

MICROBIAL ESTER PRODUCTION

CROSS-REFERENCE TO RELATED APPLICATIONS

This application cites the priority of US 62/950,564, filed on 19 December 2019
5 (pending), which is incorporated herein by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

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invention.

In this context "government" refers to the government of the United States of
America.

15 BACKGROUND

FIELD OF THE DISCLOSURE

The present disclosure relates generally to industrial biotechnology.

BACKGROUND

Although tremendous efforts have been invested for biofuel and biochemical
20 research, it is still challenging to generate robust microbial strains that can produce target
products at desirable levels. Fatty acid esters, or mono-alkyl esters, can be used as valuable
fuels such as diesel components or specialty chemicals for food flavoring, cosmetic and
pharmaceutical industries. The US market demand for fatty acid esters could reach \$4.99
billion by 2025.

25 Conventionally, esters are produced through Fischer esterification which involves
high temperature and inorganic catalysts. The reaction consumes a large amount of energy
and generates tremendous wastes, and thus is not environmentally friendly. On the other
hand, ester production through biological routes is renewable and environmentally benign.
Using current techniques, bioproduction of most of the esters is low, and is not
30 economically competitive with environmentally damaging abiotic approaches.

There is therefore a need in the art for ester bioproduction approaches with higher
production levels.

SUMMARY

Strains of microorganism have been developed having vastly improved production of organic esters, by genetically modifying the microorganisms to introduce or enhance the activity of one or both of an alcohol acyltransferase and a lipase. This has resulted in some cases in 1-3 orders of magnitude increase in ester production, with specific examples being

5 *n*-butyl acetate and *n*-butyl butyrate.

A first general embodiment is a modified microorganism capable of *n*-butyl acetate (BA) production, the microorganism capable of expressing an alcohol acyl transferase (AAT); and comprising an *n*-butanol synthesis pathway.

A second general embodiment is a modified microorganism capable of *n*-butyl butyrate (BB) production, the microorganism capable of expressing an AAT; and comprising a butyryl coenzyme A synthesis pathway and a butanol synthesis pathway.

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A third general embodiment is a genetically modified *Clostridium saccharoperbutylacetonicum* having improved BA production and designated YM028P, having NCMA designation number 202012116, deposited on 16 December 2020 at the

15 National Center for Marine Algae and Microbiota at Bigelow Laboratory for Ocean Sciences, 60 Bigelow Drive, East Boothbay, ME 04544 USA.

A fourth general embodiment is a genetically modified *Clostridium saccharoperbutylacetonicum* having improved BB production and designated YM016PB, having NCMA designation number 202012115, deposited on 16 December 2020 at the

20 National Center for Marine Algae and Microbiota at Bigelow Laboratory for Ocean Sciences, 60 Bigelow Drive, East Boothbay, ME 04544 USA.

A fifth general embodiment is a genetically modified *Clostridium saccharoperbutylacetonicum* having improved BA production and designated FJ-1301, having NCMA designation number 202012114, deposited on 16 December 2020 at the

25 National Center for Marine Algae and Microbiota at Bigelow Laboratory for Ocean Sciences, 60 Bigelow Drive, East Boothbay, ME 04544 USA.

A sixth general embodiment is a method of ester production, comprising culturing a microorganism of the first through fifth general embodiments under conditions suitable to produce an ester.

30 A seventh general embodiment is an ester-containing composition comprising an ester compound that is the product of the method of the sixth embodiment.

The foregoing presents a simplified summary in order to provide a basic understanding of some aspects of the claimed subject matter. This summary is not an

extensive overview. It is not intended to identify key or critical elements or to delineate the scope of the claimed subject matter. Its sole purpose is to present some concepts in a simplified form as a prelude to the more detailed description that is presented later.

BRIEF DESCRIPTION OF THE DRAWINGS

5 **FIG. 1** Engineering of solventogenic clostridia for fatty acid ester production. Top: Five strains out of four representative clostridial species were selected and evaluated as the host to be engineered for ester production in this study. We hypothesized that the different metabolic fluxes within different strains would make a big difference for the desirable ester production. The metabolic pathways of the four different species were represented in four
10 different colors. Bottom: Fatty acid esters could be synthesized through two primary biological pathways: one is through the esterification of fatty acid and alcohol catalyzed by lipases, and the other is through the condensation of acyl-CoA and alcohol catalyzed by alcohol acyl transferases (AATs). Key genes in the pathway: *pta*, phosphotransacetylase; *ack*, acetate kinase; *thl*, thiolase; *hbd*, beta-hydroxybutyryl-CoA dehydrogenase; *crt*,
15 crotonase; *bcd*, butyryl-CoA dehydrogenase; *adh*, alcohol dehydrogenase; *adhE*, Aldehyde-alcohol dehydrogenase; *adc*, acetoacetate decarboxylase; *ctfAB*, CoA transferase; *pth*, phosphotransbutyrylase; *buk*, butyrate kinase; *ald*, aldehyde dehydrogenase.

FIG. 2 Screening of strains and enzymes for ester synthesis. (a) Schematic representation of devices and procedures for ester fermentation. (b) Heatmap results showed the ester
20 production in various *Clostridium* strains with the overexpression of different enzymes. Empty plasmid: overexpression of pMTL82151 (for *C. saccharoperbutylacetonicum* N1-4-C, *C. pasteurianum* SD-1, *C. tyrobutyricum* *cat1::adhE1* and *cat1::adhE2*) or pTJ1 (for *C. beijerinckii* 8052) as the control; EA: ethyl acetate; EB: ethyl butyrate; BA: butyl acetate; BB: butyl butyrate. Scale bar on the right is in g/L.

25 **FIG. 3** Enhancing BA production through increasing the availability of acetyl-CoA (a & b) and dynamically expressing the *atf1* gene (c & d). (a) Introduction of isopropanol synthesis pathway (shaded in purple) and deletion of thiolase genes. The pathways in purple arrows represent the 'regeneration' of actyl-CoA. There are five annotated genes encoding thiolase in *C. saccharoperbutylacetonicum*, only two (in red) of which could be deleted (see
30 Supplementary materials). (b) The fermentation results for BA production with various mutant strains corresponding to the genetic manipulations in (a). The reported value is mean $\pm$ SD. (c) Four promoters associated with the biosynthesis of acetyl-CoA or alcohols in the pathway were selected to drive the expression of *atf1*. (d) The fermentation results for

BA production with various mutant strains in which different promoters were used to drive the expression of *atf1* as illustrated in (c). The reported value is mean $\pm$ SD. BA: butyl acetate. Key genes in the pathway: *adh*, alcohol dehydrogenase; *bdh*, butanol dehydrogenase; *ctfAB*, CoA transferase; *sadh*, secondary alcohol dehydrogenase; *hydG*, putative electron transfer protein; *pfl*, pyruvate formate lyase; *ald*, aldehyde dehydrogenase.

FIG. 4. Enhancing BA production through rational organization of the enzymes associated with BA synthesis. (a) Biological components evaluated in this study for rational organization of enzymes: (I) CC-Di-A and CC-Di-B tags, (II) PduA* scaffold, (III) MinD C-tag. (b) Schematic representation of assembling two of the three enzymes for BA synthesis with the CC-Di-A and CC-Di-B tags. (c) Schematic representation of organizing the three enzymes for BA synthesis onto the PduA* formed scaffold. (d) Schematic representation of using the MinD C-tag to draw ATF1 onto the cell membrane. (e) The fermentation results for BA production with various mutant strains corresponding to the genetic manipulations from (b)-(d). The reported value is mean $\pm$ SD. BA: butyl acetate.

FIG. 5 The deletion of the prophage genomes. (a) The gene cluster organization of the HM T prophage (P1) and another four putative prophages (P2-P5). (b) Comparison of the cell growth of prophage deleted mutants and the control N1-4-C strain. (c) Comparison of the butanol production in prophage deleted mutants and the control N1-4-C strain. (d) Comparison of the cell growth between Δ P1234 and Δ P12345. (e) Comparison of butanol production between Δ P1234 and Δ P12345; (f) Transmission electron microscopy image of clostocin O; (g) Transmission electron microscopy image of the HM T prophage particles; (h, i) Cell growth profiles of Δ P1234 and Δ P12345 with the induction (at various OD₆₀₀) using mitomycin C at 2 or 4 μ g/ml. “-” indicates that there was no cell lysis; “+” indicates that most of the cells were lysed. The value at the right side of the cell growth profile figure represents the actual OD₆₀₀ value at which mitomycin C (with the applied concentration included in the parentheses) was added for the induction.

FIG. 6 Fermentation results of the engineered strains for fatty acid ester production. (a) BA production in serum bottles using glucose (FJ-1201 and FJ-1301) or biomass hydrolysates (FJ-1201 (H)) as the substrate. (b) BA fermentation kinetics in FJ-1201 in 500-mL bioreactor using glucose as the substrate. (c) BA fermentation kinetics in FJ-1201 in 500-mL bioreactor using biomass hydrolysates as the substrate. (d) BB production in serum bottles using glucose (FJ-1202, FJ-1203, and FJ-1204) or biomass hydrolysates (FJ-1202

(H)) as the substrate. (e) BB fermentation kinetics in FJ-1202 in 500-mL bioreactor using glucose as the substrate. (f) BB fermentation kinetics in FJ-1202 in 500-mL bioreactor using biomass hydrolysates as the substrate. BA: butyl acetate; BB: butyl butyrate; Eth: ethanol; But: butanol.

- 5 **FIG. 7.** Techno-economic analysis of butyl acetate production from corn stover. a) process overview; b) total installed equipment cost; c) chemical production from each metric tonne (MT) of corn stover; d) butyl acetate production cost.

- FIG. 8** Fermentation results in serum bottles with *Clostridium saccharoperbutylacetonicum* N1-4-C and the mutant strains. (a-h): ABE fermentation results for FJ-100 as compared to
10 the control N1-4-C. The reported value is mean $\pm$ SD.

- FIG. 9.** Fermentation results in serum bottles with *Clostridium saccharoperbutylacetonicum* N1-4-C and the mutant strains with prophage deleted. (a-h): ABE fermentation results for Δ P1, Δ P2, Δ P3, Δ P4, Δ P1234 as compared to the control N1-4-C. (i-p): ABE fermentation results for Δ NP1, Δ NP2, Δ NP3, Δ NP4, Δ NP1234 as compared to the control N1-4-C. The
15 reported value is mean $\pm$ SD.

FIG. 10. Cell lysis in various prophage deletion mutants upon induction with different concentrations of mitomycin C. "I" indicates growth inhibition; "-" indicates that there was no cell lysis; "+" indicates that most of the cells were lysed; "++" indicates that all the cells were lysed.

- 20 **FIG. 11.** TEM picture of clostocin O. The magnification of (a) was 100x, while the magnification of (b) was 200x.

FIG. 12. Cell lysis in various prophage deletion mutants upon induction with different concentrations of mitomycin C. "-" indicates that there was no cell lysis; "+" indicates that most of the cells were lysed.

- 25 **FIG. 13.** HM T phage observed in the Δ P2345 strain upon induction. The magnification of (a, b) was 100x, while the magnification of (c, d) was 200x.

FIG. 14. ABE Fermentation results in serum bottles with *Clostridium saccharoperbutylacetonicum* Δ P1234 and Δ P12345. The reported value is mean $\pm$ SD.

- FIG. 15.** ABE fermentation results in 500-mL bioreactors with *Clostridium saccharoperbutylacetonicum* Δ P1234 and Δ P12345. The reported value is mean $\pm$ SD.
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FIG. 16. Sensitivity of butyl acetate production cost to different parameters. The numbers in brackets in Y-axis are the potential low, base and high values of each parameter.

DETAILED DESCRIPTION

DEFINITIONS

Unless otherwise defined, all terms (including technical and scientific terms) used herein have the same meaning as commonly understood by one of ordinary skill in the art of this disclosure. It will be further understood that terms, such as those defined in
5 commonly used dictionaries, should be interpreted as having a meaning that is consistent with their meaning in the context of the specification and should not be interpreted in an idealized or overly formal sense unless expressly so defined herein. Well known functions or constructions may not be described in detail for brevity or clarity.

With reference to the use of the word(s) “comprise” or “comprises” or “comprising”
10 in the foregoing description and/or in the following claims, unless the context requires otherwise, those words are used on the basis and clear understanding that they are to be interpreted inclusively, rather than exclusively, and that each of those words is to be so interpreted in construing the foregoing description and/or the following claims.

The term “consisting essentially of” means that, in addition to the recited elements,
15 what is claimed may also contain other elements (steps, structures, ingredients, components, etc.) that do not adversely affect the operability of what is claimed for its intended purpose. Such addition of other elements that do not adversely affect the operability of what is claimed for its intended purpose would not constitute a material change in the basic and novel characteristics of what is claimed.

20 Terms such as “at least one of A and B” should be understood to mean “only A, only B, or both A and B.” The same construction should be applied to longer list (e.g., “at least one of A, B, and C”).

The terms “about” and “approximately” shall generally mean an acceptable degree of error or variation for the quantity measured given the nature or precision of the
25 measurements. Typical, exemplary degrees of error or variation are within 20 percent (%), preferably within 10%, and more preferably within 5% of a given value or range of values. For biological systems, the term “about” refers to an acceptable standard deviation of error, preferably not more than 2-fold of a given value. Numerical quantities given herein are approximate unless stated otherwise, meaning that the term “about” or “approximately”
30 can be inferred when not expressly stated.

The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting. As used herein, the singular forms “a”,

“an” and “the” are intended to include the plural forms as well, unless the context clearly indicates otherwise.

The terms “first”, “second”, and the like are used herein to describe various features or elements, but these features or elements should not be limited by these terms. These terms are only used to distinguish one feature or element from another feature or element. Thus, a first feature or element discussed below could be termed a second feature or element, and similarly, a second feature or element discussed below could be termed a first feature or element without departing from the teachings of the present disclosure.

The term “nucleotide” as used herein refer to any such known groups, natural or synthetic. It includes conventional DNA or RNA bases (A, G, C, T, U), base analogs (e.g., inosine, 5-nitroindazole and others), imidazole-4-carboxamide, pyrimidine or purine derivatives (e.g., modified pyrimidine base 6H,8H-3,4-dihydropyrimido[4,5-c][1,2]oxazin-7-one (sometimes designated “P” base that binds A or G)) and modified purine base N6-methoxy-2,6-diaminopurine (sometimes designated “K” base that binds C or T), hypoxanthine, N-4-methyl deoxyguanosine, 4-ethyl-2'-deoxycytidine, 4,6-difluorobenzimidazole and 2,4-difluorobenzene nucleoside analogues, pyrene-functionalized LNA nucleoside analogues, deaza- or aza-modified purines and pyrimidines, pyrimidines with substituents at the 5 or 6 position and purines with substituents at the 2, 6 or 8 positions, 2-aminoadenine (nA), 2-thiouracil (sU), 2-amino-6-methylaminopurine, O-6-methylguanine, 4-thio-pyrimidines, 4-amino-pyrimidines, 4-dimethylhydrazine-pyrimidines, O-4-alkyl-pyrimidines and hydrophobic nucleobases that form duplex DNA without hydrogen bonding. Nucleobases can be joined together by a variety of linkages or conformations, including phosphodiester, phosphorothioate or methylphosphonate linkages, peptide-nucleic acid linkages.

The term “polynucleotide” as used herein refers to a multimeric compound comprising nucleotides linked together to form a polymer, including conventional RNA, DNA, LNA, BNA, copolymers of any of the foregoing, and analogs thereof.

The term “nucleic acid” as used herein refers to a single stranded polynucleotide or a duplex of two polynucleotides. Such duplexes need not be annealed at all locations, and may contain gaps or overhangs.

The term “genetically modified” means that genetic material has been altered by human intervention. In the context of a self-replicating entity such as a cell or a virus, such

alteration may have been performed on the self-replicating entity in question, or on an ancestor of the self-replicating entity from whom it acquired the alteration.

GENETICALLY MODIFIED MICROORGANISM

Strains of microorganism have been developed having vastly improved production of organic esters, by genetically modifying the microorganisms to introduce or enhance the activity of one or both of an alcohol acyl transferase and a lipase. This has resulted in some embodiments of the microorganism in 1-3 orders of magnitude increase in ester production, with specific examples being butyl acetate and butyl butyrate. AATs catalyze the addition of an acyl moiety from an acyl-coenzyme A (acyl-CoA) donor onto an alcohol acceptor. The efficiencies of various AATs vary widely depending on the alcohol and the acyl-CoA involved in the reaction. The work described herein discovered that some heterologous AATs in microorganisms catalyze the formation of butyl esters at extremely high efficiency, and that even higher efficiencies can be realized by further genetic modifications.

A first general embodiment of the microorganisms is capable of highly efficient BA production. This microorganism is capable of expressing the AAT, and has a functioning butanol synthesis pathway. Without wishing to be bound to a hypothetical model, it is believed that the AAT catalyzes transacetylation from acetyl-CoA to butanol to form the ester. The microorganism may also be characterized as having an acetyl-CoA synthesis pathway.

Although in theory any AAT could function to produce BA in this context, in preferred embodiments the AAT is one or more of: Vaat, Saat, Atf1, Eht1, and a functional homolog of any of the foregoing. The strawberry AAT Vaat (also found in banana) was elucidated by Beekwilder et al. (*Plant Physiol.* Vol. 135, 2004), having a reported amino acid sequence of SEQ ID NO: 1 (see also GenBank Accession No. AX025504.1). The strawberry AAT Saat was elucidated by Aharoni et al. (*The Plant Cell*, Vol. 12, 647-661, 2000 – incorporated herein by reference to teach the amino acid sequence), having a reported amino acid sequence of SEQ ID NO: 2 as reported in GenBank Accession No. AAG13130.1. The *Saccharomyces cerevisiae* AAT Atf1 was elucidated by Fuji et al. (*Appl. Environ. Microbiol.*, Vol. 60, 2786-2792, 1994 – incorporated herein by reference to teach the amino acid sequence), having a reported amino acid sequence of SEQ ID NO: 3 as reported in NCBI Reference Sequence NP_015022.3. Yeast ethanol hexanoyl transferase (Eht1) was elucidated by Saerens et al., (*J. Boil. Chem.*, Vol. 281, 2006– incorporated herein by

reference to teach the amino acid sequence), having a reported amino acid sequence of SEQ ID NO: 4 as reported in UniProt Accession No. P40353.

Single or multiple copies of the AAT genes may be included. Various embodiments of the microorganism comprise a nucleic acid encoding one or more AATs, which may include any of the AATs discussed above or their functional variants. A preferred embodiment of the microorganism comprises multiple nucleic acid sequences each encoding Atf1 or a functional homolog of Atf1. Another preferred embodiment of the microorganism comprises multiple genomic nucleic acid sequences each encoding Atf1 or a functional homolog of Atf1.

A second general embodiment is a microorganism capable of butyl butyrate (BB) production, the microorganism capable of expressing an AAT and comprising a butanol synthesis pathway and a butyryl coenzyme A synthesis pathway. It has been found that AATs can catalyze the condensation of butyryl coenzyme A and butanol to produce BB at high efficiency. Butyryl-coenzyme A is a four-carbon fatty acid that is the coenzyme A-activated form of butyric acid. The pathway may be endogenous or exogenous to the microorganism. One pathway was elucidated by Bennett and Rudolph (*FEMS Microbiol. Rev.* 17, 241-249, 1995) as including β -hydroxybutyryl-CoA dehydrogenase, crotonase, butyryl-CoA dehydrogenase and the α and β subunits of an electron transfer flavoprotein. In a preferred embodiment of the microorganism the AAT is Eht1 or a functional homolog of Eht1. In a further preferred embodiment of the microorganism the AAT is Saat or a functional homolog of Saat.

The microorganism may be constructed to contain a nucleic acid that encodes the AAT. Such nucleic acid may be genomic (part of the organism's genome, such as a chromosome of a bacterium) or extra-genomic (such as part of an episome, for example a bacterial plasmid). The nucleic acid encoding the AAT may be a "canonical" sequence encoding the AAT, or it may be a degenerate variant of a canonical sequence. Degenerate variants can be constructed based on an understanding of the standard three nucleotide code and its corresponding amino acids. This correspondence is understood both for RNA and for DNA. Some organisms' protein synthesis machinery employs atypical codons for certain amino acids, and this can be taken into account when designing nucleic acids that encode the AAT or its functional homologs.

Various embodiments of the microorganism may be eukaryotic or prokaryotic, such as bacteria, archaea, fungi, and protists. A preferred embodiment of the microorganism is a

prokaryote. Prokaryotes have the advantage of rapid growth, easy cultivation, and simple genetics. The prokaryote may be a bacterium or an archaean. Bacteria have the advantage of being well understood model organisms with a wide diversity of metabolic niches. Archaea have the advantage of some specialized metabolic pathways and the ability in many cases to withstand extreme industrial conditions. An embodiment of the microorganism is a fermentative bacterium. A specific preferred embodiment of the microorganism is a bacterium of genus *Clostridium*, a well characterized group of fermentative anaerobic bacteria, capable of fermentation of a wide variety of substrates. In such embodiments the bacterium may be one of *Clostridium saccharoperbutylacetonicum*, *Clostridium beijerinckii*, *Clostridium pasteurianum*, and *Clostridium tyrobutyricum*. In a particularly preferred embodiment, the bacterium is *Clostridium saccharoperbutylacetonicum*.

Lipase B (CALB) from *Candida antarctica* (*Pseudozyma Antarctica*) catalyzes the formation of esters from fatty acids (including CoA fatty acids) and alcohols, and is commercially available for biochemical applications. Lipase B increases ester production in some embodiments of the microorganism. It has a reported amino acid sequence of SEQ ID NO: 5 as Uniprot Accession No. P41365. Some embodiments of the microorganism are capable of expressing Lipase B or a functional homolog of Lipase B. Such embodiments of the microorganism may be capable of such expression owing to the presence of a nucleic acid encoding Lipase B or a functional homolog of Lipase B. The nucleic acid may take various forms, including RNA (for example, as introduced mRNA) or DNA (for example, genomic DNA, episome DNA, or plasmid DNA). Some embodiments of the microorganism capable of expressing Lipase B have one or both of an acetic acid synthesis pathway and a butyric acid synthesis pathway. Butyric acid can form butyl esters with alcohols, and acetic acid can form acetyl esters with alcohols. Examples of suitable fermentatively produced alcohols include ethanol and butanol.

