

PRODUCING BUTYL BUTYRATE FROM LIGNOCELLULOSIC BIOMASS THROUGH ESCHERICHIA COLI-CLOSTRIDIUM CO-CULTURE

FIELD OF THE INVENTION

The present invention relates to compositions comprising a genetically engineered
5 *Escherichia coli* and an acetone-butanol-ethanol (ABE)-producing *Clostridium* and methods
of their use to provide valuable chemicals, such as butyl butyrate or butyl acetate from
lignocellulosic biomass. More particularly said genetically engineered *E. coli* strain has been
transformed by at least one polynucleotide molecule comprising a CoA transferase (CTF)
gene and an alcohol acyltransferase (ATF) gene operably linked to at least one promoter.

10 BACKGROUND OF THE INVENTION

Clostridium acetone-butanol-ethanol (ABE) fermentation is a well-known process that
can be used to produce solvents such as butanol (Ebrahimi et al., 2020; Schiel-Bengelsdorf
et al., 2013). The traditional ABE process still has some limitations even though it has been
applied in large-scale operations (Lee et al., 2008). The limitations include high substrate
15 cost, solvent toxicity, and high oxygen sensitivity (Cui et al., 2020c; Dürre, 2011; Wu et al.,
2016; Xin et al., 2018).

Substrate cost accounts for approximately 70% of the total cost of ABE fermentation
(Cui et al., 2020c; Mahapatra and Kumar, 2017). Currently, edible feedstocks are being used,
and that incurs high substrate cost and raises concerns over food security (Lin et al., 2015).
20 Switching to cheaper, inedible, and abundant substrates (such as lignocellulosic biomass) is
a promising direction for improving the competitiveness of the ABE fermentation.
Lignocellulosic biomass mainly consists of cellulose, hemicellulose, and lignin, and can be
derived from agricultural wastes such as rice straw and corn stover (Saini et al., 2015a).

Solvent toxicity caused by butanol in the ABE fermentation often leads to low solvent
25 titer and high purification cost (Li et al., 2014; Yen and Wang, 2013). To alleviate butanol
toxicity, a variety of recovery methods, including gas stripping and liquid-liquid extraction,
have been employed to remove butanol from aqueous cell culture (Ha et al., 2010; Qureshi
and Blaschek, 2001). Liquid-liquid extraction showed good energy efficiency compared to
gas stripping, but extractants with a high butanol partition coefficient are usually toxic to
30 *Clostridium* (Li et al., 2010; Oudshoorn et al., 2009; Qureshi et al., 2005). Converting butanol
into its esters could be a solution to this problem. As esters are more hydrophobic, they can
be extracted from the aqueous phase more easily than butanol. Butyl butyrate is an
attractive ester product for this application because both reactants (butanol and butyrate)
can be produced in the ABE fermentation (Sreekumar et al., 2015). Butyl butyrate has been

widely used in food and cosmetic products due to its fruity fragrance, and holds the potential to be used as a good fuel source because of its compatibility with petrol and aviation kerosene (Brault et al., 2014; Jenkins et al., 2013; Noh et al., 2019).

Due to these reasons, efforts have been invested to perform in situ extractive ABE fermentation to produce butyl butyrate. Lipase-mediated esterification and in situ extraction have been used to produce butyl butyrate in *Clostridium* fermentation (Seo et al., 2017; Van Den Berg et al., 2013; Xin et al., 2016). However, these studies share some common drawbacks. For example, exogenous butyrate and lipases were supplemented due to poor production of butyrate and lipase from solventogenic *Clostridium*, which incurred additional cost (Cui et al., 2020b; Xin et al., 2016; Xin et al., 2019). Alternatively, butyl butyrate can also be produced by genetically engineered *Clostridium* that expressed an alcohol acyltransferase (ATF). In this case, butyl butyrate can be produced from the condensation of butyryl-CoA and butanol by ATF (Horton and Bennett, 2006; Noh et al., 2019). However, the titer of butyl butyrate produced in this process was relatively low (50 mg/L) (Noh et al., 2018).

In addition, both lipase-mediated and ATF-mediated production of butyl butyrate face the oxygen sensitivity limitation of *Clostridium*. To enable the growth of anaerobic *Clostridium*, anaerobic pretreatments such as purging N₂ and adding costly reducing agents are often needed, increasing the overall cost of the process (Wu et al., 2016).

Therefore, there is a need for an improved ABE fermentation method for the production of valuable chemicals such as butyl butyrate.

SUMMARY OF THE INVENTION

The present invention is directed to a co-culture strategy that yields higher butyl butyrate titer compared to other studies using genetically engineered *Clostridium* to produce butyl butyrate (Noh et al., 2018). The present invention produced 25 times more butyl butyrate (1280 mg/L vs. 50 mg/L) than the system of Noh et al., 2018.

The co-culture strategy of the present invention also avoids adding lipase and butyrate to boost butyl butyrate yield. Butyl butyrate can be produced directly from substrates.

