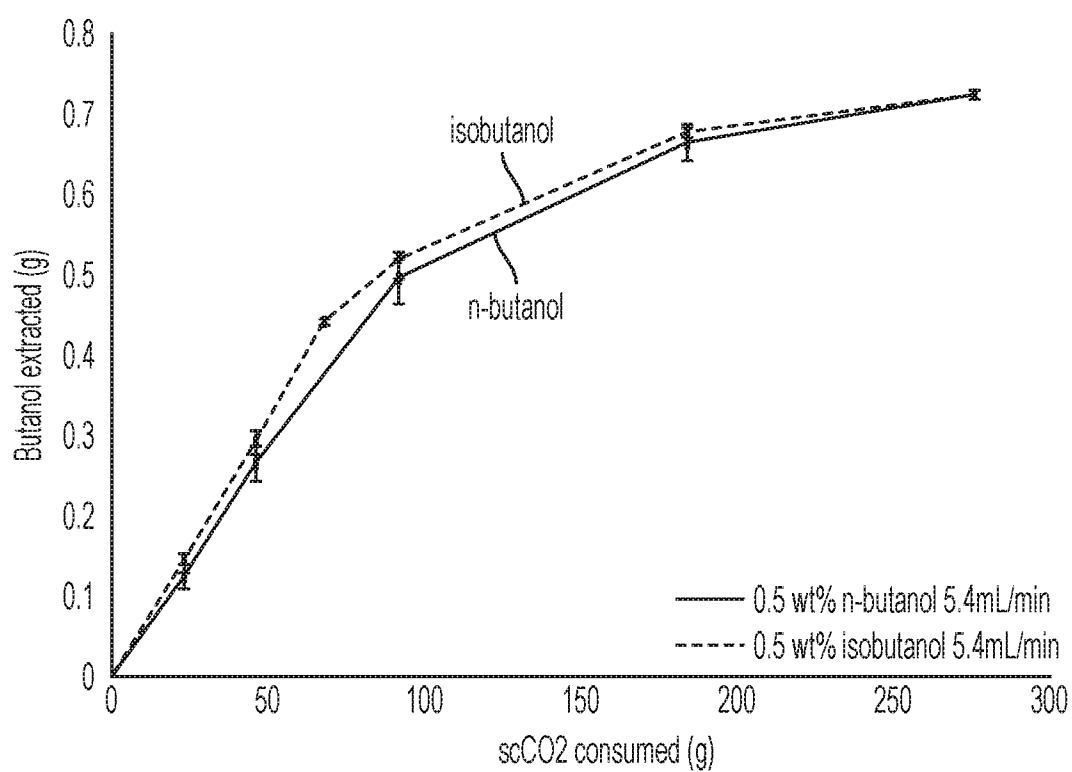


FIG. 35

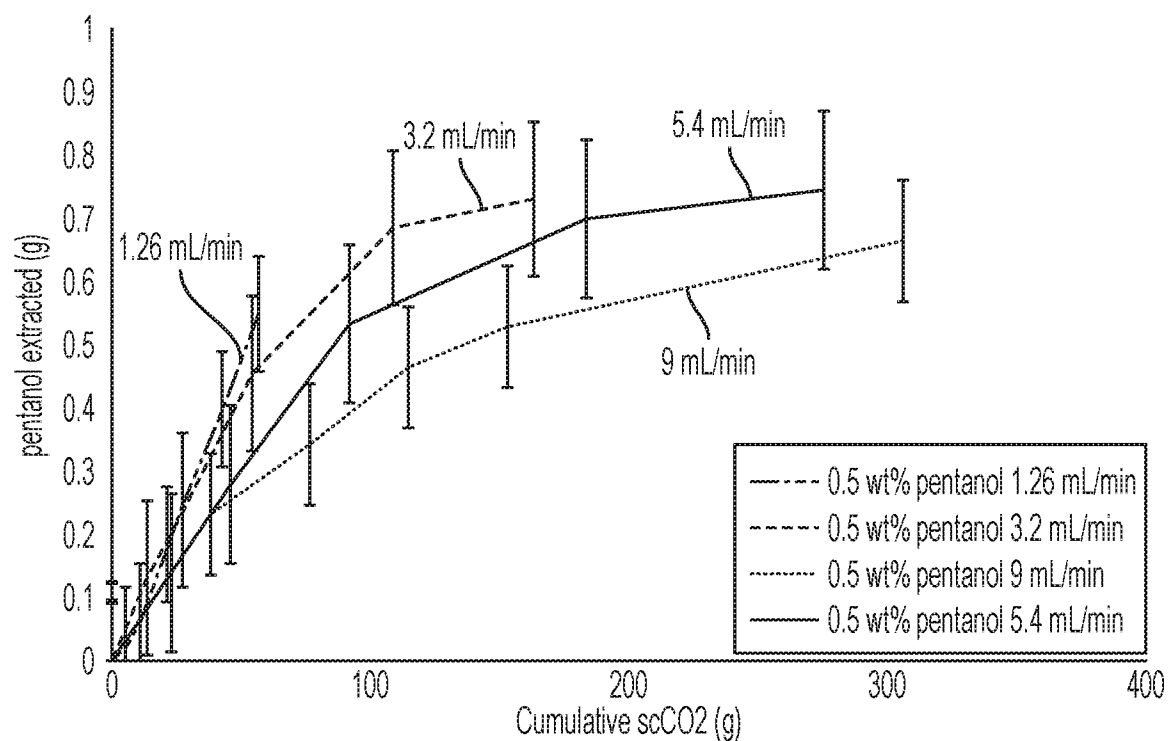


FIG. 36

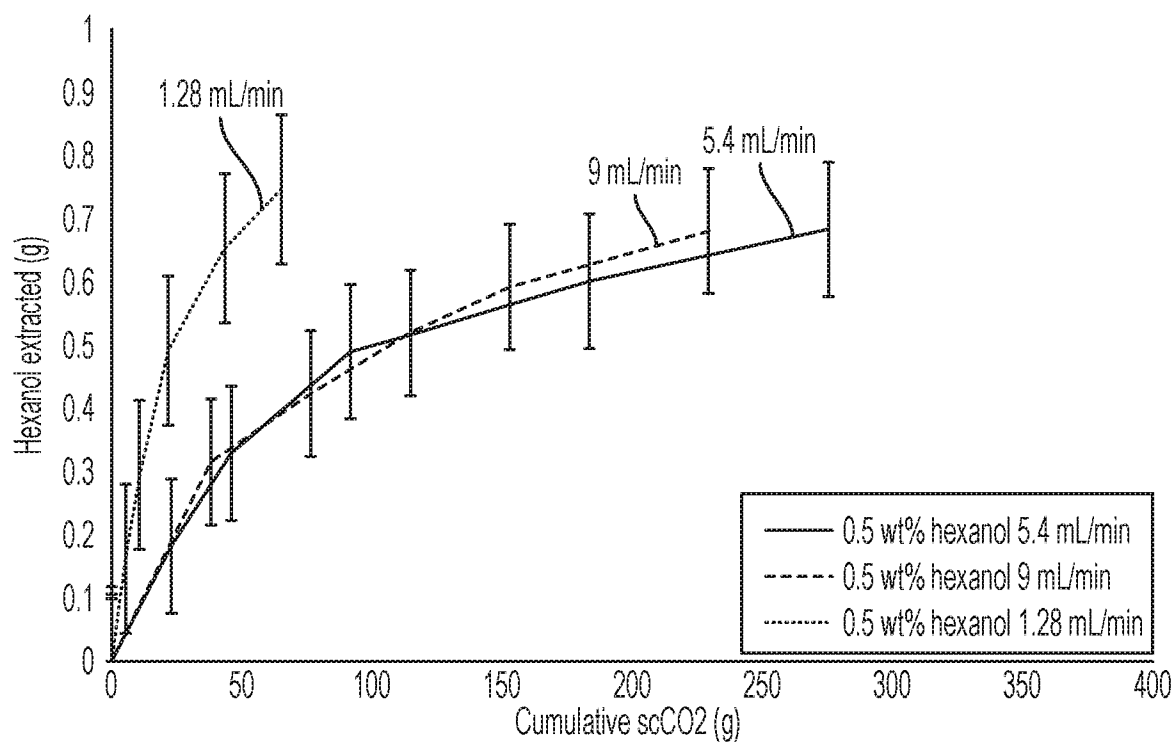


FIG. 37

P-301	C-301	C-302	C-303	C-304	T-301
Feed stock pump	CO ₂ Compressor	CO ₂ Compressor	CO ₂ Compressor	CO ₂ Compressor	CO ₂ Recycle Splitter
E-301	E-302	E-303	V-301	V-302	R-301
CO ₂ Condenser	CO ₂ Condenser	CO ₂ Condenser	Water Phase Separator	Two Phase Separator	Butanol Reactor