Enhancement of ester production can be achieved by reducing or eliminating the activity of the NADH-quinone oxidoreductase subunit G, which is a subunit of the electron transport chain complex I. The *nuoG* gene encodes the NADH-quinone oxidoreductase subunit G. Without wishing to be bound by any hypothetical model, it is believed that the availability of NADH is increased by reducing or eliminating the activity of the NADH-quinone oxidoreductase subunit G, leading to improved ester production. The *nuoG* in *C. saccharoperbutylacetonicum* is at locus tag Cspa_c47560. Some embodiments of the

microorganism have partial or complete deletions of *nuoG*. The partial deletion may be sufficient to either completely inhibit expression, reduce the activity of the resulting polypeptide, or eliminate the activity of the resulting polypeptide. In a preferred embodiment the *nuoG* gene is deleted.

5 Ester production can also be increased by the expression of secondary alcohol dehydrogenase alone or in combination with the expression of the putative electron transfer protein *hydG*. One such suitable secondary alcohol dehydrogenase, that can convert acetone into isopropanol, is the one encoded by the *sadh* gene in *C. beijerinckii* B593 (Uniprot no. A0A1S8R6K8). The *hydG* gene in the same operon as *sadh* encodes a
 10 putative electron transfer protein. Some embodiments of the microorganism are capable of expressing one or both of *hydG* and *sadh* (or functional homologs thereof). Further embodiments of the microorganism comprise a *sadh-hydG* gene cluster. One or more of the foregoing may be heterologous genes. One example of a suitable *sadh-hydG* gene cluster is of clostridial origin, such as from *C. beijerinckii* B593.

15 The AAT may be expressed using various promoters and other regulatory elements. In this context the AAT is "operatively linked" to a promoter if the promoter controls the expression of the AAT. The promoter may be adjacent to the AAT gene, or it may be remote. Some embodiments of the microorganism comprise a constitutive promoter operatively linked to the AAT. *P_{adh}* is a promoter in *Clostridium*
 20 *saccharoperbutylacetonicum*, which controls expression of *ahd*, NADPH-dependent butanol dehydrogenase at locus tag Cspa_c04380. *P_{ald}* is a promoter in *Clostridium saccharoperbutylacetonicum*, which can sense the acidic state and switch cell metabolism from acidogenesis to solventogenesis. Operatively linking *P_{adh}* or *P_{ald}* to the AAT can increase ester production in some embodiments of the microorganism.. Some
 25 embodiments of the promoter are native to the microorganism

Additional advantages can be realized by localizing the AAT at the cell membrane. Without wishing to be bound by any hypothetical model, it is believed that the cell can be protected from toxic properties of the ester if the AAT is localized at the membrane. One such approach is to fuse the AAT with a membrane-associated molecule, such as MinD.
 30 MinD is a membrane-associated protein and the localization of MinD is mediated by an 8-12 residue C-terminal membrane-targeting sequence. It is an ubiquitous ATPase that plays a crucial role in selection of the division site in eubacteria and chloroplasts. The proteins with MinD C-terminal sequence are believed to be drawn to the cell membrane. Thus, the

application of MinD C-tag can facilitate the secretion of target product and enhance its production by mitigating the intracellular toxicity as well as promoting the catalyzing process (FIG. 4a). In some embodiments of the microorganism the AAT is fused to a C-terminal membrane-targeting sequence of MinD. The AAT may be fused at any position on the AAT; in a preferred embodiment the AAT is fused at its C-terminal end to the C-terminal membrane-targeting sequence of MinD. The C-terminal membrane-targeting sequence of MinD may be part of a larger molecule, such as a whole MinD molecule. Some such embodiments of the microorganism may contain a nucleic acid encoding the AAT concatenated to a nucleic acid encoding MinD. An example of a suitable MinD tag is *Bacillus subtilis* MinD, having a canonical DNA sequence of SEQ ID NO: 6.

“Functional homologs” of a polypeptide will have some degree of identity with the wild type polypeptide. For example, it would be expected that most functional homologs having from 95-100% identity with the native polypeptide would retain at least some function. The likelihood that functionality would be retained by a homolog to the polypeptide with at least any of the following levels of sequence identity could be predicted: 70, 80, 90, 95, 99, and 99.5%. It is understood that the minimum desirable identity can be determined in some cases by identifying a known non-functional homolog to the polypeptide, and establishing that the minimum desirable identity must be above the identity between the polypeptide and the known non-functional identity.

Persons having ordinary skill in the art will also understand that the minimum desirable identity can be determined in some cases by identifying a known functional homolog to the polypeptide, and establishing that the range of desirable identity must encompass the percent identity between the polypeptide and the known non-functional level of identity.

Deletions, additions and substitutions can be selected to generate a desired polypeptide functional homolog. For example, it is not expected that deletions, additions and substitutions in a non-functional region of a polypeptide would alter the polypeptide activity. Likewise conservative substitutions or substitutions of amino acids with similar properties is expected to be tolerated in a conserved region. Of course non-conservative substitutions in these regions would be expected to decrease or eliminate the polypeptide activity.

For example, a “conservative amino acid substitution” may involve a substitution of a native amino acid residue with a nonnative residue such that there is little or no

effect on the polarity or charge of the amino acid residue at that position. Furthermore, any native residue in the polypeptide may also be substituted with alanine. Conservative amino acid substitutions also encompass non-naturally occurring amino acid residues which are typically incorporated by chemical peptide synthesis rather than by synthesis in biological systems. These include peptidomimetics, and other reversed or inverted forms of amino acid moieties. It will be appreciated by those of skill in the art that nucleic acid and polypeptide molecules described herein may be chemically synthesized as well as produced by recombinant means.

Naturally occurring residues may be divided into classes based on common side chain properties: 1) hydrophobic: norleucine, Met, Ala, Val, Leu, Ile; 2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln; 3) acidic: Asp, Glu; 4) basic: His, Lys, Arg; 5) residues that influence chain orientation: Gly, Pro; and 6) aromatic: Trp, Tyr, Phe.

For example, conservative substitutions may involve the exchange of a member of one of these classes for a member of the same class.

In making such changes, the hydropathic index of amino acids may be considered. Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics, these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5). The importance of the hydropathic amino acid index in conferring interactive biological function on a protein is understood in the art (Kyte et al., *J. Mol. Biol.*, 157:105-131, 1982). It is known that certain amino acids may be substituted for other amino acids having a similar hydropathic index or score and still retain a similar biological activity. In making conservative substitutions based upon the hydropathic index, the substitution of amino acids whose hydropathic indices are within +/- 2 may be used; in an alternate embodiment, the hydropathic indices are with +/- 1; in yet another alternate embodiment, the hydropathic indices are within +/- 0.5.

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. The greatest local average hydrophilicity of a polypeptide as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein. The following hydrophilicity values

have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0. $\pm$.1); glutamate (+3.0. $\pm$.1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5. $\pm$.1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4). In making changes based upon similar hydrophilicity values, the substitution of amino acids whose hydrophilicity values are within $\pm$ 2 may be used; in an alternate embodiment, the hydrophilicity values are with $\pm$ 1; in yet another alternate embodiment, the hydrophilicity values are within $\pm$ 0.5.

Desired amino acid substitutions (whether conservative or non-conservative) can be determined by those skilled in the art at the time such substitutions are desired. For example, amino acid substitutions can be used to identify important residues of the polypeptide, or to increase or decrease the affinity of the polypeptide with a particular binding target in order to increase or decrease the polypeptide activity.

Exemplary amino acid substitutions are set forth in Table 1.

Table 1		
Conservative Amino Acid Substitutions		
Original Amino Acid	Exemplary substitution	Preferred substitution
Ala	Val, Leu, Ile	Val
Arg	Lys, Gln, Asn	Lys
Asn	Glu	Glu
Asp	Glu	Glu
Cys	Ser, Ala	Ser
Gln	Asn	Asn
Glu	Asp	Asp
Gly	Pro, Ala	Ala
His	Asn, Gln, Lys, Arg	Arg
Ile	Leu, Val, Met, Ala, Phe, Norleucine	Leu
Leu	Ile, Val, Met, Ala, Phe, Norleucine	Ile
Lys	Arg, 1,4-diaminobutyric acid, Gln, Asn	Arg
Met	Leu, Phe, Ile	Leu
Phe	Leu, Val, Ile, Ala, Tyr	Leu
Pro	Ala, Gly	Gly
Ser	Thr, Ala, Cys	Thr
Thr	Ser	Ser
Trp	Tyr, Phe	Tyr
Tyr	Trp, Phe, Thr, Ser	Phe
Val	Ile, Met, Leu, Phe, Ala, Norleucine	Leu

For identifying suitable areas of the molecule that may be changed without destroying activity, one skilled in the art may target areas not believed to be important for activity. For example, when similar polypeptides with similar activities from the same species or from other species are known, one skilled in the art may compare the amino acid sequence of a given polypeptide to such similar polypeptides. With such a comparison, one can identify residues and portions of the molecules that are conserved among similar polypeptides. It will be appreciated that changes in areas of the polypeptide that are not conserved relative to such similar polypeptides would be less likely to adversely affect the biological activity and/or structure of the polypeptide. One skilled in the art would also know that, even in relatively conserved regions, one may substitute chemically similar amino acids for the naturally occurring residues while retaining activity (conservative amino acid residue substitutions). Therefore, even areas that may be important for biological activity or for structure may be subject to conservative amino acid substitutions without destroying the biological activity or without adversely affecting the polypeptide structure.

Further advantages can be realized by curing the microorganism of viruses in its genome. In this context "cure" means to introduce a mutation that prevents the virus from replicating or otherwise expressing viral genes. An example of curing is entirely deleting the viral genome from the microorganism. Another example is partially deleting the viral genome from the microorganism, to such an extent that viral replication is not possible. Another example is mutating a bacteriophage integrase gene, preventing functioning phage integrase from being expressed. The mutation may be an entire deletion, a partial deletion, a substitution, or an insertion so long as functioning bacteriophage integrase is not expressed. Some embodiments of the microorganism are a bacterium that has been cured of one, some, or all prophages. A preferred embodiment of the bacterium is a *Clostridium* spp. that has been cured of all native prophages. In a further preferred embodiment, the microorganism is a bacterium of genus *Clostridium* which has been cured of one or more of prophages P1, P2, P3, P4, and P5 (described further below in the examples).

The *rex* gene was identified as a redox-sensing transcriptional repressor, which modulates transcription in response to changes in cellular NADH/NAD⁺ redox state, and represses the transcription of genes related to butanol synthesis. It was originally identified by Wietzke and Bahl (*Appl. Microbiol. Biotechnol.* 96(3): 749-61, 2012) (*C. saccharoperbutylacetonicum*: Cspa_c04320). Some embodiments of the microorganism do

not express a functional redox-sensing transcriptional repressor. This lack of expression may be native to the organism, or induced through genetic modification. Such genetic modification may take any suitable form, such as a complete deletion, a partial deletion, an insertion, or a substitution.

5 Further advantages can be realized in embodiments of the microorganism capable of expressing soluble pyridine nucleotide transhydrogenase (SthA), or a functional homolog thereof, for converting NADPH to NADH. One example of a suitable SthA is from *E. coli* BL21 (SEQ ID NO: 7).

Further advantages can be realized in embodiments of the microorganism that do
 10 not express a functional *cftA1-ctfB1* gene cluster (encoding acetoacetyl-CoA:acetate/butyrate:CoA transferase). Acetoacetyl-CoA:acetate/butyrate:CoA transferase transfers CoA group from acetoacetyl-CoA to either acetate or butyrate, generating acetyl-CoA or butyryl-CoA respectively. The *ctfA1-ctfB1* gene cluster catalyzes the following two reactions: acetoacetyl-CoA + acetate → acetoacetate + acetyl-CoA, acetoacetyl-CoA +
 15 butyrate → acetoacetate + butyryl-CoA. The *ctfA1* (*atoD1*): Gene ID in *C. saccharoperbutylacetonicum* is Cspa_c21000 csr:Cspa_c21000 K01034 acetate CoA/acetoacetate CoA-transferase alpha subunit [EC:2.8.3.8 2.8.3.9] | (GenBank) *atoD1*; acetate CoA-transferase subunit alpha (N); and *ctfB1*: Gene ID in *C. saccharoperbutylacetonicum* is Cspa_c21010 csr:Cspa_c21010 K01035 acetate
 20 CoA/acetoacetate CoA-transferase beta subunit [EC:2.8.3.8 2.8.3.9] | (GenBank) *ctfB1*; butyrate--acetoacetate CoA-transferase subunit B (N). This lack of expression may be native to the organism, or induced through genetic modification. Such genetic modification may take any suitable form, such as a complete deletion, a partial deletion, an insertion, or a substitution.

25 A specific embodiment of the microorganism is a genetically modified *Clostridium saccharoperbutylacetonicum* having improved BA production and designated YM028P, having NCMA designation number 202012116, deposited on 16 December 2020 at the National Center for Marine Algae and Microbiota at Bigelow Laboratory for Ocean Sciences, 60 Bigelow Drive, East Boothbay, ME 04544 USA.

30 A specific embodiment of the microorganism is a genetically modified *Clostridium saccharoperbutylacetonicum* having improved BB production and designated YM016PB, having NCMA designation number 202012115, deposited on 16 December 2020 at the

National Center for Marine Algae and Microbiota at Bigelow Laboratory for Ocean Sciences, 60 Bigelow Drive, East Boothbay, ME 04544 USA.

A further specific embodiment of the microorganism is a genetically modified *Clostridium saccharoperbutylacetonicum* having improved BA production and designated FJ-1301, having NCMA designation number 202012114, deposited on 16 December 2020 at the National Center for Marine Algae and Microbiota at Bigelow Laboratory for Ocean Sciences, 60 Bigelow Drive, East Boothbay, ME 04544 USA.

METHOD OF ESTER PRODUCTION

A method of ester production is disclosed, comprising culturing any one of the microorganisms above under conditions suitable to produce an ester. In a preferred embodiment of the method the ester produced is one or both of BA and BB. It has been observed that organisms that have been modified as described above can generate extremely large amount of BA and BB at unprecedented levels of efficiency.

Culturing may occur in the presence of a suitable carbon source and a suitable energy source, depending on the organism. Some embodiments of the culture medium contain glucose, which is widely used by heterotrophic organisms as both a source of carbon and energy. The glucose can be part of a defined medium or part of a complex medium. Numerous such glucose media are known in the art, some of which are cataloged in R. Atlas, *Handbook of Microbiological Media, Fourth Edition*, CRC Press (2010). In a preferred embodiment the medium comprises a biomass hydrolysate. Defined media have the advantage of being of controlled composition, whereas a complex medium such as biomass hydrolysate are less expensive to produce and require no supplementation. One suitable example of hydrolysate is a corn stover hydrolysate, such as one prepared according to the method of D. Humbird et al., "Process design and economics for biochemical conversion of lignocellulosic biomass to ethanol:dilute-acid pretreatment and enzymatic hydrolysis of corn stover" (No. NREL/TP-5100-47764) National Renewable Energy Lab.(NREL), Golden, CO (United States) (2011) – which is incorporated by reference to the extent necessary to describe and enable such hydrolysates.

Culturing should be conducted at a temperature conducive to microbial metabolism, which will vary depending on the microorganism involved. Often mesophilic organisms are used in ester fermentations, and in such cases culturing occurs at mesophilic temperatures. Higher temperatures can accelerate metabolic processes, and it is contemplated that

thermophilic organisms could be used as well, in which case culturing would preferably be at thermophilic temperatures.

Ester production is generally a fermentative process. To encourage fermentation the culturing may be conducted under low oxygen conditions. Conditions may be hypoxic or anoxic, as necessary to create anaerobic conditions conducive to fermentation. It is possible that the microorganism could be engineered to ferment under aerobic conditions as well.

Such fermentation processes have been observed to generate extremely high concentrations of BA in culture, in excess of 25 g/L. Some embodiments of the culture method produce at least 1.5 g BA/L of culture. Further embodiments have been observed to produce at least 5, 7.5, 10, 12.5, 13, 14, 15, 16, 17, 18, 19, 20, or 25 g BA/L of culture. Higher concentrations of BB production have been observed than previous known methods. Some embodiments of the culture method produce at least 0.1 g BB/L of culture. Further embodiments of the culture method produce at least 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, or 1.6 g BB/L of culture.

WORKING EXAMPLE: Renewable Fatty Acid Ester Production in *Clostridium*

An earlier version of this work was published on bioRxiv on 20 August 2020 (available at <https://www.biorxiv.org/content/10.1101/2020.03.29.014746v1.full>), and is incorporated herein by reference in its entirety. The terms “we” and “our” used below refer to authors of the manuscript and persons who contributed to the work, some of whom might not be considered “inventors” of what is claimed under the laws of certain countries; the use of such terms is neither a representation nor an admission that any person is legally an inventor of what is claimed.

Abstract

Production of renewable chemicals through biological routes is considered as an urgent solution for fossil energy crisis. However, end product toxicity inhibits microbial performance and is a key bottleneck for biochemical production. To address this challenge, here we report an example of biosynthesis of high-value and easy-recoverable derivatives to alleviate endproduct toxicity and enhance bioproduction efficiency. By leveraging the natural pathways in solventogenic clostridia for co-producing acyl-CoAs, acids and alcohols as precursors, through rational screening for host strains and enzymes, systematic metabolic engineering—including rational organization of ester-synthesizing enzymes inside of the cell, and elimination of putative prophages, we developed strains that can

produce 25.3 g/L butyl acetate and 1.6 g/L butyl butyrate respectively, which were both the unprecedented levels in microbial hosts. Techno-economic analysis indicated a production cost of \$986 per metric tonne for butyl acetate production from corn stover compared to the market price of \$1,200-1,400 per metric tonne of butyl acetate, suggesting the economic competitiveness of the bioprocess.

Main

Although tremendous efforts have been invested for biofuel and biochemical research, it is still challenging to generate robust microbial strains that can produce target products at desirable levels¹. One key bottleneck is the intrinsic toxicity of end products to host cells². Therefore, the production of high-value bioproducts which can be easily recovered from fermentation might be a solution to tackle the bottleneck in bioproduction. Fatty acid esters, or mono-alkyl esters, can be used as valuable fuels such as diesel components or specialty chemicals for food flavoring, cosmetic and pharmaceutical industries³. It is projected that the US market demand for fatty acid esters could reach \$4.99 billion by 2025⁴. In addition, esters, with fatty acid and alcohol moieties, are generally hydrophobic and can easily separate from fermentation; thus the production of ester can help mitigate end product toxicity to host cells and efficient bioproduction can be achieved.

Esters are produced through Fischer esterification which involves high temperature and inorganic catalysts^{5,6}. The reaction consumes a large amount of energy and generates tremendous wastes, and thus is not environmentally friendly⁵. On the other hand, ester production through biological routes is becoming more and more attractive because it is renewable and environmentally benign. There are at least two known biological pathways for ester production: one is through esterification of fatty acid and alcohol catalyzed by lipases⁷, and the other is based on condensation of acyl-CoA and alcohol catalyzed by alcohol acyl transferases (AATs)⁵. Previously, lipases from bacteria or fungi have been employed for catalyzing esterification⁸.

In this study, we report highly efficient fatty acid ester production to unprecedented levels using engineered clostridia. We selected solventogenic clostridia to take advantage of their natural pathways for co-producing acyl-CoAs (acetyl-CoA and butyryl-CoA), fatty acids (acetate and butyrate), and alcohols (ethanol and butanol), either as intermediates or end products; we hypothesized that clostridia can be excellent microbial platforms to be engineered for efficient ester production by introducing heterologous AATs and/or lipase

genes. Indeed, through screening for host strains (from four well-known clostridial species) and enzymes (alcohol acyl transferases and lipase), systematic metabolic engineering—including organization of ester-synthesizing enzymes inside of the cell, and elimination of putative prophages, we ultimately obtained two strains which can produce
 5 25.3 g/L butyl acetate (BA) and 1.6 g/L BB respectively in extractive batch fermentations. These production levels were both highest in record to the best of our knowledge.

Results

Screening of host strains and genes for ester production

We considered clostridia as model platforms for ester production thanks to their
 10 intrinsic intermediates (fatty acids, acyl-CoAs, and alcohols) serving as precursors for ester biosynthesis (FIG. 1). We hypothesized that different flux levels of these precursors within various clostridial strains would make a big difference for the specific type(s) of ester production. Therefore, we selected five strains (from four representative species) including *C. tyrobutyricum* $\Delta cat1::adhE1$ ¹¹, *C. tyrobutyricum* $\Delta cat1::adhE2$ ¹¹, *C. pasteurianum* SD-1¹², *C.*
 15 *saccharoperbutylacetonicum* N1-4-C¹³ and *C. beijerinckii* NCIMB 8052¹⁴ to evaluate their capabilities for ester production through metabolic engineering (Table S1). We included both *C. tyrobutyricum* $\Delta cat1::adhE1$ and $\Delta cat1::adhE2$ here because they produce different levels (and thus ratios) of butanol and ethanol^{11,15}. Esters can be synthesized either through esterification of acid and alcohol catalyzed by lipase, or through condensation of
 20 acyl-CoA and alcohol catalyzed by AATs (FIG. 1). Previously, lipase B (CALB) from *Candida antarctica* has been employed for efficient ester production through esterification^{9,16}. In addition, four AATs including VAAT^{17,18}, SAAT¹⁷, ATF1^{3,10}, _ENREF_29 EHT1¹⁹ have been recruited for ester production in various hosts^{5,20-22}. Therefore, here, we evaluated all these genes in our clostridial hosts for ester production.

25 Six plasmids (pMTL-P_{cat}-*vaat*, pMTL-P_{cat}-*saat*, pMTL-P_{cat}-*atf1*, pMTL-P_{cat}-*eht1*, pMTL-P_{cat}-*lipaseB* as well as pMTL82151 as the control) were individually transformed into *C. saccharoperbutylacetonicum* N1-4-C, *C. pasteurianum* SD-1, *C. tyrobutyricum* *cat1::adhE1* and *cat1::adhE2* respectively. While pTJ1-P_{cat}-*vaat*, pTJ1-P_{cat}-*saat*, pTJ1-P_{cat}-*atf1*, pTJ1-P_{cat}-*eht1* and pTJ1-P_{cat}-*lipaseB* as well as pTJ1 were transformed into *C.*
 30 *beijerinckii* 8052. Fermentations were performed (FIG. 2a), and results were shown in FIG. 2b. Four types of esters were detected: EA, BA, ethyl butyrate (EB) and BB. Interestingly, control strains with the empty plasmid (pMTL82151 or pTJ1) also produced noticeable EA, BA and BB. This could be because: 1) the endogenous lipase in clostridia can catalyze ester

production¹³; 2) *catP* on pMTL82151 encoding a chloramphenicol acetyltransferase (belonging to the same class of enzymes as AATs) has AAT activities^{5,23}.