The co-culture can directly utilize cellobiose and xylan without the addition of any enzyme. It can also utilize cheaper lignocellulosic biomass such as agricultural waste as substrate when integrated with biomass pretreatment technology and added with cellulase. Instead of using starchy feedstocks as substrates like many other studies, the co-culture can directly use xylan and cellobiose through consolidated bioprocessing without the addition of

any enzyme (Noh et al., 2018; Xin et al., 2016) . Integrated with pretreatment technology and the addition of cellulase, this co-culture can even utilize rice straw. Compared with edible starchy substrates, these substrates are cheaper and inedible, reducing substrate cost and avoiding food security concerns. We believe other lignocellulosic biomass such as corn
5 stover can also be utilized.

In addition, the co-culture strategy enables aerobic fermentation to avoid anaerobic pretreatment. Due to the anaerobic feature of *Clostridium*, fermentation processes involving *Clostridium* usually need anaerobic pretreatments such as N₂ purging to ensure the normal growth of *Clostridium*. By contrast, the culture media and equipment do not need to undergo
10 anaerobic pretreatment in this co-culture. This is because the *E. coli* strain used in this co-culture can provide respiratory protection to *Clostridium* by consuming oxygen.

Furthermore, the co-culture strategy avoids the genetic engineering of *Clostridium*. Compared with other model bacteria such as *E. coli*, the metabolism of *Clostridium* is less understood. Besides, *Clostridium* usually needs to be manipulated at anaerobic condition.
15 Owing to these reasons, genetic engineering of *Clostridium* is often prone to failure. In the present invention, the model microorganism *E. coli* was engineered to express different enzymes, so that the tedious and risky *Clostridium* genetic engineering can be avoided.

In a first aspect the invention provides a composition for the production of butyl butyrate, comprising an acetone-butanol-ethanol (ABE)-producing *Clostridium* strain and a
20 genetically engineered *E. coli* strain, wherein said genetically engineered *E. coli* strain has been transformed by at least one polynucleotide molecule;

the at least one polynucleotide molecule comprising a CoA transferase (CTF) gene and an alcohol acyltransferase (ATF) gene operably linked to at least one promoter.

In some embodiments, the *Clostridium* strain is *C. beijerinckii* G117.

25 In some embodiments, the CoA transferase (CTF) gene is selected from the group comprising *atoDA* and *ctfAB* and the alcohol acyltransferase (ATF) gene is selected from the group comprising *aaat* (from apple), *saat* (from strawberry), *liaat* (from lavender) and *vaat* (from strawberry).

In some embodiments, the CTF gene is *ctfAB* and the ATF gene is *vaat*.

30 In some embodiments, the CTF gene is substituted by a butyrate kinase (*buk*) gene and a phosphate butyryltransferase (*ptb*) gene.

In some embodiments, the *atoDA*; *atoD* and *atoA* genes encode a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 11 and 13, respectively, or a functional sequence variant thereof; and/or

5 the *ctfAB*; *ctfA* and *ctfB* genes encode a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 15 and 17, respectively, or a functional sequence variant thereof; and/or

the *aaat* gene encodes a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 7 or a functional sequence variant thereof; and/or

10 the *saat* gene encodes a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 1 or a functional sequence variant thereof; and/or

the *liaat* gene encodes a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 5 or a functional sequence variant thereof; and/or

the *vaat* gene encodes a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 3 or a functional sequence variant thereof; and/or

15 the *buk* gene encodes a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 21 or a functional sequence variant thereof; and/or

the *ptb* gene encodes a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 19 or a functional sequence variant thereof.

20 In some embodiments, the *atoDA*; *atoD* and *atoA* genes comprise a nucleic acid sequence having at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, at least 95% sequence identity or 100% sequence identity, due to the degeneracy of the genetic code, to the polynucleotide sequence set forth in SEQ ID NO: 12 and 14, respectively; and/or

25 the *ctfAB*; *ctfA* and *ctfB* genes comprise a nucleic acid sequence having at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, at least 95% sequence identity or 100% sequence identity, due to the degeneracy of the genetic code, to the polynucleotide sequence set forth in SEQ ID NO: 16 and 18, respectively; and/or

30 the *aaat* gene comprises a nucleic acid sequence having at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, at least 95% sequence identity or 100% sequence identity, due to the degeneracy of the genetic code, to the polynucleotide sequence set forth in SEQ ID NO: 8; and/or

the *saat* gene comprises a nucleic acid sequence having at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, at least 95% sequence identity or 100% sequence identity, due to the degeneracy of the genetic code, to the polynucleotide sequence set forth in SEQ ID NO: 2; and/or

5 the *liaat* gene comprises a nucleic acid sequence having at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, at least 95% sequence identity or 100% sequence identity, due to the degeneracy of the genetic code, to the polynucleotide sequence set forth in SEQ ID NO: 6; and/or

10 the *vaat* gene comprises a nucleic acid sequence having at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, at least 95% sequence identity or 100% sequence identity, due to the degeneracy of the genetic code, to the polynucleotide sequence set forth in SEQ ID NO: 4; and/or

15 the *buk* gene comprises a nucleic acid sequence having at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, at least 95% sequence identity or 100% sequence identity, due to the degeneracy of the genetic code, to the polynucleotide sequence set forth in SEQ ID NO: 22; and/or

20 the *ptb* gene comprises a nucleic acid sequence having at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, at least 95% sequence identity or 100% sequence identity, due to the degeneracy of the genetic code, to the polynucleotide sequence set forth in SEQ ID NO: 20.