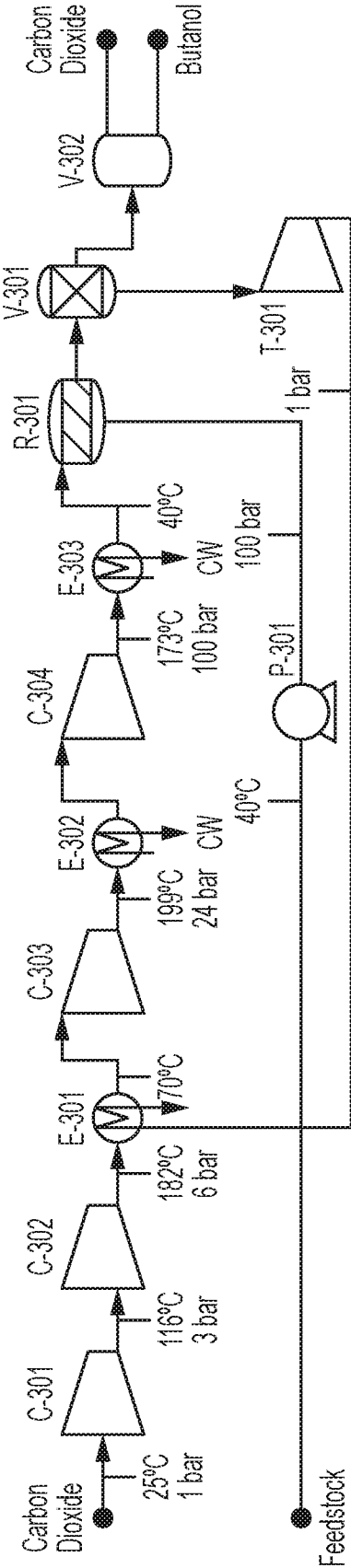


FIG. 38A

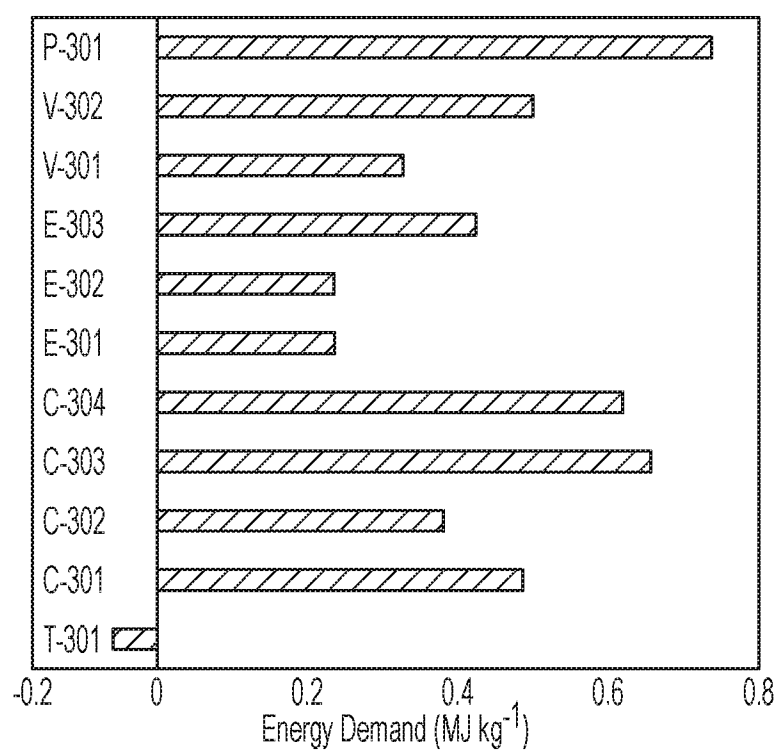


FIG. 38B

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2017/031807

A. CLASSIFICATION OF SUBJECT MATTER INV. C12P7/04 C12P7/16 C12P7/22 C12N1/21 C12M1/06 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) C12P C12N C12M		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, COMPENDEX, FSTA, BIOSIS, EMBASE, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CODY ET AL: "Energy analysis of butanol extraction using supercritical carbon dioxide", Worchester Polytechnic Institute Project report, 28 April 2016 (2016-04-28), pages 1-44, XP002771517, Worcester, Massachusetts, U.S.A. Retrieved from the Internet: URL:https://web.wpi.edu/Pubs/E-project/Available/E-project-042816-040328/unrestricted/SC_ButOH_Extraction_RC.VS.HV_2016.pdf [retrieved on 2017-06-28]	1,2, 4-10,12, 29, 31-36, 38, 40-43, 49,51
Y	* See esp. page 14 (Figure 1); the date of publication has to be verified * ----- -/--	1-10, 12-29, 31-36, 38-53
☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.		
* Special categories of cited documents :		
"A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 29 June 2017		Date of mailing of the international search report 18/07/2017
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Korsner, Sven-Erik

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2017/031807