Based on the results, it could be concluded that ATF1 is more favorable for BA production. All strains with *atf1* produced higher levels of BA compared to the same strain but with the overexpression of other genes (FIG. 2b). While VAAT, SAAT and EHT1 seemed to have better activities for BB production. Among all the strains, *C. saccharoperbutylacetonicum* FJ-004 produced the highest titer of 5.5 g/L BA. This is the highest BA production that has been reported through microbial fermentation to the best of our knowledge. *C. acetobutylicum* CaSAAT (with the overexpression of *saat* from *Fragaria xananassa*) was reported to produce 8.37 mg/L BA²². The highest BB production of 0.3 g/L was observed in *C. pasteurianum* J-5 with the overexpression of *eht1*. This is also the highest BB production reported so far directly from glucose with engineered microorganisms to the best of our knowledge, which is significantly higher than the recently reported 50.07 mg/L in an engineered *C. acetobutylicum*²². Some of our engineered strains could also produce small amount of EB with the highest of 0.02 g/L been observed in *C. pasteurianum* J-5. With the *lipaseB* overexpression, *C. tyrobutyricum* JZ-6 could generate 0.3 g/L EA. This was significantly higher than other strains tested in this work (mostly < 0.01 g/L). The mother strain *C. tyrobutyricum* $\Delta cat1::adhE1$ could produce 20.8 g/L acetate and 5.3 g/L ethanol (precursors for EA synthesis) during a batch fermentation, which might have enabled high-level EA production in *C. tyrobutyricum* JZ-6¹¹.

The production levels of BA and BB achieved above are both significantly higher than the previously reported in microbial hosts to the best of our knowledge. In comparison, the BA level is much higher than BB level, and thus has greater potentials towards economic viability. Therefore, in the following steps, we primarily focused on systematic metabolic engineering of the strain for further enhanced BA production.

Deletion of *nuoG* increased BA production

Enhancement of the pool of precursors is one common strategy to improve the production of targeted bioproduct. Butanol and acetyl-CoA are the two precursors for BA production. Therefore, we set out to increase butanol synthesis to improve BA production. The *nuoG* gene encodes the NADH-quinone oxidoreductase subunit G, which is a subunit of the electron transport chain complex I²⁵. NADH-quinone oxidoreductase can oxidize NADH to NAD⁺ and transfer protons from cytoplasm to periplasm to form a proton gradient between periplasm and cytoplasm, which can then contribute to the energy conversion²⁵.

In this study, we hypothesized that by deleting *nuoG*, BA production would be boosted because of the potentially increased butanol production. Thus, we deleted *nuoG* (*Cspa_c47560*) in N1-4-C and generated FJ-100. Further, FJ-101 was constructed based on FJ-100 for BA production. Results demonstrated that, although butanol production in FJ-100 was only slightly improved (16.5 g/L vs. 15.8 g/L in N1-4-C; FIG. 8), BA production in FJ-101 was remarkably enhanced compared to FJ-004 (7.8 g/L vs. 5.5 g/L). Based on our experiences, because *C. saccharoperbutylacetonicum* N1-4 (or N1-4-C) mother strain can naturally produce very high level butanol, it was generally very difficult to further improve butanol production in *C. saccharoperbutylacetonicum* through simple metabolic engineering strategies²⁷. This is likely the case in FJ-100 (comparing to N1-4-C). However, the increased NADH availability with *nuoG* deletion in FJ-101 would enable an enhanced 'instant' flux/availability of butanol which would serve as a precursor for BA production and thus enhance BA production in FJ-101. In this sense, the total butanol generated during the process (including the fraction serving as the precursor for BA production and the other fraction as the end product) in FJ-101 would be actually much higher than in FJ-004.

Enhancement of acetyl-CoA availability to improve BA production

At the end of fermentation with FJ-101, there was still 7.6 g/L of butanol remaining. This suggested that limited availability of intracellular acetyl-CoA was likely the bottleneck for the further improvement of BA production. To enhance the availability of acetyl-CoA, we firstly introduced isopropanol synthesis (from acetone) pathway (FIG. 3A). We hypothesized that this could pull flux from acetone to isopropanol, and thus boost the transferring of CoA from acetoacetyl-CoA to acetate driven by the CoA transferase, resulting in increased instant availability of acetyl-CoA. The *sadh* gene in *C. beijerinckii* B593 encoding a secondary alcohol dehydrogenase can convert acetone into isopropanol²⁸. The *hydG* gene in the same operon as *sadh* encodes a putative electron transfer protein. In this study, either *sadh* alone or the *sadh-hydG* gene cluster was integrated into the chromosome of FJ-100, generating FJ-200 and FJ-300, respectively. Further, by introducing pMTL-cat-*atf1*, FJ-201 and FJ-301 were obtained. Compared to FJ-101, about 50% of the acetone could be converted into isopropanol in FJ-201, while ~95% of the acetone in FJ-301 could be converted into isopropanol. The total titers of acetone plus isopropanol in FJ-301 and FJ-201 were 6.6 and 5.1 g/L respectively, both of which were higher than 4.8 g/L acetone in FJ-101. More significantly, BA production in FJ-201 and FJ-301 has been

remarkably increased compared to FJ-101, and reached 10.1 and 12.9 g/L, respectively, likely due to enhanced 'regeneration' of acetyl-CoA as described above (FIG. 3b).

Dynamic expression of *atf1* enhanced BA production

In our engineered strain, BA is synthesized through condensation of butanol and acetyl-CoA catalyzed by ATF1. The constitutively high expression of ATF1 would not necessarily lead to high BA production. For example, BA production in FJ-008 in which *atf1* was driven by the constitutive strong promoter P_{thl} from *C. tyrobutyricum* was actually much lower (3.5 g/L vs 5.5 g/L) than in FJ-004 in which *atf1* was expressed under the promoter P_{cat} from *C. tyrobutyricum*. We hypothesized that, in order to obtain more efficient BA production, the synthesis of ATF1 should be dynamically controlled and thus synchronous with the synthesis of precursors (butanol or acetyl-CoA). Therefore, for the next step, we attempted to evaluate various native promoters for *atf1* expression, and identify the one(s) that can enable an appropriately dynamic expression of ATF1 and lead to enhanced BA production.

Four promoters associated with the synthesis of BA precursors were selected to drive the *atf1* expression, and four strains were constructed correspondingly for BA production (FIG. 3C). Fermentation results were shown in FIG. 3D. Indeed, distinct results for BA production were observed in these strains. The BA level was only 1.0 and 1.1 g/L in FJ-305 and FJ-302 with P_{bdh} and P_{pfl} for *atf1* expression, respectively. P_{ald} is an important promoter in *C. saccharoperbutylacetonicum*, which can sense the acidic state and switch cell metabolism from acidogenesis to solventogenesis³¹. FJ-303 with the *atf1* expression driven by P_{ald} produced 9.9 g/L BA, which was still 20% lower than in FJ-301. Interestingly, FJ-304 in which *atf1* was expressed under P_{adh} produced 13.7 g/L BA, which was about 10.5% higher than in FJ-301. Based on the results, we speculated that the promoter of *adh* (which encodes the key enzyme catalyzing butanol and ethanol production) might have resulted in the more appropriate dynamic expression of *atf1* in line with the flux of the precursors and thus led to enhanced BA production. On the other hand, ethanol production in FJ-304 was also lower than in FJ-301 (0.3 g/L vs 0.6 g/L). The enhanced BA production in FJ-304 consumed more acetyl-CoA, and thus decreased production of ethanol, which also needed acetyl-CoA as the precursor.

Organization of BA-synthesis enzymes to enhance BA production

In this study, we evaluated several reorganization approaches to increase BA production (FIG. 4).

PduA* protein, derived from *Citrobacter freundii* Pdu bacterial microcompartment could form filaments in bacteria like *E. coli*³². The CC-Di-A and CC-Di-B are designed parallel heterodimeric coiled coils and two proteins with each of these self-assembling tags could combine and shorten the catalytic distance. The enzymes (from the same metabolic pathway), tagged with one of the coiled coils (CC-Di-A or CC-Di-B) would attach onto the formed intracellular filaments (its PduA* was tagged by the other coiled coil); thus, the organized enzymes on the filaments would improve the catalytic efficiency of the target metabolic pathway. MinD is a membrane-associated protein and the localization of MinD is mediated by an 8-12 residue C-terminal membrane-targeting sequence. The proteins with MinD C-terminal sequence were able to be drawn to the cell membrane^{32,33}. Thus, the application of MinD C-tag can facilitate the secretion of target product and enhance its production by mitigating the intracellular toxicity as well as promoting the catalyzing process (FIG. 4A).

To evaluate whether the organization of enzymes could improve BA production in our strain, three strategies were recruited: 1) assembling two of the three enzymes (enzymes associated with BA synthesis: NifJ (related to acetyl-CoA synthesis), BdhA (related to butanol synthesis) and ATF1) with the CC-Di-A and CC-Di-B tags; 2) organizing the three enzymes onto PduA* formed scaffold; or 3) introducing MinD C-tag to draw ATF1 onto the cell membrane.

Firstly, we assembled enzymes for BA synthesis by adding the CC-Di-A tag to ATF1 and the CC-Di-B tag to NifJ and BdhA (FIG. 4B). Fermentation results showed that the addition of CC-Di-A tag to the C-terminus of ATF1 in FJ-306 had significantly negative effects on BA synthesis with only 2.6 g/L BA was produced (FIG. 4E). The assembly of ATF1 together with NifJ or BdhA had even severer negative effects on BA synthesis and BA production was only 0.06 and 0.03 g/L in FJ-309 and FJ-310, respectively. Further, we organized ATF1, NifJ and BdhA onto the PduA* scaffold. PduA* was tagged with CC-Di-B, while the other three enzymes were tagged with CC-Di-A (FIG. 4C). The generated FJ-311 (harboring the scaffold and ATF1-CC-Di-A) produced 3.1 g/L BA, while FJ-312 (harboring the scaffold, ATF1-CC-Di-A and NifJ-CC-Di-A) and FJ-313 (harboring the scaffold, ATF1-CC-Di-A, NifJ-CC-Di-A and BdhA-CC-Di-A) produced slightly higher amount of BA both at 3.5 g/L. The scaffold seemed to have some positive effects on BA synthesis but didn't work as efficient as it was reported in other studies³². We speculate that the coiled coils tags (CC-Di-A or CC-Di-B) might severely impair the catalytic activity of ATF1. Furthermore, the

assembly of ATF1 together with NifJ or BdhA could further inhibit the activity of ATF1, thus resulting in significant decrease in BA production in the corresponding strains.

Moreover, we evaluated the effect of the introduction of MinD C-tag (to draw ATF1 onto the cell membrane) on BA production (FIG. 4D). Fermentation results showed that the corresponding FJ-308 strain could produce 16.4 g/L BA, which was 20% higher than FJ-304 (FIG. 4E). The FJ-307 strain (with CC-Di-A-Atf1-*MinD*) could also produce higher BA of 3.9 g/L compared to FJ-306 (2.6 g/L). All these results indicated that the addition of the cell membrane associated motif to draw ATF1 onto the cell membrane could facilitate the BA excretion and mitigate the intracellular toxicity and therefore enhance BA production. However, the assembly of BA synthetic enzymes or the organization of relevant enzymes onto the scaffold would significantly decrease BA production.

Elimination of prophages increased BA (and BB) production

During our fermentations, we noticed that the ester production of the strains was not stable and could be varied from batch to batch. It has been reported that the N1-4 (HMT) strain contains a temperate phage named HM T which could release from the chromosome even without induction³⁴. In addition, the N1-4 (HMT) strain can produce a phage-like particle clostocin O upon the induction with mitomycin C³⁵. We hypothesized that the instability of fermentations with *C. saccharoperbutylacetonicum* might be related to the prophages, and the deletion of prophages would enable more stable and enhanced production of desired endproducts. We identified five prophage-like genomes (referred here as P1-P5 respectively) within the chromosome of N1-4 (HMT) (Fig. 5a)³⁶. Based on systematic evaluation through individual and combinatory deletion of the prophages, we demonstrated that P5 is responsible for the clostocin O synthesis (Figs. 5f, h & 12)³⁵, and further confirmed that P1 was the HM T phage genome (Figs. 5g & 14). However, the phage image was different from what was described before³⁷. It was more like HM 7 (a head with a long tail), rather than HM 1 (a head with multiple short tails). Both Δ P1234 (with the deletion of P1-P4) and Δ P12345 (with the deletion of P1-P5) exhibited improved cell growth and enhanced butanol production (Figs. 5b-5e, Figs. 10, 15 & 16). Δ P12345 should be a more stable platform as there is no cell lysis at any induction conditions with mitomycin C or norfloxacin (FIG. 5i). While Δ P1234 showed similar growth and even slightly higher butanol production compared to Δ P12345 (Figs. 5d & 5e).

Thus, in a further step, we used Δ P1234 and Δ P12345 as the platform to be engineered for enhanced and more stable BA production. We deleted *nouG* and integrated

sadh-hydG cluster in both $\Delta P1234$ and $\Delta P12345$, and obtained FJ-1200 and FJ-1300 correspondingly. The plasmid pMTL-*P_{adh}-atf1-MinD* was transformed into FJ-1200 and FJ-1300, generating FJ-1201 and FJ-1301, respectively. Fermentation results showed that FJ-1201 and FJ-1301 produced 19.7 g/L and 19.4 g/L BA, respectively, which were both higher than FJ-308 (FIG. 6a & Table S3). BA production in both FJ-1201 and FJ-1301 could be completed within 48 h, resulting in a productivity of ~ 0.41 g/L/h, which was significantly higher than 0.23 g/L/h in FJ-308. BA yield in FJ-1201 reached 0.26 g/g, which was also higher than FJ-308 (0.24 g/g). We further determined BA concentrations in the fermentation broth as 0.6 g/L and 0.5 g/L respectively for the fermentation with FJ-1201 and FJ-1301. Taken together, total BA production in FJ-1201 was 20.3 g/L, which was the highest level that has ever been reported in a microbial host. It is 2400-fold higher than the highest level that has been previously reported²², and also 14.8-fold higher than that by the very recently reported *C. diolis* strain²⁴.

Besides BA production, BB production in FJ-1201 also reached 0.9 g/L, which was significantly higher than in FJ-308 (0.01 g/L) and in *C. pasteurianum* J-5 (0.3 g/L) (FIG. 2b, FIG. 6a, & Table S3). This level (0.9 g/L) was 18.6-fold higher than the highest BB production that has been previously reported (0.05 g/L in *C. acetobutylicum*)²². All these results confirmed our hypothesis that the elimination of prophages would make more robust host strain for enhanced and more stable ester production.

20 **Expression of SAAT in FJ-1200 further enhanced BB production**

As demonstrated in FIG. 2b, SAAT and EHT1 were more relevant for BB production. Therefore, to achieve higher BB production, we expressed *saat* and *eht1* in FJ-1200 and obtained FJ-1202 and FJ-1203, respectively. Fermentation showed that FJ-1202 and FJ-1203 produced 1.3 and 0.2 g/L BB, respectively (FIG. 6d). Further, we added MinD C-tag to the SAAT and introduced the recombinant gene into FJ-1200 and obtained FJ-1204 for an attempt to further improve BB production as observed for BA production in FJ-308. However, BB production in FJ-1204 was only 1.0 g/L. Notwithstanding, 1.3 g/L BB obtained in FJ-1202 is 25.8-fold higher than the highest level that has been previously reported²².

Deletion of *rex* increased BA/BB production

30 The *rex* gene was identified as a redox-sensing transcriptional repressor, which represses the transcription of genes related to butanol synthesis. The *rex* gene was deleted in FJ-1200, generating the strain YM016. Then the plasmid pMTL-*Padh-atf1-MinD* was

transformed into YM016, obtaining the strain YM016P. The fermentation results indicated that 22.0 g/L BA could be produced in a batch fermentation with YM016P.

Further systematic genome engineering to enhance BA production

Based on YM016, three copies of 'Padh-*atf1*-MinD' cassette were integrated into different loci (*ldh1*, *lacZ1* and *lacZ2*). Further, the *ctfA1*-*ctfB1* gene cluster (encoding acetoacetyl-CoA:acetate/butyrate:CoA transferase) was deleted and the obtained strain was named YM028. Then, the plasmid pMTL-*Padh-atf1*-MinD was transformed into YM028, generating YM028P for BA production. Fermentation results showed that YM028P produced 25.3 g/L BA, which was the highest BA titer that we obtained by now.

10 Ester production with biomass hydrolysates as substrate

Fermentations were carried out using biomass hydrolysates as the substrate. In the hydrolysates, besides sugars (57.4 g/L glucose and 27.2 g/L xylose) as carbon source, there were also nutrients converted from biomass (corn stover). Therefore, we tested the effect of organic nitrogen (yeast and tryptone) of various levels on ester production. Interestingly, results showed that the highest BA production of 17.5 g/L was achieved in FJ-1201 (in the extractant phase) without any exogenous nitrogen source supplemented (Table S4). In addition, 0.3 g/L BA was detected in aqueous phase, making a total BA production of 17.8 g/L in FJ-1201 (FIG. 6A & Table S4). Although this was slightly lower than when glucose was used as substrate (20.3 g/L), fermentation with hydrolysates did not need any supplementation of nutrients, which could significantly reduce production cost. We further performed fermentation in 500-mL bioreactor with pH controlled >5.0. BA production reached 16.0 g/L with hydrolysates and 18.0 g/L with glucose as substrate, both of which were lower than results from fermentation under the same conditions but with serum bottles (Figs. 6B & 6C). During bioreactor fermentation, we noticed very strong smell of BA around the reactor. We suspected that significant evaporation during fermentation with bioreactor resulted in the lower level of final BA titers as compared to fermentation with serum bottle, which was securely sealed with only minimum outlet for releasing gases. An improved bioprocess needs to be carefully designed for larger scale fermentation to minimize BA evaporation and enhance BA production and recovery.

Furthermore, we performed fermentation with FJ-1202 for BB production using hydrolysates in both serum bottle and 500-mL bioreactor. Results demonstrated that BB production in serum bottle from hydrolysates was 0.9 g/L (compared to 1.3 g/L when glucose used as substrate; FIG. 6D). BB production in bioreactor from hydrolysates reached

0.9 g/L compared to 1.6 g/L when glucose was used as substrate (Figs. 6E & 6F). The results were consistent with the case for BA production that lower-level BB was obtained when hydrolysates (compared to glucose) was used as substrate. However, interestingly, larger scale fermentation with bioreactor produced slightly higher level of BB than the fermentation under the same conditions with serum bottle, which was different from the case for BA production. This might be because BB is less evaporative (in bioreactor) than BA.

Techno-economic analysis (TEA) for BA production from biomass hydrolysates

We performed a techno-economic analysis (TEA) to evaluate the economic competitiveness of BA production from corn stover at a process capacity of 2,500 MT wet corn stover (20% moisture) per day. The whole process was developed based on the previous process using the deacetylation and disk refining (DDR) pretreatment to produce corn stover hydrolysate³⁸, which was the substrate used for our fermentation experiments to produce BA. The detailed process information is summarized in the supplementary materials. The process is composed of eight sections including feedstock handling, DDR pretreatment and hydrolysis, BA fermentation, product recovery (distillation), wastewater treatment, steam and electricity generation, utilities, and chemical and product storage (FIG. 7A). FIG. 7B shows the equipment cost distribution of each process sections, with a total installed equipment cost of \$263 million. The fermentation, steam & electricity co-generation, and wastewater treatment contribute significant percentage to the total installed equipment cost, which aligns well with previous TEA models for chemical production from biomass via fermentation^{39,40}. The total capital investment (TCI) is \$472 million by taking consideration of additional direct cost, indirect cost as well as working capitals (Table S5). From the process model, 95.2 kg of BA can be produced from 1 MT of corn stover, meanwhile significant amounts of butanol (11.1 kg), isopropanol (15.5 kg) and surplus electricity (209 kWh) are produced as coproducts (FIG. 7C). The BA production cost was estimated to be \$986/MT (FIG. 7d), which is much lower than the current

BA market price ranging between \$1,200 and \$1,400 per MT in year 2019 (based on the quotes from the industry⁴¹), showing the highly economic competitiveness of BA production using our engineered strain. By looking into the cost breakdown, the corn stover feedstock cost contributes the most (38.2%) to the BA production cost, followed by other chemicals (22.3%) and capital deprecation (18.0%) and utilities (14.5%). Sensitivity

analysis shows that corn stover price, BA yield, and BA titer are the most sensitive input parameters to the BA production cost (FIG. 16).

Discussion

Although tremendous efforts have been invested on biofuel/biochemical research worldwide, very limited success has been achieved. A key bottleneck is that the microbial host is subject to endproduct toxicity and thus desirable production efficiency cannot be obtained⁴². Our central hypothesis was that metabolically engineering of microorganisms for high-value and easy-recoverable bioproduct production can help alleviate end product toxicity and thus high titer and productivity can be achieved, with which economically viable biofuel/biochemical production can be ultimately established. Here we tested this hypothesis by engineering solventogenic clostridia for highly efficient ester (high-value and easy-recoverable) production. Based on the systematic screening of host strains and enzymes as well as multiple rounds of metabolic engineering: enriching precursors (alcohols and acetyl-CoA) for ester production, dynamically expressing heterologous ester-production pathways, organizing ester-synthesis enzymes, and improving strain robustness by eliminating putative prophages, we ultimately obtained strains for efficient production of esters in both synthetic fermentation medium and biomass hydrolysates. To the best of our knowledge, the production levels of BA and BB we achieved set up the new records.

Methods

Microorganisms and cultivation conditions

All the strains and plasmids used in this study are listed in Table S1. *C. pasteurianum* ATCC 6013 and *C. saccharoperbutylacetonicum* N1-4 (HMT) (DSM 14923) were requested from American Type Culture Collection (ATCC) and Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), respectively. *C. beijerinckii* NCIMB 8052 was provided by Dr. Hans P. Blaschek¹⁴. *C. tyrobutyricum* $\Delta cat1::adhE1$ and *C. tyrobutyricum* $\Delta cat1::adhE2$ are hyper-butanol producing mutants constructed in our lab¹¹. All the clostridial strains were grown in an anaerobic chamber (N₂-CO₂-H₂ with a volume ratio of 85:10:5) at 35 °C. Strains of *C. tyrobutyricum*, *C. saccharoperbutylacetonicum* and *C. beijerinckii* were cultivated using tryptone-glucose-yeast extract (TGY) medium⁴³, while strains of *C. pasteurianum* were cultivated using 2×YTG medium⁴⁴. When required, clarithromycin (Cla) or thiamphenicol (Tm) was supplemented into the medium at a final concentration of 30 µg/mL and 15 µg/mL, respectively. *E. coli* DH5α was used for routine

plasmid propagation and maintenance. *E. coli* CA434 was used as the donor strain for plasmid conjugation for *C. tyrobutyricum*. Strains of *E. coli* were grown aerobically at 37 °C in Luria-Bertani (LB) medium supplemented with 100 µg/mL ampicillin (Amp), 50 µg/mL kanamycin (Kan) or 34 µg/mL chloramphenicol (Cm) as needed.