It would be understood that a nucleic acid sequence may have less than 100% identity to a reference nucleic acid sequence and still encode the same amino acid sequence.

25 In some embodiments, the promoter is a strong promoter such as P_{T7}, or an anaerobic promoter, such as P_{adhE}.

Advantageously, the use of an anaerobic promotor results in automatic induction of protein expression without the need to add a chemical inducer such as IPTG.

In some embodiments, the at least one polynucleotide molecule further comprises a cellulase gene.

30 In some embodiments, said cellulase gene is expressed by another wild-type microorganism to have high cellulolytic activity and be introduced into this co-culture system

to form a three-strain co-culture. Such cellulase-expressing 'third' microorganisms can be mesophilic bacteria, such as *Clostridium cellulovorans*, or fungi.

In a second aspect, the invention provides a method of producing butyl butyrate in a co-culture, comprising the steps:

- 5 a) co-culturing a composition of any one of claims 1 to 10 in medium under conditions for conventional ABE fermentation with exception that no anaerobic treatment is used, and
- b) supplementing the medium with a carbon source selected from the group comprising glucose, xylan, cellulose and lignocellulosic biomass.

10 In some embodiments, the co-culturing step a) is performed with a starting pH of about 6.5, then the pH is allowed to drop to about 5.8 and is maintained at 5.8 for the duration of the fermentation. Such conditions improved butyl butyrate yield in co-culture fermentation conducted in a bioreactor.

Due to the anaerobic feature of *Clostridium*, fermentation processes involving
15 *Clostridium* usually need anaerobic pretreatments such as N₂ purging to ensure the normal growth of *Clostridium*. By contrast, for the present invention the culture media and equipment do not need to undergo anaerobic pretreatment. This is because the *E. coli* strain used in this co-culture can provide respiratory protection to *Clostridium* by consuming oxygen. Moreover, the co-culture also does not need aeration to provide oxygen, which
20 saves energy.

In some embodiments, the medium is supplemented with glucose at a concentration of at least 6 g/L, at least 30 g/L, at least 60 g/L, at least 90 g/L or in the range from 30 g/L to 90 g/L, preferably in the range 50 g/L to 70 g/L.

In some embodiments, the method further comprises isolating said butyl butyrate.

25 In some embodiments, the co-cultured *Clostridium* and *E. coli* are capable of increased production of butyl butyrate compared to a co-culture with a non-transformed *E. coli* cell.

In some embodiments, the lignocellulosic biomass is rice straw or corn stover, preferably rice straw.

30 In some embodiments,

- i) the medium is supplemented with cellulase; or

ii) the *E. coli* has been engineered to express cellulase,
when cellulose and/or lignocellulosic biomass are the carbon source.

In some embodiments, the cellulase is from *A. niger*.

In some embodiments, the said lignocellulosic biomass has been pretreated to
5 remove lignin in order to increase access of enzyme to cellulose and hemicellulose.

In a third aspect, the invention provides a composition for the production of butyl acetate, comprising an acetone-butanol-ethanol (ABE)-producing *Clostridium* strain and a genetically engineered *E. coli* strain, wherein said genetically engineered *E. coli* strain has been transformed by at least one polynucleotide molecule;

10 the at least one polynucleotide molecule comprising a CoA transferase (CTF) gene and an alcohol acyltransferase (ATF) gene operably linked to at least one promoter,

In some embodiments, the ATF gene is *atf1* (from *Saccharomyces cerevisiae*);

In some embodiments, the *Clostridium* strain is *C. beijerinckii* G117.

In some embodiments, the *atf1* gene encodes a polypeptide comprising the amino
15 acid sequence set forth in SEQ ID NO: 9 or a functional sequence variant thereof.

In some embodiments, the CoA transferase (CTF) gene is selected from the group comprising *atoDA* and *ctfAB*.

In some embodiments, the *atoDA*; *atoD* and *atoA* genes encode a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 11 and 13, respectively, or a
20 functional sequence variant thereof; and/or

the *ctfAB*; *ctfA* and *ctfB* genes encode a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 15 and 17, respectively, or a functional sequence variant thereof.

In a *fourth* aspect, the invention provides a method of producing butyl acetate in a co-
25 culture, comprising the steps:

- a) co-culturing a composition of any aspect of the invention in medium under conditions for conventional ABE fermentation with exception that no anaerobic treatment is used, and
- b) supplementing the medium with a carbon source selected from the group
30 comprising glucose, xylan, cellulose and lignocellulosic biomass,

wherein the co-cultured *Clostridium* and *E. coli* are capable of increased production of butyl acetate compared to a co-culture with a non-transformed *E. coli* cell.

In some embodiments, the lignocellulosic biomass is rice straw or corn stover, preferably rice straw.