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PEET ET AL: "Microbial growth under supercritical CO ₂ ", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 81, 2015, pages 2881-2892, XP002771520, * See page 2881 (Abstract/B. megaterium) * -----	31-36
X	PEET ET AL: "Draft genome sequences of supercritical CO ₂ -tolerant bacteria Bacillus subterraneus MITOT1 and Bacillus cereus MIT0214", GENOME ANNOUNCEMENTS, vol. 3, 2015, pages 1-2, XP002771518, * See the Introduction * -----	31-36
X	MARRIOTT: "Green extraction, separation and reactions using liquid or supercritical CO ₂ ", Botanix Ltd Presentation paper, 2015, pages 1-36, XP002771529, Retrieved from the Internet: URL:https://www.york.ac.uk/res/gcrn/presentations/Ray%20Marriott%20-%20Botanix.pdf [retrieved on 2017-06-28] * General overview; see pages 5, 13, 17, 25, 26, and (esp.) 30 * -----	31-36
X	PEET: "Demonstrating biocompatibility with supercritical CO": Biphasic cultivation of Bacillus spp. and probing acclimation mechanisms through proteome and lipid analysis", Massachusetts Institute of Technology (MIT) PhD thesis, 2015, pages 1-152, XP002771523, Retrieved from the Internet: URL:https://dspace.mit.edu/handle/1721.1/97795#files-area [retrieved on 2017-06-28]	31-36
Y	* See page 3 (Abstract) and pages 133-135 (Conclusions) * -----	12
Y	BRANDUARDI ET AL: "A novel pathway to produce butanol and isobutanol in Saccharomyces cerevisiae", BIOTECHNOLOGY FOR BIOFUELS, vol. 6, 2013, pages 1-12, XP021151513, * See page 2 (Figure 1) * -----	14,15, 18,45
	-/--	