5 Plasmid construction

All the plasmids used in this study are listed in Table S1, and all the primers used in this study are listed in Table S2.

The plasmids pMTL82151 and pTJ1 were used as mother vectors for heterogeneous gene expression^{45,46}. The promoter of the *cat1* gene (CTK_C06520) (*P_{cat}*) and the promoter of the *thl* gene (CTK_C01450) (*P_{thl}*) from *C. tyrobutyricum* ATCC 25755 were amplified and inserted into pMTL82151 at the *EcoRI* site, and the generated plasmids were named as pMTL82151-*P_{cat}* and pMTL82151-*P_{thl}*, respectively. Promoters of the following gene, *pflA* (Cspa_c13710) (*P_{pfl}*), *ald* (Cspa_c56880) (*P_{ald}*), *adh* (Cspa_c04380) (*P_{adh}*) and *bdh* (Cspa_c56790) (*P_{bdh}*), all from *C. saccharoperbutylacetonicum* N1-4 (HMT) were amplified and inserted into pMTL82151 at the *EcoRI* site, and the generated plasmids were named as pMTL82151-*P_{pfl}*, pMTL82151-*P_{ald}*, pMTL82151-*P_{adh}* and pMTL82151-*P_{bdh}*, respectively.

The *vaat* gene from *Fragaria vesca*, the *saat* gene from *F. ananassa* and the *atf1* gene from *S. cerevisiae* were amplified from plasmids pDL006, pDL001 and pDL004, respectively^{3,21}_ENREF_26. The *atf1'* (the codon optimized *atf1* gene), *eth1* from *S. cerevisiae*¹⁹, and *lipaseB* from *Candida antarctica*⁴⁷ were all synthesized by GenScript (Piscataway, NJ, USA). The obtained gene fragments of *vaat*, *saat*, *atf1*, *atf1'*, *eth1*, and *lipaseB* were inserted between the *BtgZI* and *EcoRI* sites in pMTL82151-*P_{cat}*, generating pMTL-*P_{cat}-vaat*, pMTL-*P_{cat}-saat*, pMTL-*P_{cat}-atf1*, pMTL-*P_{cat}-atf1'*, pMTL-*P_{cat}-eth1*, and pMTL-*P_{cat}-lipaseB*, respectively. The *atf1* gene was inserted between the *BtgZI* and *EcoRI* sites in pMTL82151-*P_{thl}*, generating pMTL-*P_{thl}-atf1*.

The *P_{cat}* promoter and the gene fragments of *vaat*, *saat*, *atf1*, *eth1*, and *lipase* were amplified and ligated into the *EcoRI* site of pTJ1, generating pTJ1-*P_{cat}-vaat*, pTJ1-*P_{cat}-saat*, pTJ1-*P_{cat}-atf1*, pTJ1-*P_{cat}-eth1* and pTJ1-*P_{cat}-lipaseB*, respectively. The *atf1* gene was inserted into the *EcoRI* site of pMTL82151-*P_{pfl}*, pMTL82151-*P_{ald}*, pMTL82151-*P_{adh}* and pMTL82151-*P_{bdh}*, generating pMTL-*P_{pfl}-atf1*, pMTL-*P_{ald}-atf1*, pMTL-*P_{adh}-atf1* and pMTL-*P_{bdh}-atf1*, respectively.

DNA sequences of *CC-Di-A*, *CC-Di-B*, *MinD* and *pduA** were synthesized by GenScript (Piscataway, NJ, USA). The MinD-tag was fused to the end of *atf1* with PCR and ligated into

the *EcoRI* site of pMTL-*P_{adh}*, generating pMTL-*P_{adh}-atf1-MinD*. In addition, the MinD-tag was fused to the end of *saat* with PCR and inserted between the *BtgZI* and *EcoRI* sites of pMTL-*P_{cat}*, generating pMTL-*P_{cat-saat-MinD}*.

The synthesized *CC-Di-A* fragment was ligated into the *EcoRI* site of pMTL-*P_{adh}*,
 5 generating pMTL-*P_{adh}-CC-Di-A*. The DNA fragments of *atf1* and *atf1-MinD* were amplified from pMTL-*P_{adh}-atf1* and pMTL-*P_{adh}-atf1-MinD* and then inserted into the *EcoRI* site of pMTL-*P_{adh}-CC-Di-A*, obtaining pMTL-*P_{adh}-A-atf1* and pMTL-*P_{adh}-A-atf1-MinD*. The *CC-Di-B* sequence with the *nifj* gene and *CC-Di-B* with the *bdhA* gene were subsequently inserted into the *KpnI* site of pMTL-*P_{adh}-A-atf1*, generating pMTL-*P_{adh}-A-atf1-B-nifj* and pMTL-*P_{adh}-A-atf1-B-nifj-B-bdhA*. The DNA fragments of *CC-Di-B-pduA**, *CC-Di-A-nifj*, *CC-Di-A-bdhA* were
 10 inserted into the *EcoRI* site of pTJ1-*P_{cat}*, generating pTJ1-*P_{cat}-B-pduA**, pTJ1-*P_{cat}-B-pduA*-A-nifj* and pTJ1-*P_{cat}-B-pduA*-A-nifj-A-bdhA*, respectively.

For the gene deletion or integration in *C. saccharoperbutylacetonicum*, all the relevant plasmids were constructed based on pYW34, which carries the customized
 15 CRISPR-Cas9 system for genome editing in *C. saccharoperbutylacetonicum*^{14,27}. The promoter P_{J23119} and the gRNA (with 20-nt guide sequence targeting on the specific gene) were amplified by two rounds of PCR with primers N-20nt/YW1342 and YW1339/YW1342 as described previously (N represents the targeted gene)²⁷. The obtained fragment was then inserted into pYW34 (digested with *BtgZI* and *NotI*) through
 20 Gibson Assembly, generating the intermediate vectors. For gene deletion, the fragment containing the two corresponding homology arms (500-1000 bp for each) for deleting the specific gene through homologous recombination was amplified and inserted into the *NotI* site of the obtained intermediate vector as described above, generating pYW34-ΔN (N represents the targeted gene). For gene integration, the fragment containing the two
 25 corresponding homology arms (~1000-bp for each), the promoter and the gene fragment to be integrated, was amplified and inserted into the *NotI* site of the obtained intermediate vector as described above, generating the final plasmid for gene integration purpose.

Fermentation with glucose as the substrate

For the fermentation for ester production, the *C. pasteurianum* strain was cultivated
 30 in Biebl medium⁴⁸ with 50 g/L glycerol as the carbon source at 35 °C in the anaerobic chamber. When the OD₆₀₀ reached ~0.8, the seed culture was inoculated at a ratio of 10% into 100 mL of the same medium in a 250-mL serum bottle and then cultivated at an agitation of 150 rpm and 30 °C (on a shaker incubator) for 72 h. The *C. beijerinckii* strain

was cultivated in TGY medium until the OD₆₀₀ reached ~0.8. Then the seed culture was inoculated at a ratio of 5% into 100 mL P2 medium along with 60 g/L glucose and 1 g/L yeast extract in a 250-mL serum bottle. The fermentation was carried out at an agitation of 150 rpm and 37 °C for 72 h⁴³. The *C. tyrobutyricum* strain was cultivated in RCM medium at 35 °C until the OD₆₀₀ reached ~1.5. Then the seed culture was inoculated at a ratio of 5% into 200 mL fermentation medium (containing: 50 g/L glucose, 5 g/L yeast extract, 5 g/L tryptone, 3 g/L (NH₄)₂SO₄, 1.5 g/L K₂HPO₄, 0.6 g/L MgSO₄·7H₂O, 0.03 g/L FeSO₄·7H₂O, and 1 g/L L-cysteine) in a 500-mL bioreactor (GS-MFC, Shanghai Gu Xin biological technology Co., Shanghai, China) and the fermentation was carried out at an agitation of 150 rpm and 37 °C for 120 h with pH controlled >6.0⁹. The *C. saccharoperbutylacetonicum* strain was cultivated in TGY medium at 35 °C in the anaerobic chamber until the OD₆₀₀ reached ~0.8. Then the seed culture was inoculated at a ratio of 5% into 100 mL P2 medium along with 60 g/L glucose and 1 g/L yeast extract in a 250-mL serum bottle. The fermentation was carried out at an agitation of 150 rpm and 30 °C for 120 h⁴³. For the fermentation at larger scales in bioreactors, it was carried out in a 500-mL fermenter (GS-MFC, Shanghai Gu Xin biological technology Co., Shanghai, China) with a working volume of 250 mL with pH controlled >5.0, at 50 rpm and 30 °C for 120 h. Samples were taken every 24 h for analysis.

For all fermentations in the serum bottle, a needle and hosepipe were connected to the top of bottle for releasing the gases produced during the fermentation. For all the fermentations for ester production, the extractant *n*-hexadecane was added into the fermentation with a ratio of 1:1 (volume of the extractant vs. volume of fermentation broth) for *in situ* ester extraction. The reported ester concentrations were the determined values in the extractant phase. All the fermentations were carried out in triplicate.

Fermentation with biomass hydrolysates as the substrate

The biomass hydrolysates was kindly provided by Dr. Daniel Schell from National Renewable Energy Laboratory (NREL) which was generated from corn stover through the innovative 'deacetylation and mechanical refining in a disc refiner (DDR)' approach⁴⁹. For the fermentation, the biomass hydrolysate was diluted and supplemented into the P2 medium as the carbon source (with final sugar concentrations of 57.4 g/L glucose and 27.2 g/L xylose). In addition, various concentrations of yeast extract (Y, g/L) and tryptone (T, g/L) were also added as the nitrogen source to evaluate their effects on the fermentation performance: 0Y+0T; 1Y+3T and 2Y+6T. The fermentation was carried out under the same

conditions as described above at 100 mL working volume in a 250-mL serum bottle. All the fermentations were carried out in triplicate.

Analytical methods

Concentrations of acetone, ethanol, butanol, acetic acid, butyric acid and glucose were measured using a high-performance liquid chromatography (HPLC, Agilent Technologies 1260 Infinity series, Santa Clara, CA) with a refractive index Detector (RID), equipped with an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA). The column was eluted with 5 mM H₂SO₄ at a flow rate of 0.6 mL/min at 25 °C. The concentration of the ester in the *n*-hexadecane phase was quantified using a gas chromatography-mass spectrometry (GC-MS, Agilent Technologies 6890N, Santa Clara, CA) equipped with an HP-5 column (60m×0.25 mm, 0.25 mm film thickness). Helium was used as the carrier gas. The initial temperature of the oven was set at 30 °C for 2 min, followed by a ramp of 10 °C/min to reach 300 °C, and a ramp of 2 °C/min to reach the final temperature of 320 °C, which was then held for 2 min. The detector was kept at 225.⁹

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- The following references are provided as an aid in understanding the subject matter provided above. No admission is made that any of the following meet the legal definition of “prior art” in any country, or that any of the following are relevant to the patentability of anything that is claimed.
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SUPPLEMENTAL MATERIALS TO EXAMPLE

25 Methods

Plasmid transformation and mutant verification

Competent cells of *C. pasteurianum* were prepared following the protocol as reported by Pyne *et al.*³. *C. pasteurianum* SD-1, an equivalent to *C. pasteurianum* Δ *cpaAIR* in which the *cpaAIR* gene (encoding the CpaAI Type II restriction endonuclease) was deleted and thus more efficient transformation was enabled³, was used as the host strain. The

30 overnight-grown seed culture was inoculated into 20 mL 2×YTG medium. When the OD₆₀₀ reached 0.3~0.4, sucrose and glycine were added to a final concentration of 0.4 M and 1.25%, respectively. When the OD₆₀₀ further reached 0.6~0.8, the cells were harvested by

centrifugation at 4,200 g and 4 °C for 10 min. The cell pellets were resuspended in 5 mL of SMP buffer (270 mM sucrose, 1mM MgCl₂ and 7mM sodium phosphate, pH 6.5) and spinned down under the same conditions. The obtained cell pellets were then resuspended in 0.6 mL of SMP buffer. 1 µg of plasmid DNA was suspended with 20 µL of 2 mM Tris-HCl (pH 8.0) and then mixed with 550 µL competent cells and 30 µL 96% cold ethanol. The mixture was transferred to a pre-chilled 4 mm electroporation cuvette and incubated on ice for 5 min. Electroporation was then applied with a voltage of 1,800 V, capacitance of 25 µF and resistance of ∞ using a Gene Pulser Xcell electroporation system (Bio-Rad Laboratories, Hercules, CA). Afterwards, the culture was transferred into 2 mL of 2×YTG medium and recovered at 35 °C for 4 h. The culture was then spread onto the 2×YTGT agar plates (2×YTG agar plates containing 15 µg/mL of thiamphenicol) for the selection of the transformants.

Competent cells of *C. beijerinckii* were prepared following the procedure as described by Wang et al.³. Briefly, the overnight cell culture was inoculated into TGY medium with an inoculation ratio of 1%. When the OD₆₀₀ reached ~0.8, the cells were harvested by centrifugation at 4,200 g and 4 °C for 10 min. The cell pellets were washed with the same volume (as the original cell culture) of ice-cold 15% glycerol and centrifuged under the same condition for 10 min. The cell pellets were resuspended with 5% volume of ice-cold 15% glycerol. Then 400 µL competent cells and ~1.0 µg of plasmid DNA were mixed and transferred into a 2-mm pre-chilled electroporation cuvette and incubated on ice for 10 min. Electroporation was carried out at 2,000 V of voltage, 25 µF of capacitance and 200 Ω of resistance. Afterwards, the cells were transferred into 1.6 mL of TGY and incubated at 35 °C for 6-8 h for recovery. The culture was then spread onto TGYC agar plates (TGY agar plates containing 30 µg/mL of clarithromycin) for the selection of transformants.

The plasmid transformation through conjugation for *C. tyrobutyricum* was performed following the procedure as described by Zhang et al.³. The donor strain *E. coli* CA434 carrying the desired plasmid was cultivated in LB medium supplemented with Cm and Kan. 3 mL of overnight-cultured *E. coli* CA434 cells were centrifuged and washed for twice (with fresh LB medium) to remove the antibiotics. The obtained donor cells were then mixed with 0.4 mL of the overnight-cultured *C. tyrobutyricum* (grown in TGY medium). The cell mixture was spotted onto the TGY agar plate and incubated in the anaerobic chamber at 37 °C for conjugation. After 24 h of cultivation, the cell lawn on the

plate was washed off using 1mL of TGY medium and then spread onto the TGY plate containing 15 µg/mL Tm and 250 µg/mL D-cycloserine (for eliminating the residual *E. coli* CA434 donor cells). The transformant colonies could be observed after 48-72 h of incubation in the anaerobic chamber.

5 Competent cells of *C. saccharoperbutylacetonicum* were prepared following the procedure as described by Herman et al., with slight modifications⁵. Briefly, the overnight-cultured cells were inoculated into fresh TGY medium. When the OD₆₀₀ of the cell culture reached ~0.8, cells were collected by centrifugation at 4,200 g and 22 °C (room temperature) for 10 min. Cell pellets were then washed with the same volume (as the
10 original cell culture) of SMP buffer. The resuspension was centrifuged again under the same conditions as described above. The cell pellets were resuspended in 5% volume of SMP buffer. After that, the plasmid (~1.0 µg) was mixed with 400 µL of competent cells and transferred into a 2 mm electroporation cuvette and incubated on ice for 30 min. Electroporation was then applied with a voltage of 1,000 V, capacitance of 25 µF and
15 resistance of 300 Ω. Subsequently, the culture was transferred into 2 mL pre-warmed TGY medium and incubated at 35 °C for 2-3 h. After that, the culture was spread onto TGYC or TGYT (TGY agar plates containing 15 µg/mL of thiamphenicol) agar plates.

The positive mutants of *C. saccharoperbutylacetonicum* with desired gene deletion or integration were identified following our previously described procedures⁵. Briefly, the
20 *C. saccharoperbutylacetonicum* transformants harboring the plasmid designed for the gene deletion or integration were incubated in TGYC liquid medium at 35 °C in the anaerobic chamber for about 24 h. The cell culture was then spread onto TGYLC plates (TGYC supplemented with 40 mM lactose). When colonies appeared on the plates, colony PCR (cPCR) was then carried out with the pair of primers of N-U/N-D (N represents the targeted
25 gene name, U represents the upstream primer flanking the target locus, and D represents the downstream primer flanking the target locus) to verify the gene deletion or gene integration. The selected mutants were then subcultured in TGY medium for 3 to 5 generations to cure the plasmid for the gene deletion/integration⁵. The obtained plasmid-free and marker-free mutant strains were used for the following steps.

30 **Prophage induction and phage harvesting**

The strain was grown in TGY medium for overnight. The cells were then transferred into fresh TGY prior to be expose to the inducing reagent mitomycin C or norfloxacin. Mitomycin C was added into the culture when the cell growth reached the desired OD₆₀₀

(0.1-0.2, 0.2-0.3, 0.3-0.4 or 0.4-0.5). The cells of some of the cultures grew too fast at the early stage for the induction purpose, and thus they were not induced under every above mentioned OD₆₀₀ conditions in this work. For the induction, generally mitomycin C at final concentrations of 2 µg/mL and 4 µg/mL was used⁷. Besides, 1 µg/mL and 3 µg/mL of mitomycin C were also tried for the induction in ΔP234. After 30 min of treatment at 35 °C, the cells were harvested via centrifugation at 4,000 g for 5 min and resuspended at the same volume of TGY fresh medium. Then the OD₆₀₀ was monitored carefully during the following 3-5 h.

The seed culture with the OD₆₀₀ of around 0.2, 0.4 and 0.6 was treated using norfloxacin at the final concentrations of 0.3, 1, 3, 6, 9 3 µg/mL at 35 °C for 24 h⁸. The final OD₆₀₀ was measured after the treatment.

The cells were collected via centrifugation at 4,000 g for 20 min, and then filtered through the 0.2 µm filter to obtain the supernatants. The supernatants were centrifugated at 20,000 rpm for 3h, and the obtained precipitation (containing the phages) was then resuspended by 1/30 volume of ddH₂O.

Transmission election microscopy (TEM)

20 µL prepared phage sample was applied to a mesh copper grid and settled for 2 min. Then the liquid was blotted off with a filter paper. The negative stain of 2% phosphotungstic acid (PTA) was applied to a mesh copper grid and settled for 30 s. Then the liquid was blotted off with a filter paper followed by air dry. The prepared samples were then used for TEM observation under a Zeiss EM10 transmission electron microscope (Carl Zeiss AG, Oberkochen, Germany) at an accelerating voltage of 60 kV.

Techno-economic analysis (TEA)

A comprehensive TEA model was developed to evaluate the economic feasibility of BA production from corn stover using the deacetylation and disk refining (DDR) pretreatment. The model originally developed to produce ethanol⁹ was modified to produce BA by mainly substituting the fermentation and distillation unit operations. The processing capacity was set at 2,500 wet metric tonnes (MT, 20% moisture) of corn stover per day. The process was assumed to run 350 days (8,410 hours) per year; thus the annual corn stover consumption is 875,000 wet MT per year. The composition of corn stover was 35.05% cellulose, 19.53% hemicellulose, 15.76% lignin, 4.93% ash, 3.10% of protein, and 21.63% other solids on a dry basis¹⁰. All process was simulated using the software SuperPro Designer (Intelligen Inc., NJ).

The whole process can be divided into eight sections, including feedstock handling, DDR pretreatment and hydrolysis, fermentation, product recovery (distillation), wastewater treatment, steam and electricity cogeneration, utilities, and chemical and product storage. In the process, corn stover is milled at the pre-processing plant and delivered to the feed handling section from a uniform corn stover supply system¹⁹. Received corn stover is added with water to obtain a 25% solid slurry. The slurry is added with sodium hydroxide at a loading of 40 kg/MT dry corn stover, heated to 80 °C and held for 2 hours to remove acetyl groups from corn stover. The alkali-treated corn stover is then washed by using the same amount of added water, followed by dewatering using screw-type presses to remove excess water to attain 40% solids content for the subsequent disk refining and enzymatic hydrolysis. The parameters of disk refining are adapted from a previous optimization study using wet disk mills². The electricity consumption of disk milling is 212 kWh per dry MT of corn stover. The details of the DDR process are described in Chen et al. 2015². The pretreated corn stover is then cooled and sent to hydrolysis tanks where it is hydrolyzed into monomeric sugars by cellulase at 48 °C for 84 hr. The enzyme loading for the enzymatic hydrolysis is 19 mg protein/g cellulose according to a previous study². After the enzymatic hydrolysis, 10% of the hydrolysate is split off to seed fermenters for production of seed culture, and the rest 90% of the hydrolysates is fermented into BA and coproducts in large fermenters at 32 °C, where hexadecane is added to the fermenter at 1:1 ratio (v/v) to extract BA from the fermentation broth. The fermentation takes 96 hours to convert all hydrolyzed sugars to BA and coproducts butanol and isopropanol. The key parameters for the conversion and fermentation yields are summarized in Table S6. The fermentation beer is then sent to the decanter to separate the extractant phase and the aqueous phase, which are separately pumped to the distillation section to recovery BA and coproducts butanol and isopropanol. The distillation stillage is press filtered to separate insoluble solids for combustion to produce steam and electricity, whereas the pressed filtrate is sent to the waste treatment section. The remaining sections of the model, including wastewater treatment, steam and electricity cogeneration, and utilities, inherited most of the original designs from the NREL process model^{20 19}.