5 BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows a conceptual design for producing butyl butyrate from glucose in an *E. coli*-*C. beijerinckii* co-culture. In a medium that did not undergo anaerobic treatment, *E. coli* first grew by consuming glucose (carbon and energy source) and oxygen. After oxygen was depleted, *C. beijerinckii* started to grow and convert glucose into butanol and butyrate. Meanwhile, the lactate produced by *E. coli* was consumed by *C. beijerinckii*. The generated butanol and butyrate were further condensed into butyl butyrate by the CTF- and ATF-expressing *E. coli*. Pyr: pyruvate. AcCoA: acetyl-CoA. BuCoA: butyryl-CoA. CTF: CoA transferase. ATF: alcohol acyltransferase.

Figure 2A-C shows the process of validating the design for the butyl butyrate production. (A) Validating the metabolic pathway for the conversion of butanol and butyrate into butyl butyrate by using *E. coli* mono-culture. The cells were cultured in 8 mL of the aerobic DSMY medium (20 g/L glucose, 3 g/L butanol and 3 g/L butyrate) in a 20-mL closed serum bottle. An overlay of hexadecane (4 mL) was added. Various *E. coli* strains were tested, and only the strain expressing both *atoDA* and *saat* (YHECE1.1) produced butyl butyrate. *atoDA* encodes an *E. coli* CoA transferase (CTF); *saat* encodes an alcohol acyltransferase (ATF) from strawberry. The two genes were expressed in one operon under T7 promoter when they were co-expressed. (B) Butyl butyrate production by *E. coli*-*C. beijerinckii* co-culture in a 160-mL serum bottle containing 30 mL of the aerobic DSMY medium (40 g/L glucose) and 15 mL of hexadecane. The *C. beijerinckii* strain used was G117. YHECE1.1 was replaced by its parent strain (MG1655DE3) to establish proper controls. When G117 was mono-cultured as a control, oxygen was removed and the inoculation was done under an anaerobic condition. (C) The illustration of the YHECE1.1-G117 co-culture. YHECE1.1 seed culture was prepared in LB medium under aerobic condition; G117 seed culture was prepared in the anaerobic DSMY medium under anaerobic condition; the seed cultures were used to inoculate the aerobic DSMY medium in a closed serum bottle. A layer of hexadecane (50%, v/v) was included to extract butyl butyrate. After oxygen was depleted, ester was produced through the extractive fermentation. Error bars in the figure indicate standard error (n=3).

Figure 3A-G shows the profiles of metabolites except butyl butyrate during co-culture of YHECE1.1 and G117. The co-cultures were processed in closed serum bottles containing the DSMY medium containing 40 g/L glucose. The medium and headspace contained oxygen. The *C. beijerinckii* strain used was G117. YHECE1.1 was replaced by its parent strain (MG1655DE3) to establish proper controls. When G117 was mono-cultured as a control, oxygen was removed and the inoculation was done under anaerobic condition. Time profiles of (A) glucose, (B) butanol, (C) butyrate, (D) acetate, (E) succinate, (F) lactate, and (G) acetone. Error bars in the figure indicate standard error (n=3).

Figure 4A-C shows the process of screening variants of key genes to improve butyl butyrate production. (A) Two pathways for converting butyrate into butyryl-CoA. *atoDA* encodes an *E. coli* CoA transferase (CTF); *ctfAB* encodes a *C. acetobutylicum* CTF. AtoDA or CtfAB converts butyrate into butyryl-CoA directly by using acetyl-CoA (AcCoA) as the CoA donor. *buk* encodes a *C. acetobutylicum* butyrate kinase. *ptb* encodes a *C. acetobutylicum* phosphate butyryltransferase. Buk catalyzes the phosphorylation of butyrate by using ATP, yielding a butyryl phosphate (butyryl-P). Ptb catalyzes the condensation of butyryl-P with free CoA. (B) Butyl butyrate production by *E. coli* (hosting *saat* and different butyryl-CoA producing genes in the same cassette under P_{T7}) mono-culture in closed serum bottles containing 8 mL of the aerobic DSMY medium (containing 20 g/L glucose, 3 g/L butanol and 3 g/L butyrate) and 4 mL of hexadecane. *saat* encodes an alcohol acyltransferase (ATF) from strawberry. (C) Butyl butyrate production by *E. coli* (hosting *ctfAB* and different *atf* genes in the same cassette under P_{T7}) mono-culture under the same condition as (B). *liaat* encodes an ATF from lavender; *vaat* encodes an ATF from strawberry; *aaat* encodes an ATF from apple, *atf1* encodes an ATF from *Saccharomyces cerevisiae*. The strain expressing *ctfAB* and *vaat* produced the most butyl butyrate. Error bars in the figure indicate standard error (n=3).

Figure 5A-B shows anaerobic promoter screening for ester production. (A) Anaerobic promoter screening operation. *E. coli* was pre-cultured and then inoculated into closed serum bottles containing aerobic DSMY and glucose. The fluorescence signal at different time point was quantified by a microplate reader. (B) Characterization of the anaerobic promoters by using a fluorescence protein reporter. The fluorescence of *E. coli* strains expressing *ilov* under different promoters was measured. Specific fluorescence was obtained by dividing fluorescence by optical density at 600 nm (OD₆₀₀). WT: parent strain MG1655DE3. Error bars in the figure indicate standard error (n=3).