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2017/031807

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SHEPPARD ET AL: "Retro-biosynthetic screening of a modular pathway design achieves selective route for microbial synthesis of 4-methyl-pentanol", NATURE COMMUNICATIONS, vol. 5, 2014, pages 1-10, XP002771521, * See page 3 (Figure 1) *	14,21
A	KORNELI ET AL: "Getting the big beast to work - Systems biotechnology of Bacillus megaterium for novel high-value proteins", JOURNAL OF BIOTECHNOLOGY, vol. 163, 2013, pages 87-96, XP028975073, * See pages 87-88 (Introduction) *	1-53
X,P	FREEDMAN: "Surveying and harnessing the genetic, (meta)genomic, and metabolic potential of the deep carbonated biosphere", Massachusetts Institute of Technology (MIT) PhD thesis, June 2016 (2016-06), pages 1-190, XP002771522, Cambridge, Massachusetts, U.S.A. Retrieved from the Internet: URL:https://dspace.mit.edu/handle/1721.1/104482 [retrieved on 2017-06-28] * Very relevant; see i.a. the Figures and SR7 *	1-53
X,P	BOOCK ET AL: "Systems biology towards a continuous platform for biofuels production: Engineering an environmentally-isolated Bacillus strain for biofuel production and recovery under supercritical CO2", 2017 Genomic Sciences Program Annual PI meeting / Abstract book Abstract of a presentation, February 2017 (2017-02), pages 315-316, XP002771526, Retrieved from the Internet: URL:https://www.ornl.gov/gsp2017/2017-GSP-Abstract-Book.pdf [retrieved on 2017-06-28]	1,2, 5-11, 14-18, 21, 30-37, 40-45, 49,51
Y,P	* Note that further oral disclosures at the meeting might also count as prior art, if applicable (see SR7) *	1-53

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INTERNATIONAL SEARCH REPORT

International application No

PCT/US2017/031807

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	FREEDMAN ET AL: "Systems biology towards a continuous platform for biofuels production: Heterologous gene expression and isobutanol synthesis in <i>B. megaterium</i> SR7 and biofuel extraction under supercritical CO ₂ ", 2017 Genomic Sciences Program Annual PI meeting / Abstract book Abstract of a presentation, February 2017 (2017-02), pages 317-318, XP002771527, Retrieved from the Internet: URL:https://www.ornl.gov/gsp2017/2017-GSP-Abstract-Book.pdf [retrieved on 2017-06-28]	1,2, 5-11, 14-18, 21, 30-37, 40-45, 49,51
Y,P	* Note that further oral disclosures at the meeting might also count as prior art, if applicable (see SR7) *	1-53
Y,P	DISPIRITO ET AL: "Semi-continuous extraction of bio-alcohols using supercritical carbon dioxide", Worcester Polytechnic Institute Project report, 24 March 2017 (2017-03-24), pages 1-89, XP002771525, Worcester, Massachusetts, U.S.A. Retrieved from the Internet: URL:https://web.wpi.edu/Pubs/E-project/Available/E-project-032317-124330/unrestricted/Stolz_DiSpirito MQP_Report.pdf [retrieved on 2017-06-29] * See page 2 (Abstract) and sections 2.2, 2.3.1, 2.3.3, and 2.3.4 *	1-8,16, 17,22, 23,41
Y,P	VEETIL ET AL: "Can microbially derived advanced biofuels ever compete with conventional bioethanol ? A critical review", BIORESOURCES, vol. 11, 28 September 2016 (2016-09-28), pages 10711-10755, XP002771603, * See pages 10718-10719 (ketoisovalerate/ <i>L. lactis</i>) with references to normal prior art) *	18,19, 30,45,46

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2017/031807

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
L	CONLON ET AL: "Extraction of bio-butanol using supercritical carbon dioxide", Worcester Polytechnic Institute Project report, 2017, pages 1-67, XP002771528, Worcester, Massachusetts, U.S.A. Retrieved from the Internet: URL:https://web.wpi.edu/Pubs/E-project/Available/E-project-042816-093354/unrestricted/MQP_FINAL_REPORT.pdf [retrieved on 2017-06-28] * The date of publication is not indicated and has to be verified; see pages 59-62 * -----	