The energy and mass balance and flow rate information for the process were generated to determine the capital and operating costs. The purchased equipment costs were determined based on previous literature, particularly from Humbird et al. (2011)¹⁹ and Chen et al. (2015)². The cost of the product recovery (distillation) section was mainly

determined by the embedded cost estimator of SuperPro Designer. The purchased equipment costs were scaled using the exponential scaling equation with exponents ranged between 0.5 and 0.8 depending on the type of equipment¹⁰. The equipment cost obtained in previous years is adjusted to the year of 2019 using the plant cost index from chemical engineering magazine. The total capital investment (TCI) was calculated as a sum of direct and indirect costs, which were determined based on the installed equipment costs. Direct costs included installed equipment cost, site development (9% of inside-battery-limits (ISBL) equipment cost), warehouse (4.0% of ISBL), and additional piping (4.5% of ISBL). Indirect cost is the sum of proratable costs (10% of total direct cost (TDC)), field expenses (10% of TDC), home office and construction (20% of TDC), project contingency (10% of TDC), and other costs (10% of TDC). Working capital was assumed to be 5% of the fixed capital investment. A summary of the total operating cost is listed in Table S5. The total operating cost included both fixed and variable operating costs. Fixed operating costs include labor and various overhead items and variable operating costs include raw material costs, utility costs, and co-product credits. The detailed variable and fixed operating costs are summarized in Table S7.

The BA production cost was calculated based on the methods described in prior studies^{11, 12}. For the process model, BA was assigned as the main product and butanol, isopropanol, and electricity were assigned as the coproducts, which were sold to generate coproduct credits. Sensitivity analyses were also performed at $\pm 20\%$ variation range to evaluate the most influential variables on the BA production cost.

Results

Evaluation of codon-optimization of *atf1* for BA synthesis

Different microorganisms have different codon usage preferences. The *atf1* gene was originally from *S. cerevisiae* and its genetic codon usage might not be preferable for the *C. saccharoperbutylacetonicum* host. Thus, a codon optimized *atf1* gene (designated as *atf*) was synthesized and evaluated for potentially improved expression and BA production in the *C. saccharoperbutylacetonicum* host strain. However, fermentation results demonstrated that FJ-007 (carrying *atf1'* rather than *atf1*) actually generated slightly lower concentration of BA than FJ-004 (5.0 g/L vs. 5.5 g/L, Table S8). The result was unexpected, but not totally surprising. Similar cases have been reported previously where the original natural gene showed better efficiency than the codon-optimized counterpart for desirable biochemical production¹³. We speculate that the natural gene might be able to transcribe

into more stable mRNA structure, and thus lead to higher translation level than the codon-optimized gene¹³. In addition, it has been recently reported that codon-optimized genes could bring about toxicity to the host cells¹⁴.

Enhancement of acetyl-CoA availability to improve BA production

5 In the pathway, thiolase is the enzyme that converts acetyl-CoA into acetoacetyl-CoA (Fig. 3a), and the attenuation of the thiolase activity might be able to repress the acetyl-CoA flux towards butanol (and other products) and lead to the accumulation of intracellular acetyl-CoA, which could be beneficial for enhanced BA production. Therefore, we attempted to delete the thiolase gene to further increase the acetyl-CoA availability. There
10 are five annotated genes encoding thiolase in *C. saccharoperbutylacetonicum*: *Cspa_c06180*, *Cspa_c17310*, *Cspa_c20520*, *Cspa_c20890*, *Cspa_c50320*. After numerous attempts, we were only able to delete *Cspa_c20890* and *Cspa_c50320* individually in FJ-300, generating the mutant strains FJ-400 and FJ-500, respectively. By introducing the vector pMTL-cat-*atf1* into these two strains for BA production, FJ-401 and FJ-501 were obtained, respectively.
15 Fermentation results indicated that BA production was actually slightly lower in FJ-401 (10.8 g/L) and FJ-501 (9.8 g/L) than that in FJ-301 (Fig. 3b). The result was unexpected. Acetoacetyl-CoA is one of the key metabolites for the cell; the repression for its biosynthesis might impair the cell metabolism and thus lead to decreased BA production.

The elimination of prophages increased cell growth and butanol production

20 During our fermentation process, we noticed that the performance for ester production of the strains was not very stable and could be varied from batch to batch. Our industrial collaborator also observed that *C. saccharoperbutylacetonicum* often had instable performance for ABE production in the continuous fermentation process (data not shown). It has been previously reported that the N1-4 (HMT) strain contains a temperate phage
25 named HM T which could release from the chromosome even without induction¹⁵. In addition, the N1-4 (HMT) strain can produce a phage-like particle clostocin O with the induction of mitomycin C. We hypothesized that the instability of the fermentation with *C. saccharoperbutylacetonicum* might be related to the existence of prophages, and the deletion of these prophages and clostocin O encoding sequences would improve the
30 stability of the strain and thus enable more stable and enhanced production of the desired endproduct (BA here). The online program PHAST was used to predict the prophage sequences in N1-4 (HMT)¹⁶, with four possible prophage genomes were identified: the HM T prophage (renamed as P1) as well as three other putative prophages which were named

as P2, P3 and P4 here (Fig. 5a). Besides, one additional incomplete prophage genome (without integrase gene) was found and named as P5.

We firstly constructed the mutant with single deletion of each prophage genome and generated the $\Delta P1$, $\Delta P2$, $\Delta P3$ and $\Delta P4$ strains. Because there are genes within the prophage genome possibly responsible for the normal cell metabolism, we also constructed the mutant with the deletion of only the integrase gene (without the integrase, the prophage cannot release from the chromosome), obtaining the $\Delta NP1$, $\Delta NP2$, $\Delta NP3$ and $\Delta NP4$ strains. In addition, we also constructed the mutant $\Delta P1234$ (with the deletion of all four prophage genomes) and $\Delta NP1234$ (with the deletion of all four integrase genes).

Fermentations were first conducted in the serum bottle to investigate the effects of the elimination of prophages on butanol production in the mutant strains. As shown in Figs. 5b, 5c & S3, $\Delta P4$ and $\Delta P1234$ showed increased cell growth and butanol production, while all the other eight mutants did not demonstrate significance difference in terms of the cell growth and solvent production compared to the mother strain. $\Delta P4$ and $\Delta P1234$ reached the maximum OD₆₀₀ of 17.9 and 17.6, which were 15.5% and 13.5% higher than that of the control N1-4-C strain, respectively. The butanol production in $\Delta P4$ and $\Delta P1234$ reached 17.1 and 16.8 g/L respectively, which were also higher than that of the control N1-4-C strain (16.0 g/L).

To further study the individual prophage, we constructed the triple-deletion mutants $\Delta P234$, $\Delta P134$, $\Delta P124$, $\Delta P123$. Phage induction experiments of $\Delta P234$, $\Delta P134$, $\Delta P124$, $\Delta P123$ and $\Delta P1234$ with mitomycin C revealed that all the mutants exhibited cell lysis (FIG. 10). Transmission electron microscopy (TEM) results indicated that all the mutants produced the tail-like particles, which showed likely the same appearance as clostocin O as reported previously² (FIG. 11). We were not able to observe the HM T phage in the supernatant of $\Delta P234$. It might be because there were too many clostocin O particles in the view which made it difficult to observe the HM T phage.

Based on the above results, we tentatively concluded that P5 might be responsible for the production of clostocin O. To verify this hypothesis and obtain a more robust strain for bioproduction, P5 was deleted in $\Delta P234$, $\Delta P134$, $\Delta P124$, $\Delta P123$ and $\Delta P1234$, obtaining $\Delta P2345$, $\Delta P1345$, $\Delta P1245$, $\Delta P1235$ and $\Delta P12345$. The induction experiments indicated that the cell lysis was detected in $\Delta P2345$ with the addition of 4 $\mu\text{g/mL}$ of mitomycin C at the OD₆₀₀ of 0.2-0.5 (FIG. 12), while no cell lysis was detected in any other mutants at any conditions with the treatment using mitomycin C or norfloxacin (data not shown).

Furthermore, after induction, phage-like particles were only observed in the supernatant of Δ P2345 (Fig. 5g & FIG. 13) and they were likely the HM T phages. However, the phage image was different from what was described before¹². It was more like HM 7 (a head with a long tail), rather than HM 1 (a head with multiple short tails). It is worthwhile to mention that this is the first time that an image of the HM T phage has been reported.

After the deletion of P5, no clostocin O particle was observed in the supernatant of Δ P12345, which confirmed that P5 indeed encoded clostocin O. In addition, as mentioned above, no cell lysis was observed in Δ P12345 with induction (Fig. 5i & FIG. 12), suggesting that Δ P12345 could be a more stable platform to be engineered for enhanced ester production. On the other hand, we showed above that Δ P1234 grew faster and produced more butanol than the control N1-4-C strain. Therefore, we further compared the fermentation performance of Δ P1234 vs Δ P12345 in both serum bottles and bioreactors (Figs. 5d, 5e, S8, & S9). Results showed that the further deletion of P5 in Δ P12345 did not result in significant difference in cell growth or butanol production when compared to Δ P1234; actually the butanol production in Δ P12345 was slightly lower than in Δ P1234.

Discussion

We firstly set out to screen the host strains and ester synthesis genes for specific ester production. With the combination of five clostridial strains (*C. saccharoperbutylacetonicum* N1-4-C, *C. pasteurianum* ester SD-1, *C. beijerinckii* 8052, *C. tyrobutyricum* *cat1::adhE1* and *cat1::adhE2*) and five ester synthesis genes (*vaat*, *saat*, *atf1*, *eht1* and *lipaseB*), we obtained very promising results. Most of the engineered strains could produce EA, BA and BB at the same time (Fig. 2), and some of the strains could also produce small amount of EB. *C. saccharoperbutylacetonicum* FJ-004 produced 5.5 g/L BA, which was the highest BA production level that has ever been reported¹³. *C. pasteurianum* J-5 produced 0.3 g/L BB, which was also significantly higher than the previously reported level of 0.05 g/L in an engineered *C. acetobutylicum* strain^{13, 14}. The results confirmed our hypothesis that solventogenic clostridia are outstanding platforms to be engineered for ester production. Because the BA production in FJ-004 was significantly higher than the production levels of other esters, we decided to focus on further improving BA production in *C. saccharoperbutylacetonicum* through systematic metabolic engineering.

Butanol and acetyl-CoA are the two precursors for BA synthesis. The enhancement of the intracellular pool of these two precursors in the host could help improve BA production. We thus firstly deleted *nuoG* to save NADH and improve butanol and thus BA

production. Our fermentation results showed that FJ-101 with the deletion of *nuoG* had increased BA production to 7.8 g/L. However, there was still 7.6 g/L butanol remaining at the end of fermentation with FJ-101; it was thus reasonable to speculate that the availability of acetyl-CoA was the bottleneck for further improving BA production. We tried two strategies to improve intracellular acetyl-CoA availability. One was for the enhanced 'regeneration' of acetyl-CoA, and the other was for 'blocking' the pathway that consumes acetyl-CoA. Comparatively, the former seemed a better strategy. By introducing a heterologous isopropanol synthesis pathway to promote the 'regeneration' of intracellular acetyl-CoA, the FJ-301 strain could produce up to 12.9 g/L BA (Fig. 3b).

The dynamic expression of the heterologous pathway to be synchronous with the production of the precursors could be highly beneficial for the production of the target bioproduct. On the other hand, the imbalance of intracellular metabolism and the accumulation of toxic precursors would harm the cells and lead to decreased production of the target product. We hypothesized that the appropriate regulation of the BA synthesis enzyme using the native promoter of the host strain could achieve the similar effect as DSRS. In this work, four native promoters associated with BA precursors formation were selected and evaluated to control the *atf1* gene expression (Fig. 3c). Results indicated that the *atf1* gene controlled by the P_{adh} promoter showed 10.5% increase in BA production compared with the control FJ-301 strain (Fig. 3d). The P_{adh} promoter is responsible for the ethanol and butanol synthesis. The synchronous expression of alcohol dehydrogenase and ATF1 remarkably increased BA production.

Spatial organization of the enzymes associated with BA synthesis is another strategy that we employed to enhance BA production. The cross-link of the enzymes associated with BA synthesis or anchoring these enzymes onto a synthetic scaffold (PduA*) was not able to improve the BA production; while anchoring the ATF1 enzyme to the cell membrane by adding a MinD C-tag to the C-terminus of the enzyme led to significantly increased BA production. The obtained FJ-308 produced 16.4 g/L BA, which was 20% more than that in FJ-304 (Fig. 4). The attachment of ATF1 to the cell membrane facilitated the excretion of BA from the cells, which could mitigate the intracellular toxicity caused by BA and meanwhile boost the BA synthesis.

During the fermentation, the performance of the strain for BA production was not stable, and remarkable cell lysis was also observed at the end of the fermentation. We speculated that the instability of the strain could be because of the prophages existing in

the chromosome of *C. saccharoperbutylacetonicum*. Based on analysis, we identified four putative prophages P1-P4 and one incomplete prophage genome P5 in the genome of *C. saccharoperbutylacetonicum* N1-4 (HMT). P5 was demonstrated to be responsible for the synthesis of clostocin O (Figs. 5F & S5). Ultimately, we obtained two mutant strains Δ P1234 and Δ P12345, both of which grew faster and produced more butanol than the wild type strain (Figs. 5b-e, S3, S8, & S9). Thus, we further constructed the BA-producing strains FJ-1201 and FJ-1301 respectively based on Δ P1234 and Δ P12345. Fermentation results demonstrated that FJ-1201 could produce 20.3 g/L BA (Fig. 6A & Table S3), which was the highest production level of BA known to us in any microbial biocatalyst host¹³. The BA yield in FJ-1201 reached 0.26 g/g, which was also significantly higher than the initial BA-producing strain FJ-004 (0.07 g/g). Thus, the deletion of prophages from *C. saccharoperbutylacetonicum* could not only increase the cell growth (and stability) but also the production of the desired end products (butanol or BA).

In addition, we also noticed that the BB production in FJ-1201 reached 0.9 g/L, which was significantly higher than that in *C. pasteurianum* J-5 (0.3 g/L, the highest BB production level based on our initial screening of the strains and enzymes for ester production) (Fig. 6A & Table S3). We further overexpressed *saat* (instead of *atf1*) in the strain, and obtained the FJ-1202 strain in which the BB production reached unprecedented 1.3 g/L (Fig. 6D).

Both the BA-producing *C. saccharoperbutylacetonicum* FJ-1201 and the BB-producing *C. saccharoperbutylacetonicum* FJ-1202 performed well when biomass hydrolysates was used as the substrate for the fermentation. FJ-1201 could produce 17.8 g/L BA and FJ-1202 could produce 0.9 g/L BB from biomass hydrolysates (with no need to supplement any exogenous nitrogen source). Although these levels were slightly lower than when glucose was used as the substrate for the fermentation with the same strain, the operation eliminated the requirement of yeast extract and tryptone and thus would significantly decrease the cost of the bioprocess for fatty acid ester production.

Table S1 Strains and plasmids used in this study

Strains and plasmids	Relevant genotype and characteristics	Source
Strains		
<i>C. saccharoperbutylacetonicum</i> N1-4 (HMT)	DSM 14923 (= ATCC 27021), wild-type strain	DSMZ
<i>C. tyrobutyricum</i> Δ cat1::adhE1	ATCC 25755, cat1::adhE1	⁴
<i>C. tyrobutyricum</i> Δ cat1::adhE2	ATCC 25755, cat1::adhE2	⁴
<i>C. pasteurianum</i> SD-1	ATCC 6013 with the deletion of <i>cpaAI</i> gene	Lab stock
<i>Clostridium beijerinckii</i> NCIMB 8052	Wild-type strain	²²
<i>C. saccharoperbutylacetonicum</i>	Δ Csp_135p (with the endogenous plasmid Csp_135p)	²³

N1-4-C	eliminated)	
<i>C. saccharoperbutylacetonicum</i> FJ-001	N1-4-C harboring pMTL82151	This study
<i>C. saccharoperbutylacetonicum</i> FJ-002	N1-4-C harboring pMTL-P _{cat} - <i>vaat</i>	This study
<i>C. saccharoperbutylacetonicum</i> FJ-003	N1-4-C harboring pMTL-P _{cat} - <i>saat</i>	This study
<i>C. saccharoperbutylacetonicum</i> FJ-004	N1-4-C harboring pMTL-P _{cat} - <i>atfI</i>	This study
<i>C. saccharoperbutylacetonicum</i> FJ-005	N1-4-C harboring pMTL-P _{cat} - <i>ehlI</i>	This study
<i>C. saccharoperbutylacetonicum</i> FJ-006	N1-4-C harboring pMTL-P _{cat} - <i>lipaseB</i>	This study
<i>C. saccharoperbutylacetonicum</i> FJ-007	N1-4-C harboring pMTL-P _{cat} - <i>atfI</i> '	This study
<i>C. saccharoperbutylacetonicum</i> FJ-008	N1-4-C harboring pMTL-P _{thi} - <i>atfI</i>	This study
<i>C. saccharoperbutylacetonicum</i> FJ-100	N1-4-C, Δ <i>nuoG</i>	This study
<i>C. saccharoperbutylacetonicum</i> FJ-101	FJ-100 harboring pMTL-P _{cat} - <i>atfI</i>	This study
<i>C. saccharoperbutylacetonicum</i> FJ-200	FJ-100 with P _{thi} - <i>sadh</i> integrated at <i>Cspa_c21540</i> site	This study
<i>C. saccharoperbutylacetonicum</i> FJ-201	FJ-200 harboring pMTL-P _{cat} - <i>atfI</i>	This study
<i>C. saccharoperbutylacetonicum</i> FJ-300	FJ-100 with P _{thi} - <i>sadh</i> - <i>hydG</i> integrated at <i>Cspa_c21540</i> site	This study
<i>C. saccharoperbutylacetonicum</i> FJ-301	FJ-300 harboring pMTL-P _{cat} - <i>atfI</i>	This study
<i>C. saccharoperbutylacetonicum</i> FJ-302	FJ-300 harboring pMTL-P _{pfI} - <i>atfI</i>	This study
<i>C. saccharoperbutylacetonicum</i> FJ-303	FJ-300 harboring pMTL-P _{ald} - <i>atfI</i>	This study
<i>C. saccharoperbutylacetonicum</i> FJ-304	FJ-300 harboring pMTL-P _{adh} - <i>atfI</i>	This study
<i>C. saccharoperbutylacetonicum</i> FJ-305	FJ-300 harboring pMTL-P _{bdh} - <i>atfI</i>	This study
<i>C. saccharoperbutylacetonicum</i> FJ-306	FJ-300 harboring pMTL-P _{adh} -A- <i>atfI</i>	This study
<i>C. saccharoperbutylacetonicum</i> FJ-307	FJ-300 harboring pMTL-P _{adh} -A- <i>atfI</i> -MinD	This study
<i>C. saccharoperbutylacetonicum</i> FJ-308	FJ-300 harboring pMTL-P _{adh} - <i>atfI</i> -MinD	This study
<i>C. saccharoperbutylacetonicum</i> FJ-309	FJ-300 harboring pMTL-P _{adh} -A- <i>atfI</i> -B- <i>nifJ</i>	This study
<i>C. saccharoperbutylacetonicum</i> FJ-310	FJ-300 harboring pMTL-P _{adh} -A- <i>atfI</i> -B- <i>nifJ</i> -B- <i>bdhA</i>	This study
<i>C. saccharoperbutylacetonicum</i> FJ-311	FJ-300 harboring pTJ1-P _{cat} -B- <i>pduA</i> * and pMTL-P _{adh} -A- <i>atfI</i>	This study
<i>C. saccharoperbutylacetonicum</i> FJ-312	FJ-300 harboring pTJ1-P _{cat} -B- <i>pduA</i> *-A- <i>nifJ</i> and pMTL-P _{adh} -A- <i>atfI</i>	This study
<i>C. saccharoperbutylacetonicum</i> FJ-313	FJ-300 harboring pTJ1-P _{cat} -B- <i>pduA</i> *-A- <i>nifJ</i> -A- <i>bdhA</i> and pMTL-P _{adh} -A- <i>atfI</i>	This study
<i>C. saccharoperbutylacetonicum</i> FJ-400	FJ-300 derivative, Δ <i>Cspa_c20890</i>	This study
<i>C. saccharoperbutylacetonicum</i> FJ-401	FJ-400 harboring pMTL-P _{cat} - <i>atfI</i>	This study
<i>C. saccharoperbutylacetonicum</i> FJ-500	FJ-300 derivative, Δ <i>Cspa_c50320</i>	This study
<i>C. saccharoperbutylacetonicum</i> FJ-501	FJ-500 harboring pMTL-P _{cat} - <i>atfI</i>	This study
<i>C. saccharoperbutylacetonicum</i> FJ-1100	Δ P1234 derivative with the deletion of <i>nuoG</i>	This study
<i>C. saccharoperbutylacetonicum</i> FJ-1200	FJ-1100 derivative with the insertion of P _{thi} - <i>sadh</i> - <i>hydG</i>	This study