Figure 6A-E shows production outcomes of butyl butyrate from glucose by using a simplified *E. coli*-*C. beijerinckii* co-culture process. The *E. coli* strain (YHECE1.11.4) used an

anaerobic promoter (*P_{adhE}*) to express *ctfAB* and *vaat*. In the process, a low inoculum size was used and no chemical inducer added. The co-culture (YHECE1.11.4 and G117) was done in closed serum bottles containing 30 mL of the aerobic DSMY medium (containing 60 g/L glucose) and 15 mL of hexadecane. Time profiles of (A) butyl butyrate, butyl acetate, (B) glucose, butyrate, butanol, (C) succinate, lactate, acetate, and acetone. (D) OD₆₀₀ and relative abundance of YHECE1.11.4 and G117 at various time points. (E) Time profile of dissolved oxygen level in the first 60 mins. Dissolved oxygen (DO) was measured by a DO probe (Applikon Biotechnology, The Netherlands). The DO level at the beginning of the fermentation was defined as 100%. Error bars in the figure indicate standard error (n=3).

Figure 7A-H shows YHECE1.11.4 (MG1655DE3 expressing *ctfAB* and *vaat* under *P_{T7}*)-G117 co-culture using xylan or cellulose as carbon sources. The co-cultures were processed in closed serum bottles containing 30 mL of DSMY, 15 mL of hexadecane, and 60 g/L carbon sources (xylan or cellulose). (A) Microbial interactions in the co-culture using xylan as carbon source. G117 can secrete xylanase to hydrolyze xylan into xylose which can be directly metabolized by G117 and YHECE1.11.4. (B) Xylanase secretion by G117 in the co-culture. SDS-PAGE was performed to observe xylanase in the cell culture supernatant. Lane 1: YHECE1.11.4-G117 co-culture using xylan as carbon source. Lane 2: YHECE1.11.4-G117 co-culture using glucose as carbon source. Lane 3: Protein standard. Lane 4: G117 anaerobic mono-culture using glucose as carbon source. Lane 5: YHECE1.11.4 mono-culture using glucose as carbon source. Xylanase secretion by G117 was only triggered when xylan was present. Time profiles of (C) butyl butyrate, (D) xylose, butyrate, butanol, (E) succinate, lactate, acetate, and acetone in YHECE1.11.4-G117 co-culture using 60 g/L xylan as carbon source. Time profiles of (F) butyl butyrate, (G) cellobiose, glucose, butyrate, butanol, (H) succinate, lactate, acetate, and acetone in YHECE1.11.4-G117 co-culture using 60 g/L cellulose as carbon source with the addition of 500 U cellulase from *Aspergillus niger*. Error bars in the figure indicate standard error (n=3).

Figure 8A-C shows G117-YHECE1.11.4 co-culture using 60 g/L cellobiose as carbon source without the addition of enzymes. The co-culture was processed in serum bottles containing 30 mL of the aerobic DSMY, 60 g/L cellobiose, and 15 mL of hexadecane. Time profiles of (A) butyl butyrate, (B) cellobiose, butyrate, butanol, (C) succinate, lactate, acetate, and acetone. Error bars in the figure indicate standard error (n=3).

Figure 9A-B shows cellulase secretion using *E. coli*. MG1655DE3 was engineered to express an endoglucanase from *Bacillus* (Cel-CD, accession: M84963, 1494 to 2618) under *PT7*. Cells were cultured in the aerobic DSMY medium or LB containing antibiotic with a starting OD₆₀₀ of 0.05 at 37 °C/225 rpm. When OD₆₀₀ achieved 0.5, 80 mM IPTG was

added. Then cells were cultured at 37 °C/225 rpm for 24 h. Then the cell culture was centrifuged and filtered. The filtrate was used for SDS-PAGE and enzymatic activity measurement. (A) SDS-PAGE results of Cel-CD secretion at aerobic condition in the aerobic DSMY medium (Lane 1) and LB (Lane 3). Lane 2 is protein marker. Cel-CD was only
 5 secreted when cells were cultured in LB. The molecular weight of Cel-CD is 41.87 kDa. (B) Enzymatic activity of secreted Cel-CD. Culture medium (0.5 mL) was added to 2 mL of phosphate-buffered saline containing 1 % sodium carboxymethyl cellulose in a test tube. The reaction mixture was incubated at 37 °C for 15 min, then 2 mL DNS reagent was added and the mixture was boiled for 7 min before it was diluted to a final volume of 12.5 mL. The
 10 enzyme activity was measured with a spectrophotometer at 540 nm. 10 U of Cellic Ctec2 was used as a standard. Relative enzymatic activity was obtained by dividing the absorbance of secreted enzymes by the absorbance of Cellic Ctec2 (Novozymes).

Figure 10A-H shows production outcomes of butyl butyrate from pretreated rice straw by using the *E. coli*-*C. beijerinckii* co-culture. Fermentations were carried out in serum
 15 bottles (A-C) or bioreactors (D-G). Time profiles of (A) butyl butyrate, butyl acetate, (B) saccharides, butyrate, butanol, (C) succinate, lactate, acetate, and acetone of the YHECE1.11.4-G117 co-culture in serum bottles. The co-cultures were grown in 30 mL of the aerobic DSMY medium, containing 60 g/L pretreated rice straw and 83.3 U/mL *Aspergillus niger* cellulase (C1184, Sigma-Aldrich). A hexadecane overlay (15 mL) was added. The pH
 20 of the cell cultures was adjusted to 6.3 manually every 12 h. Error bars in A-C indicate standard error (n=3). Time profiles of (D) butyl butyrate, butyl acetate, (E) saccharides, butyrate, butanol, (F) succinate, lactate, acetate, and acetone of the YHECE1.11.4-G117 co-culture in bioreactors (Applikon; vessel size: 500 mL). The co-cultures were grown in 100 mL of the aerobic DSMY medium, containing 60 g/L pretreated rice straw and 83.3 U/mL *A.*
 25 *niger* cellulase. A hexadecane overlay (50 mL) was added. The initial pH was 6.5 and was allowed to decrease to 5.8. Then the pH was maintained at 5.8 throughout the fermentation. Error bars in D-E indicate standard error (n=2; two bioreactors were run independently). (G) An image of one bioreactor at the end of the fermentation. (H) The effect of pH on butyl butyrate production of the YHECE1.11.4 mono-culture. The experiments were conducted in
 30 closed serum bottles containing 8 mL of the aerobic DSMY medium (containing 20 g/L glucose, 3 g/L butanol and 3 g/L butyrate) and 4 mL of hexadecane. Initial pH of the cell cultures was adjusted to 5.0, 5.5, 6.0, or 6.5. The pH was not adjusted throughout the experiments. Samples were taken after 24 h. Error bars in the figure indicate standard error (n=3).

DETAILED DESCRIPTION OF THE INVENTIONDefinitions

Certain terms employed in the specification, examples and appended claims are collected here for convenience.

5 As used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise.

As used herein, ranges can be expressed as from "about" one particular value, and/or to "about" another particular value. When such a range is expressed, another embodiment includes from the one particular value and/or to the other particular value.
10 Similarly, when values are expressed as approximations, by use of the antecedent "about," it will be understood that the particular value forms another embodiment.

The terms "amino acid" or "amino acid sequence," as used herein, refer to an oligopeptide, peptide, polypeptide, or protein sequence, or a fragment of any of these, and to naturally occurring or synthetic molecules. Where "amino acid sequence" is recited herein to
15 refer to an amino acid sequence of a naturally occurring protein molecule, "amino acid sequence" and like terms are not meant to limit the amino acid sequence to the complete native amino acid sequence associated with the recited protein molecule.

As used herein, the term "functional sequence variant" refers to an amino acid sequence (reference or wild-type sequence) that is altered by one or more amino acids
20 without abolishing or substantially altering the polypeptide activity of the non-variant reference. The variant may have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties (e.g., replacement of leucine with isoleucine). More rarely, a variant may have "non-conservative" changes (e.g., replacement of glycine with tryptophan). Analogous minor variations may also include amino acid deletions or
25 insertions, or both. In particular, the variants may be naturally occurring or may be recombinant or synthetically produced. More in particular, the variant may be of at least 70%, at least 80%, at least 90%, or at least 95% sequence identity to the reference sequence. Guidance in determining which amino acid residues may be substituted, inserted, or deleted without abolishing enzyme/catalytic activity may be found using
30 computer programs well known in the art, for example, DNASTAR software.

The phrases "nucleic acid" or "nucleic acid sequence," as used herein, refer to an oligonucleotide, nucleotide, polynucleotide, or any fragment thereof, to DNA or RNA of genomic or synthetic origin which may be single-stranded or double-stranded and may

represent the sense or the antisense strand, to peptide nucleic acid (PNA), or to any DNA-like or RNA-like material.

As a result of the degeneracy of the genetic code, a number of coding sequences can be produced which encode, for example, the same CoA transferase (CTF) polypeptide sequence. For example, the triplet codon CGT encodes the amino acid arginine. Arginine is alternatively encoded by CGA, CGC, CGG, AGA and AGG. Therefore it is appreciated that such substitutions of synonymous codons in the coding region fall within the nucleotide sequence variants that are covered by the invention.

As used herein, the term “operably linked” means that the components to which the term is applied are in a relationship that allows them to carry out their inherent functions under suitable conditions. For example, a control sequence which is “operably linked” to a protein coding sequence is ligated thereto, so that expression of the protein coding sequence is achieved under conditions compatible with the transcriptional activity of the control sequences. By way of an example, a first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein-coding regions, in the same reading frame.

As used herein, the term “comprising” or “including” is to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude the presence or addition of one or more features, integers, steps or components, or groups thereof. However, in context with the present disclosure, the term “comprising” or “including” also includes “consisting of”. The variations of the word “comprising”, such as “comprise” and “comprises”, and “including”, such as “include” and “includes”, have correspondingly varied meanings. Bibliographic references mentioned in the present specification are for convenience listed in the form of a list of references and added at the end of the examples. The whole content of such bibliographic references is herein incorporated by reference but their mention in the specification does not imply that they form part of the common general knowledge.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention.