<i>C. saccharoperbutylacetonicum</i> FJ-1201	FJ-1200 harboring pMTL-P _{adh} -atf1-MinD	This study
<i>C. saccharoperbutylacetonicum</i> FJ-1300	ΔP12345 derivative with the deletion of <i>nuoG</i> and insertion of P _{thi} -sadh-hydG	This study
<i>C. saccharoperbutylacetonicum</i> FJ-1301	FJ-1300 harboring pMTL-P _{adh} -atf1-MinD	This study
<i>C. saccharoperbutylacetonicum</i> FJ-1202	FJ-1200 harboring pMTL-P _{cat} -saat	This study
<i>C. saccharoperbutylacetonicum</i> FJ-1203	FJ-1200 harboring pMTL-P _{cat} -eht1	This study
<i>C. saccharoperbutylacetonicum</i> FJ-1204	FJ-1200 harboring pMTL-P _{cat} -saat-MinD	This study
<i>C. saccharoperbutylacetonicum</i> YM016	FJ-1200 derivative with the deletion of <i>rex</i>	This study
<i>C. saccharoperbutylacetonicum</i> YM016PB	YM016 harboring pMTL-P _{cat} -saat	This study
<i>C. saccharoperbutylacetonicum</i> YM028	YM016 derivative, Δ <i>ldh1</i> ::P _{adh} -atf1-MinD, Δ <i>lacZ1</i> ::P _{adh} -atf1-MinD, Δ <i>lacZ2</i> ::P _{adh} -atf1-MinD, Δ <i>ctfA1</i> - <i>ctfB1</i>	This study
<i>C. saccharoperbutylacetonicum</i> YM028P	YM028 harboring pMTL-P _{adh} -atf1-MinD	This study
<i>C. saccharoperbutylacetonicum</i> ΔP1	N1-4-C strain with the deletion of prophage HM T (P1)	This study
<i>C. saccharoperbutylacetonicum</i> ΔP2	N1-4-C strain with the deletion of putative phage P2	This study
<i>C. saccharoperbutylacetonicum</i> ΔP3	N1-4-C strain with the deletion of putative phage P3	This study
<i>C. saccharoperbutylacetonicum</i> ΔP4	N1-4-C strain with the deletion of putative phage P4	This study
<i>C. saccharoperbutylacetonicum</i> ΔNP1	N1-4-C strain with the deletion of <i>Cspa_c09880</i> (NP1) gene	This study
<i>C. saccharoperbutylacetonicum</i> ΔNP2	N1-4-C strain with the deletion of <i>Cspa_c26510</i> (NP2) gene	This study
<i>C. saccharoperbutylacetonicum</i> ΔNP3	N1-4-C strain with the deletion of <i>Cspa_c36800</i> (NP3) gene	This study
<i>C. saccharoperbutylacetonicum</i> ΔNP4	N1-4-C strain with the deletion of <i>Cspa_c57380</i> (NP4) gene	This study
<i>C. saccharoperbutylacetonicum</i> ΔNP1234	N1-4-C strain with the deletion of <i>Cspa_c09880</i> , <i>Cspa_c26510</i> , <i>Cspa_c36800</i> and <i>Cspa_c57380</i>	This study
<i>C. saccharoperbutylacetonicum</i> ΔP123	N1-4-C strain with the deletion of putative prophages P1, P2 and P3	This study
<i>C. saccharoperbutylacetonicum</i> ΔP124	N1-4-C strain with the deletion of putative prophages P1, P2 and P4	This study
<i>C. saccharoperbutylacetonicum</i> ΔP134	N1-4-C strain with the deletion of putative prophages P1, P3 and P4	This study
<i>C. saccharoperbutylacetonicum</i> ΔP234	N1-4-C strain with the deletion of putative prophages P2, P3 and P4	This study
<i>C. saccharoperbutylacetonicum</i> ΔP1234	N1-4-C strain with the deletion of putative prophages P1, P2, P3 and P4	This study
<i>C. saccharoperbutylacetonicum</i> ΔP2345	N1-4-C strain with the deletion of putative prophages P2, P3, P4 and P5	This study
<i>C. saccharoperbutylacetonicum</i> ΔP1345	N1-4-C strain with the deletion of putative prophages P1, P3, P4 and P5	This study
<i>C. saccharoperbutylacetonicum</i> ΔP1245	N1-4-C strain with the deletion of putative prophages P1, P2, P4 and P5	This study
<i>C. saccharoperbutylacetonicum</i> ΔP1235	N1-4-C strain with the deletion of putative prophages P1, P2, P3 and P5	This study
<i>C. saccharoperbutylacetonicum</i> ΔP12345	N1-4-C strain with the deletion of putative prophages P1, P2, P3, P4 and P5	This study
<i>C. pasteurianum</i> J-1	SD-1 harboring pMTL82151	This study
<i>C. pasteurianum</i> J-2	SD-1 harboring pMTL-P _{cat} -vaat	This study
<i>C. pasteurianum</i> J-3	SD-1 harboring pMTL-P _{cat} -saat	This study
<i>C. pasteurianum</i> J-4	SD-1 harboring pMTL-P _{cat} -atf1	This study
<i>C. pasteurianum</i> J-5	SD-1 harboring pMTL-P _{cat} -eht1	This study
<i>C. pasteurianum</i> J-6	SD-1 harboring pMTL-P _{cat} -lipaseB	This study

<i>Clostridium beijerinckii</i> F-1	NCIMB 8052 harboring pTJ1	This study
<i>Clostridium beijerinckii</i> F-2	NCIMB 8052 harboring pTJ1-P _{cat} - <i>vaat</i>	This study
<i>Clostridium beijerinckii</i> F-3	NCIMB 8052 harboring pTJ1-P _{cat} - <i>saat</i>	This study
<i>Clostridium beijerinckii</i> F-4	NCIMB 8052 harboring pTJ1-P _{cat} - <i>atfI</i>	This study
<i>Clostridium beijerinckii</i> F-5	NCIMB 8052 harboring pTJ1-P _{cat} - <i>ehl1</i>	This study
<i>Clostridium beijerinckii</i> F-6	NCIMB 8052 harboring pTJ1-P _{cat} - <i>lipaseB</i>	This study
<i>C. tyrobutyricum</i> JZ-1	Δ cat1::adhE1 harboring pMTL82152	This study
<i>C. tyrobutyricum</i> JZ-2	Δ cat1::adhE1 harboring pMTL-P _{cat} - <i>vaat</i>	This study
<i>C. tyrobutyricum</i> JZ-3	Δ cat1::adhE1 harboring pMTL-P _{cat} - <i>saat</i>	This study
<i>C. tyrobutyricum</i> JZ-4	Δ cat1::adhE1 harboring pMTL-P _{cat} - <i>atfI</i>	This study
<i>C. tyrobutyricum</i> JZ-5	Δ cat1::adhE1 harboring pMTL-P _{cat} - <i>ehl1</i>	This study
<i>C. tyrobutyricum</i> JZ-6	Δ cat1::adhE1 harboring pMTL-P _{cat} - <i>lipaseB</i>	This study
<i>C. tyrobutyricum</i> JZ-7	Δ cat1::adhE2 harboring pMTL82152	This study
<i>C. tyrobutyricum</i> JZ-8	Δ cat1::adhE2 harboring pMTL-P _{cat} - <i>vaat</i>	This study
<i>C. tyrobutyricum</i> JZ-9	Δ cat1::adhE2 harboring pMTL-P _{cat} - <i>saat</i>	This study
<i>C. tyrobutyricum</i> JZ-10	Δ cat1::adhE2 harboring pMTL-P _{cat} - <i>atfI</i>	This stud
<i>C. tyrobutyricum</i> JZ-11	Δ cat1::adhE2 harboring pMTL-P _{cat} - <i>ehl1</i>	This study
<i>C. tyrobutyricum</i> JZ-12	Δ cat1::adhE2 harboring pMTL-P _{cat} - <i>lipaseB</i>	This study
<i>E. coli</i> CA434	hsd20(rB-, mB-), recA13, rpsL20, leu, proA2, with IncPb conjugative plasmid R702	²⁴
<i>E. coli</i> DH5 α	F ⁻ , ϕ 80d/lacZ Δ M1, Δ (lacZYA-argF)U169, <i>deoR</i> , <i>recA1</i> , <i>endA1</i> , <i>hsdR17</i> (r _K ⁻ , m _K ⁻), <i>phoA</i> , <i>supE44</i> , λ ⁻ <i>thi-1</i> , <i>gyrA96</i> , <i>relA1</i>	NEB
Plasmids		
pYW34	CAK <i>ori</i> , Amp ^r , Erm ^r , Plac::Cas9, gRNA	³
pMTL82151	pBP1 <i>ori</i> , <i>catP</i> , ColE1, <i>tra</i>	²⁵
pYW34- Δ nuoG	Derivative of pYW34 for <i>nuoG</i> deletion	This study
pYW34- <i>sadh</i>	Derivative of pYW34 for P _{thl} - <i>sadh</i> integration	²⁶
pYW34- <i>sadh-hydG</i>	Derivative of pYW34 for P _{thl} - <i>sadh-hydG</i> integration	²⁶
pYW34- Δ CspA c06180	Derivative of pYW34 for <i>CspA</i> c06180 deletion	This study
pYW34- Δ CspA c17310	Derivative of pYW34 for <i>CspA</i> c17310 deletion	This study
pYW34- Δ CspA c20520	Derivative of pYW34 for <i>CspA</i> c20520 deletion	This study
pYW34- Δ CspA c20890	Derivative of pYW34 for <i>CspA</i> c20890 deletion	This study
pYW34- Δ CspA c50320	Derivative of pYW34 for <i>CspA</i> c50320 deletion	This study
pYW34- Δ P1	Derivative of pYW34 for P1 phage deletion	This study
pYW34- Δ P2	Derivative of pYW34 for P2 phage deletion	This study
pYW34- Δ P3	Derivative of pYW34 for P3 phage deletion	This study
pYW34- Δ P4	Derivative of pYW34 for P4 phage deletion	This study
pYW34- Δ P5	Derivative of pYW34 for P5 phage deletion	This study
pYW34- Δ NP1	Derivative of pYW34 for <i>CspA</i> c09880 deletion	This study
pYW34- Δ NP2	Derivative of pYW34 for <i>CspA</i> c26510 deletion	This study
pYW34- Δ NP3	Derivative of pYW34 for <i>CspA</i> c36800 deletion	This study
pYW34- Δ NP4	Derivative of pYW34 for <i>CspA</i> c57380 deletion	This study
pMTL82151-P _{cat}	=pJZ98-P _{cat} 1, pMTL82151 derivative, containing P _{cat} for gene overexpression purpose	⁴
pMTL82151-P _{thl}	pMTL82151 derivative, containing P _{thl} for gene overexpression purpose	Lab stock
pMTL82151-P _{pfl}	pMTL82151 derivative, containing P _{pfl} for gene overexpression purpose	This study
pMTL82151-P _{bld}	pMTL82151 derivative, containing P _{bld} for gene overexpression purpose	This study
pMTL82151-P _{adh}	pMTL82151 derivative, containing P _{adh} for gene overexpression purpose	This study
pMTL82151-P _{bdh}	pMTL82151 derivative, containing P _{bdh} for gene overexpression purpose	This study
pMTL-P _{cat} - <i>vaat</i>	pMTL82151-P _{cat} derivative, with <i>vaat</i> inserted downstream of P _{cat}	This study
pMTL-P _{cat} - <i>saat</i>	pMTL82151-P _{cat} derivative, with <i>saat</i> inserted downstream of P _{cat}	This study
pMTL-P _{cat} - <i>atfI</i>	pMTL82151-P _{cat} derivative, with <i>atfI</i> inserted downstream of P _{cat}	This study
pMTL-P _{cat} - <i>ehl1</i>	pMTL82151-P _{cat} derivative, with <i>ehl1</i> inserted downstream of P _{cat}	This study
pMTL-P _{cat} - <i>lipaseB</i>	pMTL82151-P _{cat} derivative, with <i>lipaseB</i> inserted	This study

	downstream of P _{cat}	
pMTL-P _{cat} - <i>atfI</i> '	pMTL82151-P _{cat} derivative, with <i>atfI</i> ' inserted downstream of P _{cat}	This study
pMTL-P _{thl} - <i>atfI</i>	pMTL82151-P _{thl} derivative, with <i>atfI</i> inserted downstream of P _{thl}	This study
pMTL-P _{pfl} - <i>atfI</i>	pMTL82151-P _{pfl} derivative, with <i>atfI</i> inserted downstream of P _{pfl}	This study
pMTL-P _{bld} - <i>atfI</i>	pMTL82151-P _{bld} derivative, with <i>atfI</i> inserted downstream of P _{bld}	This study
pMTL-P _{adh} - <i>atfI</i>	pMTL82151-P _{adh} derivative, with <i>atfI</i> inserted downstream of P _{adh}	This study
pMTL-P _{bdh} - <i>atfI</i>	pMTL82151-P _{bdh} derivative, with <i>atfI</i> inserted downstream of P _{bdh}	This study
pMTL-P _{adh} -CC-Di-A	pMTL82151-P _{adh} derivative, CC-Di-A inserted downstream of P _{adh}	This study
pMTL-P _{adh} - <i>atfI</i> -MinD	pMTL82151-P _{adh} derivative, with <i>atfI</i> -MinD inserted downstream of P _{adh}	This study
pMTL-P _{cat} - <i>saat</i> -MinD	pMTL82151-P _{cat} derivative, with <i>saat</i> -MinD inserted downstream of P _{cat}	This study
pMTL-P _{adh} -A- <i>atfI</i>	pMTL82151-P _{adh} derivative, with CC-Di-A- <i>atfI</i> fragment inserted downstream of P _{adh}	This study
pMTL-P _{adh} -A- <i>atfI</i> -MinD	pMTL82151-P _{adh} derivative, with CC-Di-A- <i>atfI</i> -MinD fragment inserted downstream of P _{adh}	This study
pMTL-P _{adh} -A- <i>atfI</i> -B- <i>nifJ</i>	pMTL-P _{adh} -A- <i>atfI</i> derivative, with CC-Di-B- <i>nifJ</i> fragment inserted downstream of the <i>atfI</i> gene	This study
pMTL-P _{adh} -A- <i>atfI</i> -B- <i>nifJ</i> -B- <i>bdhA</i>	pMTL-P _{adh} -A- <i>atfI</i> -B- <i>nifJ</i> derivative, with CC-Di-B- <i>bdhA</i> fragment inserted downstream of the <i>nifJ</i> gene	This study
pMTL-P _{adh} -A- <i>atfI</i> -MinD-B- <i>nifJ</i>	pMTL-P _{adh} -A- <i>atfI</i> -MinD derivative, with CC-Di-B- <i>nifJ</i> fragment inserted downstream of the <i>atfI</i> -MinD gene	This study
pMTL-P _{adh} -A- <i>atfI</i> -MinD-B- <i>nifJ</i> -B- <i>bdhA</i>	pMTL-P _{adh} -A- <i>atfI</i> -MinD-B- <i>nifJ</i> derivative, with CC-Di-B- <i>bdhA</i> fragment inserted downstream of the <i>atfI</i> -MinD gene	This study
pTJ1-P _{cat} -B- <i>pduA</i> *	pTJ1-P _{cat} derivative with the insertion of CC-Di-B- <i>pduA</i> *	This study
pTJ1-P _{cat} -B- <i>pduA</i> *-A- <i>nifJ</i>	pTJ1-P _{cat} -B- <i>pduA</i> * derivative with the insertion of CC-Di-A- <i>nifJ</i>	This study
pTJ1-P _{cat} -B- <i>pduA</i> *-A- <i>nifJ</i> -A- <i>bdhA</i>	pTJ1-P _{cat} -B- <i>pduA</i> *-A- <i>nifJ</i> derivative with the insertion of CC-Di-A- <i>bdhA</i>	This study
pTJ1	pYL102E derivative, Amp ^r , Erm ^r	22
pTJ1-P _{cat}	pTJ1 derivative, with the insertion of P _{cat} promoter	This study
pTJ1-P _{cat} - <i>vaat</i>	pTJ1 derivative, with the insertion of P _{cat} - <i>vaat</i>	This study
pTJ1-P _{cat} - <i>saat</i>	pTJ1 derivative, with the insertion of P _{cat} - <i>saat</i>	This study
pTJ1-P _{cat} - <i>atfI</i>	pTJ1 derivative, with the insertion of P _{cat} - <i>atfI</i>	This study
pTJ1-P _{cat} - <i>ehlI</i>	pTJ1 derivative, with the insertion of P _{cat} - <i>ehlI</i>	This study
pTJ1-P _{cat} - <i>lipaseB</i>	pTJ1 derivative, with the insertion of P _{cat} - <i>lipaseB</i>	This study
pDL004	pETite* carrying <i>atfI</i> from <i>S. cerevisiae</i>	27
pDL001	pETite* carrying <i>saat</i> from <i>Fragaria ananassa</i>	28
pDL006	pETite* carrying <i>vaat</i> from <i>F. vesca</i>	27
pYW34-Δ <i>rex</i>	Derivative of pYW34 for <i>rex</i> deletion	
pYW34-Δ <i>ldhI</i> ::P _{adh} - <i>atfI</i> -MinD	Derivative of pYW34 for <i>ldhI</i> deletion and P _{adh} - <i>atfI</i> -MinD cassette integration	
pYW34-Δ <i>lacZ1</i> ::P _{adh} - <i>atfI</i> -MinD	Derivative of pYW34 for <i>lacZ1</i> deletion and P _{adh} - <i>atfI</i> -MinD cassette integration	
pYW34-Δ <i>lacZ2</i> ::P _{adh} - <i>atfI</i> -MinD	Derivative of pYW34 for <i>lacZ2</i> deletion and P _{adh} - <i>atfI</i> -MinD cassette integration	
pYW34-Δ <i>ctfA1</i> - <i>ctfB1</i>	Derivative of pYW34 for <i>ctfA1</i> - <i>ctfB1</i> deletion	

Table S2 – Primers Used In Working Example

Primers	Sequence (5' to 3')	SEQ ID	Purpose
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		NO.	
nuoG-UF	TGATATGACTAATAATTAGCGGCCGCTAGTATTTTCA GGAATTTCTCCAAT	8	pYW34- <i>ΔnuoG</i>
nuoG-UR	TTGAAAGACATTAAGAGGGGCAACGATTAACATGA TAAATG	9	
nuoG-DF	TTAATCGTTGCCCCTCTTAATGTCTTTCAAACATAAT ACC	10	
nuoG-DR	ACTAGTAACCATCACACTGTAAGATAACCTTTGAATT GTTACGC	11	
nuoG-U	ATTGCTTGAATTTCTGCTTCAAAAC	12	
nuoG-D	TAGATAACATGATATCCAAGGTCGC	13	
nuoG-20nt	GCTCAGTCCTAGGTATAATGCTAGCTTTACTTCATCT TTTGAAGCGTTTTAGAGCTAGAAATAGCAAG	14	
YW1139	GTCATAAACTTGCTCAACTGATATGATCTTCCATAAC TTAAC	15	gRNA amplificati on
YW1142	ATATAGGTAATCGCTTTTATAAGGGCCCGATCGGTCT TGCCTTGCTCGTCG	16	
Cspa_c06180-UF	TGATATGACTAATAATTAGCGGCCGCCCCCATTAGA TACTAAAGAAACTCA	17	pYW34- <i>ΔCspa_c06180</i>
Cspa_c06180-UR	CTAAACTCTTTCAACAACACTACGTCTCTCATGTTTGAC CTC	18	
Cspa_c06180-DF	ATGAGAGACGTAGTTGTTGAAAGAGTTTAGTATACA AGTT	19	
Cspa_c06180-DR	ACTAGTAACCATCACACTGGGTTTGTAAAAGCAGTTT ATTCATC	20	
Cspa_c06180-U	GTTTTTGAGAGATTAGGTGGAAAGG	21	
Cspa_c06180-D	TCCCTACTGCATAGATTCTCTGCATT	22	
Cspa_c06180-20nt	GCTCAGTCCTAGGTATAATGCTAGCTCAAAAAGTAAA TGTTAATGGGTTTTAGAGCTAGAAATAGCAAG	23	
Cspa_c17310-UF	TGATATGACTAATAATTAGCGGCCGCCAGACAGTGG CCTTATCAATAGGAG	24	pYW34- <i>ΔCspa_c17310</i>
Cspa_c17310-UR	TTATAATCTTTCTACAACACTACTTCTTTCATTTATGCTC CT	25	
Cspa_c17310-DF	ATGAAAGAAGTAGTTGTAGAAAGATTATAATAATAG ATAA	26	
Cspa_c17310-DR	ACTAGTAACCATCACACTGCCTCTTTTTTCTTTTTCAT TGCTTC	27	
Cspa_c17310-U	TCAGGGACAGCAGTTCATGTAGTAA	28	
Cspa_c17310-D	TTATGGTAAAGCCTAATACAAACCC	29	
Cspa_c17310-20nt	GCTCAGTCCTAGGTATAATGCTAGCAAGAAAGTTAA	30	

	TGTAAGTGGGTTTTAGAGCTAGAAATAGCAAG		
Cspa_c20520-UF	TGATATGACTAATAATTAGCGGCCGCAAGACATTAA AGATTCTGCAACAGC	31	pYW34- Δ Cspa_c20 520
Cspa_c20520-UR	TTATTCTCTTTCAACTACTACATCTTTCATTTTTTATT CCTC	32	
Cspa_c20520-DF	ATGAAAGATGTAGTAGTTGAAAGAGAATAATTGAAT TTAA	33	
Cspa_c20520-DR	ACTAGTAACCATCACACTGATGCAAGATACACAAGC TATAGGAT	34	
Cspa_c20520-U	TTTTATGTCATAGCAATTGAGGGAA	35	
Cspa_c20520-D	TTGTTCTTTATATTTTTCATTGGGC	36	
Cspa_c20520-20nt	GCTCAGTCCTAGGTATAATGCTAGCCTTGCAACCTTG TGTATTGGGTTTTAGAGCTAGAAATAGCAAG	37	
Cspa_c20890-UF	TGATATGACTAATAATTAGCGGCCGCAATAACTGAT GTAGCCAGTGCAACT	38	pYW34- Δ Cspa_c20 890
Cspa_c20890-UR	TCAATTGCATCTTTCTACTACTTCTTTCATTTTTAACT CC	39	
Cspa_c20890-DF	ATGAAAGAAGTAGTAGAAAGATGCAATTGATAGAG GAATG	40	
Cspa_c20890-DR	ACTAGTAACCATCACACTGAATTTTCTTACCGCTACA AAGATCA	41	
Cspa_c20890-U	AATATTTGCTGAACTTGATGACGTA	42	
Cspa_c20890-D	ACCATTTAATACATAATGGTCACCT	43	
Cspa_c20890-20nt	GCTCAGTCCTAGGTATAATGCTAGCTGTAAATGATG CAAGATGGGTTTTAGAGCTAGAAATAGCAAG	44	
Cspa_c50320-UF	TGATATGACTAATAATTAGCGGCCGCTGACAATAAT GTGTTTAAATTCCT	45	pYW34- Δ Cspa_c50 320
Cspa_c50320-UR	GTGGAAAGTGTATATATTTAAAAGGAGCTAAGGATGA ACTAT	46	
Cspa_c50320-DF	TTAGCTCCTTTTAAATATATACACTTTCCACTTATAAA ACT	47	
Cspa_c50320-DR	ACTAGTAACCATCACACTGCTATTTTAGGATATGTTG ATAATGC	48	
Cspa_c50320-U	TTTCATTGATTTTATCCATCTTCTT	49	
Cspa_c50320-D	TTAATCCTATTTATTCAGATGAAGA	50	
Cspa_c50320-20nt	GCTCAGTCCTAGGTATAATGCTAGCAACTGTGCTACT TGAAAATAGTTTTAGAGCTAGAAATAGCAAG	51	
P1-UF	TGATATGACTAATAATTAGCATCAACCAGGGAATTT ATAAGAAGC	52	pYW34- Δ P1