EXAMPLES

Standard molecular biology techniques known in the art and not specifically described were generally followed as described in Green and Sambrook, Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York (2012).

5 Example 1: Materials and Methods

1.1 Bacterial strains and plasmids

A list of strains and plasmids used is shown in Table 1. *E. coli* DH5 α was used for molecular cloning. *E. coli* MG1655DE3 was used as a host for recombinant protein expression.

10 **Table 1: Strains and plasmids**

	Description	Sources
PLASMIDS		
pECE1.1	pMB1-P _{T7} - <i>atoDA</i> - <i>saat</i> -T7t	herein
pECE1.1.1	pMB1-P _{T7} - <i>atoDA</i> -T7t	herein
pECE1.1.2	pMB1-P _{T7} - <i>saat</i> -T7t	herein
pECE1.2	pMB1-P _{T7} - <i>ctfAB</i> - <i>saat</i> -T7t	herein
pECE1.4	pMB1-P _{T7} - <i>ctfAB</i> - <i>atf1</i> -T7t	herein
pECE1.4.2	pMB1-P _{T7} - <i>ctfAB</i> - <i>aaat</i> -T7t	herein
pECE1.4.3	pMB1-P _{T7} - <i>ctfAB</i> - <i>vaat</i> -T7t	herein
pECE1.4.5	pMB1-P _{T7} - <i>ctfAB</i> - <i>liaat</i> -T7t	herein
pECE1.5	pMB1-P _{T7} - <i>ptb</i> - <i>buk</i> - <i>saat</i> -T7t	herein
pECE1.10.1	pMB1-P _{T7} - <i>ilov</i> -T7t	herein
pECE1.10.2	pMB1-P _{thrC3} - <i>ilov</i> -T7t	herein
pECE1.10.3	pMB1-P _{ldhA} - <i>ilov</i> -T7t	herein
pECE1.10.4	pMB1-P _{adhE} - <i>ilov</i> -T7t	herein
pECE1.10.5	pMB1-P _{nar} - <i>ilov</i> -T7t	herein
pECE1.11.4	pMB1-P _{adhE} - <i>ctfAB</i> - <i>vaat</i> -T7t	herein
STRAINS		
G117	<i>C. beijerinckii</i> G117	(Yan et al., 2016)
MG1655DE3	<i>E. coli</i> MG1655 (DE3)	Lab collection
YHECE1.1	MG1655DE3 hosting pECE1.1	herein

YHECE1.1.1	MG1655DE3 hosting pECE1.1.1	herein
YHECE1.1.2	MG1655DE3 hosting pECE1.1.2	herein
YHECE1.2	MG1655DE3 hosting pECE1.2	herein
YHECE1.4	MG1655DE3 hosting pECE1.4	herein
YHECE1.4.2	MG1655DE3 hosting pECE1.4.2	herein
YHECE1.4.3	MG1655DE3 hosting pECE1.4.3	herein
YHECE1.4.5	MG1655DE3 hosting pECE1.4.5	herein
YHECE1.5	MG1655DE3 hosting pECE1.5	herein
YHECE1.10.1	MG1655DE3 hosting pECE1.10.1	herein
YHECE1.10.2	MG1655DE3 hosting pECE1.10.2	herein
YHECE1.10.3	MG1655DE3 hosting pECE1.10.3	herein
YHECE1.10.4	MG1655DE3 hosting pECE1.10.4	herein
YHECE1.10.5	MG1655DE3 hosting pECE1.10.5	herein
YHECE1.11.4	MG1655DE3 hosting pECE1.11.4	herein

Plasmid construction

A GT standard method was used to construct all the plasmids (Ma et al., 2019). Genes and primers used for plasmid construction were summarized in Table 2.

5 **Table 2: Genes, primers, and barcodes used for plasmid construction**

Gene	Accession number/source	
<i>saat</i>	AX025475	
<i>atf1</i>	P40353	
<i>vaat</i>	AX025504	
<i>liaat</i>	AIW81432.1	
<i>aaat</i>	AX025508	
<i>atoDA</i>	P76458 and P76459	
<i>ctfAB</i>	P33752 and P23673	
<i>ptb-buk</i>	P58255 and Q45829	
<i>ilov</i>	pTKEI-tLOV (Addgene)	
Primer	Sequences	SEQ ID NO.
<i>Psaat-f</i>	G*gaaaagattgaggtcagtat	25
<i>Psaat-r</i>	A*aattaaggtcttggggag	26
<i>PatoDA-f</i>	G*aaaacaaaattgatgacattacaa	27
<i>PatoDA-r</i>	A*taaatcaccccggtgcg	28
<i>PctfAB-f</i>	G*aactctaaaataattagatttgaa	29
<i>PctfAB-r</i>	A*aacagccatgggtctaag	30
<i>Pptb-buk-f</i>	G*attaagagtttaataaattatc	31
<i>Pptb-buk-r</i>	A*ttgtattccttagcttttct	32