P1-UR	AGCTGCTAATTTAGTATCAGTCTTCATTTAATGGAAT TTACATTAA	53	
P1-DF	TAAATGAAGACTGATACTAAATTAGCAGCTGAAAAT GAAAGT	54	
P1-DR	ACTAGTAACCATCACACTGGCCCCAAAATTCCTTTAAC TTGCTCGTA	55	
P1-U	TATTAGTGCAAAATGGCTTTATGAC	56	
P1-D	GCATCTGAATCCATTATTTTAGTTT	57	
P1-20nt	GCTCAGTCCTAGGTATAATGCTAGCGATCATATATTG CTAAACATGTTTTAGAGCTAGAAATAGCAAG	58	
P2-UF	TGATATGACTAATAATTAGCGAAGAGAAAGAAAGCA AATATTACC	59	pYW34- ΔP2
P2-UR	ATAGCTCCTATCTTTGTGTTAAAAATGTGTTAATAAT AAA	60	
P2-DF	TTAACACATTTTTAACACAAAGATAGGAGCTATATAT GAAA	61	
P2-DR	ACTAGTAACCATCACACTGGCTTTAACTTCGATCCTC TTAGCACCT	62	
P2-U	CCAAGTGAAAAAATTGAATTTGATA	63	
P2-D	AATTTTTATTAAGACCCTGCCGCTA	64	
P2-20nt	GCTCAGTCCTAGGTATAATGCTAGCGGAGCTGCAAC AACTATAAAGTTTTAGAGCTAGAAATAGCAAG	65	pYW34- ΔP3
P3-UF	TGATATGACTAATAATTAGC GCTTAATATTGTCTATTGATACCGA	66	
P3-UR	GAATAAAAGATGATTAGATATGCTCGAATTTGATTA TTATAA	67	
P3-DF	AATTCGAGCATATCTAATCATCTTTTATTCTATACAC ATTA	68	
P3-DR	ACTAGTAACCATCACACTGGCTTATAAATCTTTATGG GGCAAGAGG	69	
P3-U	TTTTCCAAATAATTTGCTTAATCCT	70	
P3-D	CAAGACAATAATTATCGAAAAACA	71	pYW34- ΔP4
P3-20nt	GCTCAGTCCTAGGTATAATGCTAGCTCACCTCCATAT TTTCTTCTGTTTTAGAGCTAGAAATAGCAAG	72	
P4-UF	TGATATGACTAATAATTAGCCAGAGCTTTGTGTAATT GATGTAAC	73	
P4-UR	TATATTATCAAGCAAATAACCCGTAAGTAACAATTA AGCT	74	
P4-DF	TTACTTACGGGTATTGCTTGATAATATATTTAAAT ATA	75	

P4-DR	ACTAGTAACCATCACACTGGCCTAATGAAGCCAATA TGGAGAACT	76	pYW34- ΔP5
P4-U	ATGCAACATACCGGAAAAGGTAAAC	77	
P4-D	AATAGATAGGTTGCAAAAGGAAATG	78	
P4-20nt	GCTCAGTCCTAGGTATAATGCTAGCTCTCTTAAATCA CTCATTATGTTTTAGAGCTAGAAATAGCAAG	79	
P5-UF	TGATATGACTAATAATTAGCATTTGTATGAAGGTAGC GGTTGAGG	80	
P5-UR	TAAGATAGTTTAGTTTCCTTTACAAATTAATGTATTT TTTAA	81	
P5-DF	TTAATTTGTAAAGGAACTAACTATCTTAGAAGAC AGAC	82	
P5-DR	ACTAGTAACCATCACACTGGCCTTAGGAATGATATT CGAAAAAGCA	83	
P5-U	TCTTTCCTTATAAAGAGTATCGTTTCTAA	84	
P5-D	CAATTAAAGTATGAACAAAAGCATGG	85	
P5-20nt	GCTCAGTCCTAGGTATAATGCTAGCTTCAAAATTACA AGCTTCATGTTTTAGAGCTAGAAATAGCAAG	86	pYW34- ΔNP1
Cspa_c09880-UF	TGATATGACTAATAATTAGCAATTTCAAGCATAATG GTTATTTCA	87	
Cspa_c09880-UR	ATGAAAGCAGCTATTAAAGGCGCTCTTAATGTAGC GCCC	88	
Cspa_c09880-DF	TTAAAGAGCGCCTTTAATAGCTGCTTTCATAGTATAC CTCC	89	
Cspa_c09880-DR	ACTAGTAACCATCACACTGGCTCTTAATCAGTCTTCA TTTAATGGA	90	
Cspa_c09880-U	GGAGATGCTTTTTATGATAAAGAAA	91	
Cspa_c09880-D	ATATCATTAATACTGATAATGGCGG	92	
Cspa_c09880-20nt	GCTCAGTCCTAGGTATAATGCTAGCCATTAACCAGTC TTGGTGCAGTTTTAGAGCTAGAAATAGCAAG	93	
Cspa_c26510-UF	TGATATGACTAATAATTAGCGAAAGCCTCAGGATGG AAAGATAGT	94	pYW34- ΔNP2
Cspa_c26510-UR	ATGGCAAATAAACTTTAAAAATGTGTTAATAATAA AAATAGC	95	
Cspa_c26510-DF	TTAACACATTTTTAAAGTTTTATTTGCCATAAAATCA TCC	96	
Cspa_c26510-DR	ACTAGTAACCATCACACTGGCAGAAAACAGAAAGG ATTAATCAAC	97	
Cspa_c26510-U	GCTGTGCTGGTATCTAAAATAAAGG	98	

Cspa_c26510-D	AGGTGAAATTATTGTGTTAGGTGA	99	pYW34- ΔNP3
Cspa_c26510-20nt	GCTCAGTCCTAGGTATAATGCTAGCGGAATAGTTACT TTTAAGCAGTTTTAGAGCTAGAAATAGCAAG	100	
Cspa_c36800-UF	TGATATGACTAATAATTAGCAGCTGACAAATTA GTTAGTGAA	101	
Cspa_c36800-UR	CTAATTTAGCAACGCAGTCTTTACAGCCATAAGTAA AACC	102	
Cspa_c36800-DF	ATGGCTGTAAAGACTGCGTTGCTAAATTAGAAATAT AAAC	103	
Cspa_c36800-DR	ACTAGTAACCATCACACTGGCGGAATGTTTATACCA GTCCTATTCA	104	
Cspa_c36800-U	AGAAAAATACAGAAATGATCTTCAA	105	
Cspa_c36800-D	GGTACTTAATTTCTCTTTGGTTTC	106	
Cspa_c36800-20nt	GCTCAGTCCTAGGTATAATGCTAGCTGATATAGATTA TGATAATAGTTTTAGAGCTAGAAATAGCAAG	107	
Cspa_c57380-UF	TGATATGACTAATAATTAGCAATTTCTGAACATTTTA AAGGAAGA	108	pYW34- ΔNP4
Cspa_c57380-UR	TTTAAATATATTATCCTTATAACTTGCCATAAATACC ACC	109	
Cspa_c57380-DF	ATGGCAAGTTATAAGGATAATATATTTAAATATAAA CCTC	110	
Cspa_c57380-DR	ACTAGTAACCATCACACTGGCCTAAGGAAGAAAACA AAATAGCCAA	111	
Cspa_c57380-U	TACATAAATTCTTTCCAGAAGGGAT	112	
Cspa_c57380-D	GCCCAATTAAGGAGTAGAAG	113	
Cspa_c57380-20nt	GCTCAGTCCTAGGTATAATGCTAGCATATTTAAATGT TACCCACCGTTTTAGAGCTAGAAATAGCAAG	114	
pMTL-KZR	AGATCTCCATGGACGCGTGACGTCG	115	
P _{bld} -F	TCCATATGACCATGATTACGTTAAATAAATTTCTTA AAAAACAATAT	116	pMTL8215 1-P _{bld}
P _{bld} -R	ATCCCCGGGTACCGAGCTCGAATTCGACTAATCCTCC TTATGATTTAAAA	117	
P _{bdh} -F	TCCATATGACCATGATTACGGTTAAGCCACTACCAG GATCAATCA	118	pMTL8215 1-P _{bdh}
P _{bdh} -R	ATCCCCGGGTACCGAGCTCGAATTCCTTTTCTCC TCTTACACACGCA	119	
P _{adh} -F	TCCATATGACCATGATTACGACAGGAGATAGCAAAT ACAGAGCTA	120	pMTL8215 1-P _{adh}
P _{adh} -R	ATCCCCGGGTACCGAGCTCGAATTCATTTTAATAAC CCTCCTCAAAATA	121	

P _{pfl} -F	TCCATATGACCATGATTACGTTAGATCCTAAAAAATA TGGCGTTA	122	pMTL8215 1-P _{pfl}
P _{pfl} -R	ATCCCCGGGTACCGAGCTCGAATTCAAATTAACCAC CTCCCAAATTGAAA	123	
vaat-F	ATATAATTTATGAAAGGGTGGTTTTTATGGAGAAAA TTGAGGTCAGT	124	pMTL-P _{cat} - <i>vaat</i>
vaat-R	CGACTCTAGAGGATCCCCGGGTACCGAATTCTCAAT ATCTTGAAATTAGCGTC	125	
saat-F	ATATAATTTATGAAAGGGTGGTTTTTATGGAGAAAA TTGAGGTCAGT	126	pMTL-P _{cat} - <i>saat</i>
saat-R	CGACTCTAGAGGATCCCCGGGTACCGAATTCTTAAA TTAAGGTCTTTGGAGATGC	127	
atf1-F	ATATAATTTATGAAAGGGTGGTTTTTATGAATGAAAT CGATGAGAAAAATCA	128	pMTL-P _{cat} - <i>atf1</i>
atf1-R	CGACTCTAGAGGATCCCCGGGTACCGAATTCCTAAG GGCCTAAAAGGAGAGCTTTGT	129	
eht1-F	ATATAATTTATGAAAGGGTGGTTTTTATGTCAGAAAGT ATCTAAGTGG	130	pMTL-P _{cat} - <i>eht1</i>
eht1-R	CGACTCTAGAGGATCCCCGGGTACCGAATTCTTATA CAACCAACTCGTCAAAC	131	
lipaseB-F	ATATAATTTATGAAAGGGTGGTTTTTATGAGAAAAAG TATTTTTAAGATC	132	pMTL-P _{cat} - <i>lipaseB</i>
lipaseB-R	CGACTCTAGAGGATCCCCGGGTACCGAATTCCTAAG GAGTAACAATTCCTGAG	133	
atf1'-F	ATATAATTTATGAAAGGGTGGTTTTTATGAACGAGAT CGACGAGAAAAACC	134	pMTL-P _{cat} - <i>atf1</i>
atf1'-R	CGACTCTAGAGGATCCCCGGGTACCGAATTCTTACG GGCCTAACAGCAGCGC	135	
atf1-thl-F	ATATAATTTATGAAAGGGTGGTTTTTATGAATGAAAT CGATGAGAAAAATCA	136	pMTL-P _{thl} - <i>atf1</i>
atf1-thl-R	CGACTCTAGAGGATCCCCGGGTACCGAATTCCTAAG GGCCTAAAAGGAGAGCTTTGT	137	
atf1-bld-F	AATCATAAGGAGGATTAGTCATGAATGAAATCGATG AGAAAAATCA	138	pMTL8215 1-P _{bld} - <i>atf1</i>
atf1-bdh-F	GTGTAAGAGGAGGAAAAAGAAATGAATGAAATCGAT GAGAAAAATCA	139	pMTL8215 1-P _{bdh} - <i>atf1</i>
atf1-adh-F	TGAGGAGGGTTATTAATAATTATGAATGAAATCGATG AGAAAAATCA	140	pMTL8215 1-P _{adh} - <i>atf1</i>
atf1-pfl-F	ATTTGGGAGGTGGTTAATTTATGAATGAAATCGATG AGAAAAATCA	141	pMTL8215 1-P _{pfl} - <i>atf1</i>
atf1-promoter-R	ATCCCCGGGTACCGAGCTCGAATTCCTAAGGGCCTA	142	

	AAAGGAGAGCTTTGT		
atf1-MinD-R1	AGCCATCATCCCTTTGTTTTGTTCTCCAAGACTTGC AACGGCGATCCCGAACCCTAGTAGGGCCTAAAAGG AGAGCTT	143	Adding MinD-tag to <i>atf1</i> 3' end
atf1-MinD-R2	GATCCCCGGGTACCGAGCTCTTAGGAACGTACACCG AAAAATGATTTGATTTT	144	
saat-MinD-R1	GCCATCATCCCTTTGTTTTGTTCTCCAAGACTTGCA ACGGCGATCCCGAACCCTAGTAATTAAGGTCTTTG GAGATGC	145	Adding MinD-tag to <i>saat</i> 3' end
saat-MinD-R2	CTAGAGGATCCCCGGGTACCGAGCTCTTAGGAACGT ACACCGAAAAATGATTTGATTTTAGCCATCATCCCTT TGTTTTG	146	
adh-A-F	TGAGGAGGGTTATTAATAATTATGGGTAGCGAAATTG CCGCGCTGG	147	CC-Di-A amplificati on
adh-A-R	ATTTTCTCATCGATTTCATTCATATGGCTGCCGCGC GGCACCA	148	
B-nifJ-F1	ACTGAAAAAGAAAAACGCTGCGCTGAAACAGAAAA TTGCCGCGCTGAAACAGATGAGAAAAATGAAACTA TGATG	149	CC-Di-B- <i>nifJ</i> amplificati on
B-nifJ-F2	TTTAGGCCCTTAGGAGCTCGAAAGAGGGGTCAACGC GATGGGTAGCAAAATCGCCGCACTGAAAAAGAAAA ACGCTGCG	150	
B-nifJ-R	TCGACTCTAGAGGATCCCCGGGTACCCTATTGTTGAT TAGCTAACTTCTTG	151	
B-bdhA-F1	ACTGAAAAAGAAAAACGCTGCGCTGAAACAGAAAA TTGCCGCGCTGAAACAGATGATGAGATTTACATTAC CAAGAG	152	CC-Di-B- <i>bdhA</i> amplificati on
B-bdhA-F2	GTTAGCTAATCAACAATAGGAGGAGGGTTATTAATA TTATGGGTAGCAAAATCGCCGCACTGAAAAAGAAAA ACGCTGCG	153	
B-bdhA-R	TCGACTCTAGAGGATCCCCGGGTACCTTAAAAATCA ACCTTATTTCCATAA	154	
pMTL-TJ1-F	TTGTAAAACGACGCCAGTGGTAGACTTTAAGGATG GAACCTTTG	155	Amplificati on of fragments from pMTL8215 1-based plasmids
pMTL-TJ1-R	TTCTAACGCGTCACCTAAAGAGATCTCCATGGACGC GTGACGTCG	156	
rex-g1-all-F	AAGAAGAAAATAGAAATttgacagctagctcagtcctaggtataatG CTAGCTTGGAGATTTCTGGACAACAAGTTTATAGAGCT AGAAATA	157	pYW34- Δ_{rex}
rex-g1-all-R	TATTTCTAGCTCTAAAACCTTGTTGTCCGAAATCTCCA AGCTAGCATTATACCTAGGACTGAGCTAGCTGTCAA ATTTCTATTTTCTTCTT	158	

rex-Up-F	tatgactaataattagcgccgcGATATGTAGAGGATGTGATCAA GCTTT	159	
rex-Up-R	CCAAATAGCTCTTACTCCGCTTCGCTTTAAATCCCT ATTGTCCATCT	160	
rex-Down-F	TGGACAATAGGGATTTAAAGCGAAGGCGGAGTAAG AGCTATTTGG	161	
rex-Down-R	gtaaccatcacactggcgccgcTCTGAAATATCTGCTCCAGCAA CAA	162	
ldh1-g1-all-F	AAGAAGAAAATAGAAATttgacagctagctcagtcctaggataatG CTAGCTCAAGATTAATAATATGTCGTGTTTTAGAGCTA GAAATA	163	pYW34- Δ ldh1::P _{adh} -atf1-MinD
ldh1-g1-all-R	TATTTCTAGCTCTAAAACACGACATATTTTAATCTTG AGCTAGCATTATACCTAGGACTGAGCTAGCTGTCAA ATTTCTATTTTCTTCTT	164	
ZH-ldh1-Up-F	tatgactaataattagcgccgcTCTGCCAGTTGTCACTTGAGGTT TT	165	
ZH-ldh1-Up-R	GCTCTGTATTTGCTATCTCCTGCCATTACTTCTCCCAT AGCTTTATCCAT	166	
ZH-ldh1-Down-F	ATTTTTCGGTGTACGTTCCCTAAGGAGTTGTAGGAATT GTAAATCCAGTG	167	
ZH-ldh1-Down-R	gtaaccatcacactggcgccgcCTTGTGTTTCTCTATCTGATGCT GGA	168	
atf1-ZH-ldh1-F	AAAGCTATGGGAGAAGTAATGGCAGGAGATAGCAA ATACAGAGCTA	169	
atf1-ZH-ldh1-R	GATTTACAATTCTACAACCTCCTTAGGAACGTACACC GAAAAATGATTGATTTT	170	
lacZ1-g1-all-F	AAGAAGAAAATAGAAATttgacagctagctcagtcctaggataatG CTAGCTATAGCCTTTGTGACCGTGAGTTTTAGAGCTA GAAATA	171	
lacZ1-g1-all-R	TATTTCTAGCTCTAAAACCTCACGGTCACAAAGGCTAT AGCTAGCATTATACCTAGGACTGAGCTAGCTGTCAA ATTTCTATTTTCTTCTT	172	
lacZ1-Up-N-F	tatgactaataattagcgccgcCTTGTAGTCATTTTCAGCGTGTTT CG	173	pYW34- Δ lacZ1::P _{ad} h-atf1- MinD
lacZ1-Up-N-R	TCTTTAATTGCCACTTCTTGAAGCGGCCGCTCTCCTT TGAACATACCTCTCCAC	174	
lacZ1-Down-N-F	GGAGAGGGTATGTTCAAAGGAGAGCGGCCGCTTCAA GAAGTGGCAATTAAAGAGGG	175	
lacZ1-Down-N-R	gtaaccatcacactggcgccgcCTCCCATGGTACCTCCTTATTG AAA	176	
atf1-ZH-lacZ1-F	tatgttcaaaggagagcgccgcCAGGAGATAGCAAATACAGAG	177	

	CTA		
atf1-ZH-lacZ1-R	ttgccacttcttgaagcggccgcTTAGGAACGTACACCGAAAAAT GATTTGATTTT	178	
lacZ2-g1-all-F	AAGAAGAAAATAGAAATttgacagctagctcagtcctaggtataatG CTAGCAAGTATCCTAAATTACAAGGGTTTTAGAGCT AGAAATA	179	pYW34- Δ lacZ2::P _{ad} h-atf1- MinD
lacZ2-g1-all-R	TATTTCTAGCTCTAAAACCCTTGTAATTTAGGATACT TGCTAGCATTATACCTAGGACTGAGCTAGCTGTCAA ATTTCTATTTTCTTCTT	180	
lacZ2-Up-N-F	ctttgtgatagactaataattaACACGAGATATAGACGAAAGGAG G	181	
lacZ2-Up-N-R	TATTCCATGCTGCTTAAATCTGCGGCCGCATTGCTG CCATCATTTCTTTAGG	182	
lacZ2-Down-N-F	TAAAGGAAATGATGGCAGCAATGCGGCCGCAGATTT TAAGCAGCATGGAATAGGC	183	
lacZ2-Down-N-R	atccactagtaaccatcacactgGCAAAACCTGGTGTAAACATC CTT	184	
atf1-ZH-lacZ2-F	aatgatggcagcaatgcgccgcCAGGAGATAGCAAATACAGAG CTA	185	
atf1-ZH-lacZ2-R	tgctgcttaaaatctgcgccgcTTAGGAACGTACACCGAAAAAT GATTTGATTTT	186	
atoD1ctfB1-g1-all-F	AAGAAGAAAATAGAAATttgacagctagctcagtcctaggtataatG CTAGCTCTTGGAAGTGAATTTGAAAGTTTTAGAGCTA GAAATA	187	pYW34- Δ ctfA1- ctfB1
atoD1ctfB1-g1-all-R	TATTTCTAGCTCTAAAACCTTCAATTTTCAGTTCCAAG AGCTAGCATTATACCTAGGACTGAGCTAGCTGTCAA ATTTCTATTTTCTTCTT	188	
atoD1ctfB1-Up-N-F	ctttgtgatagactaataattaAGGGGTTAGTTACGCTTCGCCT	189	
atoD1ctfB1-Up-N-R	TCCACCACTTACTCTATCCTTCTGCGGCCGCATAATG GAGCCTCAATGGAAGCGATA	190	
atoD1ctfB1-Down-N-F	CGCTTCCATTGAGGCTCCATTATGCGGCCGCAGAAAG GATAGAGTAAGTGGTGGATTT	191	
atoD1ctfB1-Down-N-R	atccactagtaaccatcacactgTATCCAAAGTTGTAAACCTCCTA ACG	192	

Table S3: Butyl acetate production in the mutant strains with prophage deleted from the chromosome

	FJ-1201 48 h	FJ-1201 72 h	FJ-1301 48 h	FJ-1301 72 h
EA	<0.01	<0.01	<0.01	<0.01
BA	19.3±0.5	19.7±1.7	19.1±0.5	19.4±0.7

BB	0.9±0.1	0.7±0.1	0.8±0.0	0.7±0.0
BA (aqueous phase)	0.6±0.1	0.6±0.1	0.5±0.0	0.5±0.1

*EA: ethyl acetate; BA: butyl acetate; BB: butyl butyrate. All values are in g/L.

Table S4 FJ-1201 ester production using biomass hydrolysates as the substrate with or without supplementation of exogenous organic nitrogen source*

	0Y+0T[#]	1Y+3T	2Y+6T
EA	<0.01	<0.01	<0.01
BA	17.5±0.2	16.9±0.5	16.2±0.7
BB	0.1±0.0	0.2±0.1	0.2±0.1
BA (aqueous phase)	0.3±0.0	0.2±0.0	0.2±0.0

5 *EA: ethyl acetate; BA: butyl acetate; BB: butyl butyrate. All values are in g/L.

*Y=Yeast, T=Tryptone; 0Y+0T: 0 g/L Y and 0 g/L T; 1Y+3T: 1 g/L Y and 3 g/L T; 2Y+6T: 2 g/L Y and 6 g/L T.

Table S5. Project total capital investment (\$ million) for the processes

	Purchased Cost	Installed cost
Feedstock handling	15.5	26.4
Pretreatment & hydrolysis	18.0	29.8
Fermentation	36.6	55.2
Production recovery & upgrading	6.6	12.6
Product & chemical Storage	1.3	2.2
Wastewater treatment	59.8	59.8
Electricity & steam generation	34.4	68.4
Utilities	4.5	8.3
Total Installed Equipment cost		262.7
Other Direct Cost		17.1
Total Direct Cost (TDC)		279.8
Total Indirect Costs (TIC)		167.9
Fixed Capital Investment (FCI)		447.6
Land		1.8
Working Capital		22.4
Total Capital Investment (TCI)		471.8

10 Table S6. Key operation parameters for the corn stover conversion and butyl acetate fermentation considered in the techno-economic analysis (TEA)

Pretreatment and hydrolysis

Sodium hydroxide loading	40 kg/MT dry corn stover
Deacetylation incubation temperature	80 °C
Disk milling energy consumption	212 kWh/MT dry corn stover
Enzymatic hydrolysis solids loading	20%
Enzyme loading	19 mg protein/g cellulose
Cellulose hydrolysis efficiency	82%
Hemicellulose hydrolysis efficiency	74%

Fermentation

Hexadecane load	1:1 by volume
Fermentation time	96 hours
BA yield	0.25 g BA/g consumed sugar
Butanol yield	0.03 g butanol/g consumed sugar
Isopropanol yield	0.04 g isopropanol/g consumed sugar
Additional nutrients	None (based on verified experiments)

Table S7. Summary of key raw material costs

Item	Cost (\$)
Raw materials and utilities	
Corn stover (20% moisture)	51.5/MT ^a
Sodium hydroxide	116/MT ^b
Cellulase	4,240/MT ^a
Hexadecane	4,000/MT ^b
Wastewater treatment chemicals	4.51/MT ^b
Boiler Chemicals	8,336/MT ^a
Cooling tower chemicals	3,668/MT ^a
Sulfuric acid	41.0/MT ^b
Freshwater	0.22/MT ^a
Electricity	0.065/kWh ^a
Natural gas	185/MT ^b
Co-product credits	
Butanol	900/MT ^b
Isopropanol	1150/MT ^b
Surplus Electricity	0.065/kWh ^b
Fixed Operating Costs	
Labor costs	2,500,000 ^c
Labor burden	90% of labor cost
Maintenance	3% of ISBL
Property insurance	0.7% of fixed capital investment

^aThe price of corn stover and other chemicals were from the previous literature including Humbird et al. (2011)¹⁰, Chen et al. (2015)⁹, and Dalle Ave and Adams (2018)²⁹;

5 ^bData from different sources, including the ICIS chemical price report and industrial quotes;

^cAssuming 50 employees with an average annual salary of \$50,000 per employee.

Table S8 Butyl acetate production in the engineered strains for the evaluation of the effect of gene codon optimization

	FJ-004	FJ-007
EA	<0.01	<0.01
BA	5.5±0.5	5.0±0.2
BB	0.01±0.00	< 0.01
Glucose consumption	79.3±0.1	76.7±2.4

Lactate	0.00	0.7±0.1
Acetate	0.3±0.00	0.2±0.1
Ethanol	1.0±0.0	1.0±0.0
Acetone	5.0±0.2	3.6±0.2
Butyrate	0.1±0.0	0.00
Butanol	7.8±0.0	9.5±0.7

* EA: ethyl acetate; BA: butyl acetate; BB: butyl butyrate. All values are in g/L.

Supplemental References

References

- 5 The following references are provided as an aid in understanding the subject matter provided above. No admission is made that any of the following meet the legal definition of “prior art” in any country, or that any of the following are relevant to the patentability of anything that is claimed.
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EXEMPLARY EMBODIMENTS

5 The following are non-limiting examples of specific embodiments of the subject matter disclosed above. This disclosure specifically but non-exclusively supports claims to these embodiments:

Emb. 1. A modified microorganism capable of butyl acetate (BA) production, the microorganism capable of expressing an alcohol acyl transferase (AAT); and comprising a
10 butanol synthesis pathway.

Emb. 2. Any one of the microorganisms above, wherein the AAT is selected from one or more of: Vaat, Saat, Atf1, Eht1, and a functional homolog of any of the foregoing.

Emb. 3. Any one of the microorganisms above, wherein the AAT is Vaat having at least 70% sequence identity with SEQ ID NO: 1.

15 Emb. 4. Any one of the microorganisms above, wherein the AAT is Saat having at least 70% sequence identity with SEQ ID NO: 2.

Emb. 5. Any one of the microorganisms above, wherein the AAT is Atf1 having at least 70% sequence identity with SEQ ID NO: 3.

Emb. 6. Any one of the microorganisms above, wherein the AAT is Eht1 having at
20 least 70% sequence identity with SEQ ID NO: 4.

Emb. 7. Any one of the microorganisms above, comprising an acetyl CoA synthesis pathway.

Emb. 8. Any one of the microorganisms above, comprising multiple nucleic acid sequences each encoding Atf1 or a functional homolog of Atf1.

25 Emb. 9. Any one of the microorganisms above, comprising multiple genomic nucleic acid sequences each encoding Atf1 or a functional homolog of Atf1.

Emb. 10. A modified microorganism capable of butyl butyrate (BB) production, the microorganism capable of expressing an alcohol acyl transferase (AAT); and comprising a butanol synthesis pathway and a butyryl coenzyme A synthesis pathway.

30 Emb. 11. The microorganism of embodiment 0, wherein the AAT is Eht1 or a functional homolog of Eht1.

Emb. 12. The microorganism of any one of embodiments 0-0, wherein the AAT is Saat or a functional homolog of Saat.

- Emb. 13. Any one of the microorganisms above, comprising the AAT enzyme.
- Emb. 14. Any one of the microorganisms above, comprising a nucleic acid encoding the AAT.
- Emb. 15. Any one of the microorganisms above, comprising a genomic nucleic acid
5 encoding the AAT.
- Emb. 16. Any one of the microorganisms above, comprising a plasmid encoding the AAT.
- Emb. 17. Any one of the microorganisms above, wherein the microorganism is a prokaryote.
- 10 Emb. 18. Any one of the microorganisms above, wherein the microorganism is fermentative.
- Emb. 19. Any one of the microorganisms above, wherein the microorganism is a bacterium of genus *Clostridium*.
- Emb. 20. Any one of the microorganisms above, wherein the microorganism is
15 selected from *Clostridium saccharoperbutylacetonicum*, *Clostridium beijerinckii*, *Clostridium pasteurianum*, and *Clostridium tyrobutyricum*.
- Emb. 21. Any one of the microorganisms above, capable of expressing Lipase B or a functional homolog of Lipase B.
- Emb. 22. Any one of the microorganisms above, capable of expressing a functional
20 homolog of Lipase B having at least 70% sequence identity with SEQ ID NO: 5.
- Emb. 23. Any one of the microorganisms above, capable of expressing Lipase B or a functional homolog of Lipase B, and comprising an acetic acid synthesis pathway.
- Emb. 24. Any one of the microorganisms above, capable of expressing Lipase B or a functional homolog of Lipase B, and comprising a butyric acid synthesis pathway.
- 25 Emb. 25. Any one of the microorganisms above, comprising a nucleic acid encoding Lipase B or a functional homolog of Lipase B.
- Emb. 26. Any one of the microorganisms above, comprising a nucleic acid encoding Lipase B or a functional homolog of Lipase B having at least 70% sequence identity with SEQ ID NO: 5.
- 30 Emb. 27. Any one of the microorganisms above, having reduced or eliminated NuoG activity.
- Emb. 28. Any one of the microorganisms above, capable of expressing a heterologous Sadh or a functional homolog thereof.

- Emb. 29. Any one of the microorganisms above, comprising a heterologous nucleic acid encoding a Sadh or a functional homolog of a Sadh.
- Emb. 30. Any one of the microorganisms above, comprising a *sadh-hydG* gene cluster.
- Emb. 31. Any one of the microorganisms above, comprising an exogenous AAT gene
5 operatively linked to promoter P_{adh} .
- Emb. 32. Any one of the microorganisms above, comprising an exogenous AAT gene operatively linked to a promoter P_{adh} native to the microorganism.
- Emb. 33. Any one of the microorganisms above, comprising an exogenous AAT gene operatively linked to promoter P_{ald} .
- 10 Emb. 34. Any one of the microorganisms above, comprising an exogenous AAT gene operatively linked to promoter P_{ald} native to the microorganism.
- Emb. 35. Any one of the microorganisms above, comprising an AAT that is localized at the cell membrane.
- Emb. 36. Any one of the microorganisms above, wherein the AAT is fused to a C-
15 terminal membrane-targeting sequence of MinD.
- Emb. 37. Any one of the microorganisms above, wherein the AAT is fused to a C-terminal membrane-targeting sequence of MinD encoded by SEQ ID NO: 6.
- Emb. 38. Any one of the microorganisms above, wherein the AAT is fused to an 8-12 residue C-terminal membrane-targeting sequence of MinD.
- 20 Emb. 39. Any one of the microorganisms above, wherein the AAT is fused at its C-terminal end to the C-terminal membrane-targeting sequence of MinD.
- Emb. 40. Any one of the microorganisms above, comprising a nucleic acid encoding a polypeptide comprising an alcohol acyltransferase and a C-terminal membrane-targeting sequence of MinD.
- 25 Emb. 41. Any one of the microorganisms above, wherein the microorganism has been cured of a prophage.
- Emb. 42. Any one of the microorganisms above, wherein the microorganism has been cured of all native prophages.
- Emb. 43. Any one of the microorganisms above, wherein the microorganism has been
30 cured of one or more prophages by inactivation of an integrase gene of the one or more prophages.

- Emb. 44. Any one of the microorganisms above, wherein the microorganism has been cured of one or more prophages by inactivation of an integrase gene of the one or more prophages through the partial or entire deletion of the integrase gene.
- Emb. 45. Any one of the microorganisms above, wherein the microorganism has been
5 cured of one or more prophages by deletion of the one or more prophages.
- Emb. 46. Any one of the microorganisms above, wherein the microorganism is a bacterium of genus *Clostridium*, and wherein the microorganism has been cured of one or more of prophages P1, P2, P3, P4, and P5.
- Emb. 47. Any one of the microorganisms above, wherein the microorganism is a
10 bacterium of genus *Clostridium*, and wherein the microorganism has been cured of all of prophages P1, P2, P3, and P4.
- Emb. 48. Any one of the microorganisms above, wherein said microorganism does not express a functional redox-sensing transcriptional repressor Rex.
- Emb. 49. Any one of the microorganisms above, capable of expressing soluble pyridine
15 nucleotide transhydrogenase (SthA) or a functional homolog thereof.
- Emb. 50. Any one of the microorganisms above, capable of expressing soluble pyridine nucleotide transhydrogenase (SthA) having at least 70% sequence identity with SEQ ID NO: 7.
- Emb. 51. Any one of the microorganisms above, wherein said microorganism does not
20 express a functional *cftA1-ctfB1* gene cluster.
- Emb. 52. Any one of the microorganisms above, wherein the functional homolog has only exemplary substitutions from Table 1 compared to the AAT, Lipase B, SthA, or Sadh.
- Emb. 53. Any one of the microorganisms above, wherein the functional homolog has only preferred substitutions from Table 1 compared to the AAT, Lipase B, SthA, or Sadh.
- Emb. 54. Any one of the microorganisms above, wherein the functional homolog has a
25 amino acid sequence identity level to the AAT, Lipase B, SthA, or Sadh of at least 70, 75, 70, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 99.5, 99.9, or 100%.
- Emb. 55. A genetically modified *Clostridium saccharoperbutylacetonicum* having improved BA production and designated YM028P, having NCMA designation number
30 202012116, deposited on 16 December 2020 at the National Center for Marine Algae and Microbiota at Bigelow Laboratory for Ocean Sciences, 60 Bigelow Drive, East Boothbay, ME 04544 USA.

- Emb. 56. A genetically modified *Clostridium saccharoperbutylacetonicum* having improved BB production and designated YM016PB, having NCMA designation number 202012115, deposited on 16 December 2020 at the National Center for Marine Algae and Microbiota at Bigelow Laboratory for Ocean Sciences, 60 Bigelow Drive, East Boothbay, ME
5 04544 USA.
- Emb. 57. A genetically modified *Clostridium saccharoperbutylacetonicum* having improved BA production and designated FJ-1301, having NCMA designation number 202012114, deposited on 16 December 2020 at the National Center for Marine Algae and Microbiota at Bigelow Laboratory for Ocean Sciences, 60 Bigelow Drive, East Boothbay, ME
10 04544 USA.
- Emb. 58. A method of ester production, comprising culturing any one of the microorganisms above under conditions suitable to produce an ester.
- Emb. 59. Any one of the methods above, comprising culturing any one of the microorganisms above in a medium containing glucose.
- 15 Emb. 60. Any one of the methods above, comprising culturing any one of the microorganisms above in a medium containing a biomass hydrolysate.
- Emb. 61. Any one of the methods above, comprising culturing any one of the microorganisms above in a medium containing a corn stover hydrolysate.
- Emb. 62. Any one of the methods above, wherein culturing occurs at mesophilic
20 temperatures.
- Emb. 63. Any one of the methods above, wherein culturing occurs under anaerobic conditions.
- Emb. 64. Any one of the methods above, wherein the ester is at least one of BA and BB.
- Emb. 65. Any one of the methods above, producing at least about 1.5 g BA/L of culture.
- 25 Emb. 66. Any one of the methods above, producing at least 1.5 g BA/L of culture.
- Emb. 67. Any one of the methods above, producing at least about 5, 7.5, 10, 12.5, 13, 14, 15, 16, 17, 18, 19, 20, or 25 g BA/L of culture.
- Emb. 68. Any one of the methods above, producing at least 5, 7.5, 10, 12.5, 13, 14, 15, 16, 17, 18, 19, 20, or 25 g BA/L of culture.
- 30 Emb. 69. Any one of the methods above, producing at least about 0.1 g BB/L of culture.
- Emb. 70. Any one of the methods above, producing at least 0.1 g BB/L of culture.
- Emb. 71. Any one of the methods above, producing at least about 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. or 1.6 g BB/L of culture.

Emb. 72. Any one of the methods above, producing at least 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. or 1.6 g BB/L of culture.

CONCLUSIONS

It is to be understood that any given elements of the disclosed embodiments of the invention may be embodied in a single structure, a single step, a single substance, or the like. Similarly, a given element of the disclosed embodiment may be embodied in multiple structures, steps, substances, or the like. The foregoing description and accompanying drawings illustrate and describe certain processes, machines, manufactures, and compositions of matter, some of which embody the invention(s). Such descriptions or illustrations are not intended to limit the scope of what can be claimed, and are provided as aids in understanding the claims, enabling the making and use of what is claimed, and teaching the best mode of use of the invention(s). If this description and accompanying drawings are interpreted to disclose only a certain embodiment or embodiments, it shall not be construed to limit what can be claimed to that embodiment or embodiments. Any examples or embodiments of the invention described herein are not intended to indicate that what is claimed must be coextensive with such examples or embodiments. Where it is stated that the invention(s) or embodiments thereof achieve one or more objectives, it is not intended to limit what can be claimed to versions capable of achieving all such objectives. Any statements in this description criticizing the prior art are not intended to limit what is claimed to exclude any aspects of the prior art. Additionally, the disclosure shows and describes certain embodiments of the processes, machines, manufactures, compositions of matter, and other teachings disclosed, but it is to be understood that the teachings of the present disclosure are capable of use in various other combinations, modifications, and environments and is capable of changes or modifications within the scope of the teachings as expressed herein. Any section headings herein are provided only for consistency with the suggestions of 37 C.F.R. § 1.77 or otherwise to provide organizational queues. These headings shall not limit or characterize the invention(s) set forth herein.

CLAIMS

The following is claimed:

1. A modified microorganism capable of butyl acetate (BA) production, the microorganism capable of expressing an alcohol acyl transferase (AAT); and comprising a butanol synthesis pathway.
2. The modified microorganism of claim 1, wherein the AAT is selected from one or more of: Vaat, Saat, Atf1, Eht1, and a functional homolog of any of the foregoing.
3. The modified microorganism of claim 1, capable of expressing all of: Vaat, Saat, Atf1, and Eht1.
4. The modified microorganism of claim 1, wherein the AAT is Vaat having at least 70% sequence identity with SEQ ID NO: 1.
5. The modified microorganism of claim 1, wherein the AAT is Saat having at least 70% sequence identity with SEQ ID NO: 2.
6. The modified microorganism of claim 1, wherein the AAT is Atf1 having at least 70% sequence identity with SEQ ID NO: 3.
7. The modified microorganism of claim 1, wherein the AAT is Eht1 having at least 70% sequence identity with SEQ ID NO: 4.
8. The modified microorganism of claim 1, comprising an acetyl CoA synthesis pathway.
9. The modified microorganism of claim 1, comprising multiple nucleic acid sequences each encoding Atf1 or a functional homolog of Atf1.
10. A modified microorganism capable of butyl butyrate (BB) production, the microorganism capable of expressing an alcohol acyl transferase (AAT); and comprising a butanol synthesis pathway and a butyryl coenzyme A synthesis pathway.
11. The microorganism of claim 10, wherein the AAT is Eht1 or a functional homolog of Eht1.
12. The microorganism of claim 10, wherein the AAT is Saat or a functional homolog of Saat.
13. Any one of the microorganisms of claims 1 and 10, wherein the microorganism is a prokaryote.

14. Any one of the microorganisms of claims 1 and 10, wherein the microorganism is fermentative.
15. Any one of the microorganisms of claims 1 and 10, wherein the microorganism is a bacterium of genus *Clostridium*.
16. Any one of the microorganisms of claims 1 and 10, wherein the microorganism is selected from *Clostridium saccharoperbutylacetonicum*, *Clostridium beijerinckii*, *Clostridium pasteurianum*, and *Clostridium tyrobutyricum*.
17. Any one of the microorganisms of claims 1 and 10, capable of expressing Lipase B or a functional homolog of Lipase B.
18. Any one of the microorganisms of claims 1 and 10, capable of expressing a functional homolog of Lipase B having at least 70% sequence identity with SEQ ID NO: 5.
19. Any one of the microorganisms of claims 1 and 10, capable of expressing Lipase B or a functional homolog of Lipase B, and comprising an acetic acid synthesis pathway.
20. Any one of the microorganisms of claims 1 and 10, capable of expressing Lipase B or a functional homolog of Lipase B, and comprising a butyric acid synthesis pathway.
21. Any one of the microorganisms of claims 1 and 10, having reduced or eliminated NuoG activity.
22. Any one of the microorganisms of claims 1 and 10, capable of expressing a heterologous Sadh or a functional homolog thereof.
23. Any one of the microorganisms of claims 1 and 10, comprising a *sadh-hydG* gene cluster.
24. Any one of the microorganisms of claims 1 and 10, comprising an exogenous AAT gene operatively linked to promoter P_{adh} .
25. Any one of the microorganisms of claims 1 and 10, comprising an exogenous AAT gene operatively linked to promoter P_{ald} .
26. Any one of the microorganisms of claims 1 and 10, comprising an AAT that is localized at the cell membrane.
27. Any one of the microorganisms of claims 1 and 10, wherein the AAT is fused to a C-terminal membrane-targeting sequence of MinD.
28. Any one of the microorganisms of claims 1 and 10, wherein the AAT is fused to a C-terminal membrane-targeting sequence of MinD encoded by SEQ ID NO: 6.
29. Any one of the microorganisms of claims 1 and 10, wherein the AAT is fused to an 8-12 residue C-terminal membrane-targeting sequence of MinD.

30. Any one of the microorganisms of claims 1 and 10, wherein the microorganism has been cured of a prophage.
31. Any one of the microorganisms of claims 1 and 10, wherein the microorganism has been cured of all native prophages.
32. Any one of the microorganisms of claims 1 and 10, wherein the microorganism has been cured of one or more prophages by deletion of the one or more prophages.
33. Any one of the microorganisms of claims 1 and 10, wherein the microorganism is a bacterium of genus *Clostridium*, and wherein the microorganism has been cured of one or more of prophages P1, P2, P3, P4, and P5.
34. Any one of the microorganisms of claims 1 and 10, wherein the microorganism is a bacterium of genus *Clostridium*, and wherein the microorganism has been cured of all of prophages P1, P2, P3, P4, and P5.
35. Any one of the microorganisms of claims 1 and 10, wherein said microorganism does not express a functional redox-sensing transcriptional repressor Rex.
36. Any one of the microorganisms of claims 1 and 10, capable of expressing soluble pyridine nucleotide transhydrogenase (SthA) or a functional homolog thereof.
37. Any one of the microorganisms of claims 1 and 10, capable of expressing soluble pyridine nucleotide transhydrogenase (SthA) having at least 70% sequence identity with SEQ ID NO: 7.
38. Any one of the microorganisms of claims 1 and 10, wherein said microorganism does not express a functional *cftA1-ctfB1* gene cluster.
39. Any one of the microorganisms of claims 1 and 10, wherein the functional homolog has only preferred substitutions from Table 1 compared to the AAT, Lipase B, SthA, or Sadh.
40. A genetically modified *Clostridium saccharoperbutylacetonicum* having improved BA production and designated YM028P, having NCMA designation number 202012116, deposited on 16 December 2020 at the National Center for Marine Algae and Microbiota at Bigelow Laboratory for Ocean Sciences, 60 Bigelow Drive, East Boothbay, ME 04544 USA.
41. A genetically modified *Clostridium saccharoperbutylacetonicum* having improved BB production and designated YM016PB, having NCMA designation number 202012115, deposited on 16 December 2020 at the National Center for Marine

Algae and Microbiota at Bigelow Laboratory for Ocean Sciences, 60 Bigelow Drive, East Boothbay, ME 04544 USA.

42. A genetically modified *Clostridium saccharoperbutylacetonicum* having improved BA production and designated FJ-1301, having NCMA designation number 202012114, deposited on 16 December 2020 at the National Center for Marine Algae and Microbiota at Bigelow Laboratory for Ocean Sciences, 60 Bigelow Drive, East Boothbay, ME 04544 USA.
43. A method of ester production, comprising culturing any one of the microorganisms of claims 1, 10, and 40-42 under conditions suitable to produce an ester.
44. The method of claim 43, comprising culturing any one of the microorganisms above in a medium containing glucose.
45. The method of claim 43, comprising culturing any one of the microorganisms above in a medium containing a biomass hydrolysate.
46. The method of claim 43, comprising culturing any one of the microorganisms above in a medium containing a corn stover hydrolysate.
47. The method of claim 43, wherein the ester is at least one of BA and BB.
48. The method of claim 43, producing at least 1.5 g BA/L of culture.
49. The method of claim 43, producing at least 25 g BA/L of culture.
50. The method of claim 43, producing at least 0.1 g BB/L of culture.
51. The method of claim 43, producing at least 1.6 g BB/L of culture.