<i>Patf1-f</i>	G*aatgaaatcgatgagaaaaatc	33
<i>Patf1-r</i>	A*agggcctaaaaggagag	34
<i>Paaat-f</i>	G*agttttccgtacttcaagt	35
<i>Paaat-r</i>	A*ttgactagtactacgcag	36
<i>Pvaat-f</i>	G*gagaaaattgaggttctatca	37
<i>Pvaat-r</i>	A*gtaacgggagataagtgct	38
<i>Pliaat-f</i>	G*gctatgatcatcacaaaaca	39
<i>Pliaat-r</i>	A*agtggtccagttgttatagttt	40
<i>Pilov-f</i>	G*tccatcgaaaaaaactcg	41
<i>Pilov-r</i>	A*atggtgatggtgatggtg	42
Barcodes	Sequence	SEQ ID NO.
RBS1	TagaaataattttgtttaactttaagaaggagatatacatatG	43
RBS4stop	TaaattaattgtctttttcaggtgaaggttcccatG	44
N31	TgatgggctgaagggttaaG	45

1.2 Media and culture conditions

1.2.1 Culture media

Luria–Bertani (LB) medium was used for the cell growth of *E. coli* DH5 α and the preculture of MG1655DE3 and its derivatives. Ampicillin (100 μ g/mL) was added into the media where appropriate.

A modified anaerobic defined salt medium containing 5 g/L yeast extract (termed as anaerobic DSMY) and a specific amount of carbon source was used for the mono-culture of G117 (Tables 3 and 4) (Cui et al., 2020a).

10 **Table 3: Trace element solution (1L)**

Chemicals used	Amount	
	ml	g
HCl (25% solution, w/w)	10	-
FeCl ₂ ·4H ₂ O	-	1.5
CoCl ₂ ·6H ₂ O	-	0.19
MnCl ₂ ·4H ₂ O	-	0.1
ZnCl ₂	-	0.07
H ₃ BO ₃	-	0.006
Na ₂ MoO ₄ ·2H ₂ O	-	0.036
NiCl ₂ ·6H ₂ O	-	0.024
CuCl ₂ ·2H ₂ O	-	0.002

Table 4: Na₂SeO₃–Na₂WO₄ Solution (1L)

Chemicals used	Amount	
	ml	g
Na ₂ SeO ₃ ·5H ₂ O	-	0.006
Na ₂ WO ₄ ·2H ₂ O	-	0.008

NaOH	-	0.5
------	---	-----

For recombinant protein expression in *E. coli* mono-culture and *E. coli*-G117 co-culture, anaerobic DSMY was prepared without reducing agents and N₂ purging (termed as aerobic DSMY). A specific amount of carbon source was supplemented where appropriate.

5 1.2.2 Culture conditions of DH5α

E. coli DH5α was cultured aerobically at 37 °C/225 rpm in Luria-Bertani (LB) medium. Agar (15 g/L) was added before the sterilization for preparing LB agar plates. Ampicillin (100 µg/mL) was added where appropriate.

1.2.3 Ester production by the mono-cultures of *E. coli* strains hosting P_{T7} plasmids

10 For MG1655DE3 strains hosting P_{T7} plasmids, cells were firstly precultured in LB medium with 100 µg/mL ampicillin at 37 °C/225 rpm overnight. Then the overnight-cultured cells were inoculated into 30 mL of aerobic DSMY with 20 g/L glucose and 100 µg/mL ampicillin in a 125-mL shake flask to reach an initial OD₆₀₀ of 0.05. Cells were cultivated at 37 °C/225 rpm. When the OD₆₀₀ achieved 0.5, β-D-1-thiogalactopyranoside (IPTG) was
15 added in a working concentration of 0.1 mM to induce the protein expression. Cells were then cultured at 30 °C/225 rpm for protein expression for 8 h. Then 8 mL of cell culture was transferred into a 20-mL serum bottle with a butyl stopper. Subsequently, butanol and sodium butyrate were added in a working concentration of 3 g/L and 3.8 g/L respectively. After pH was adjusted to 6.5, 4 mL hexadecane was added as extractant. This time point
20 was designated as the start of the fermentation (0 h). Cells were then cultured at 30 °C/225 rpm. Samples from the hexadecane layer and aqueous layer were taken after 24 h. The pH was not adjusted throughout the experiments. Experiments were done in triplicates.

1.2.4 Ester production by the mono-cultures of *E. coli* strains hosting the anaerobic promoter plasmids

25 For MG1655DE3 strains hosting anaerobic promoter plasmids, cells were precultured in LB medium with 100 µg/mL ampicillin at 37 °C/225 rpm overnight. Then the precultured cells were inoculated into a 20-mL serum bottle containing 8 mL of aerobic DSMY with 20 g/L glucose for to induce gene expression. The initial OD₆₀₀ was 0.5. After that, 3 g/L butanol and 3.8 g/L sodium butyrate were added. After pH was adjusted to 6.5, 4 mL of hexadecane
30 was added as extractant. This time point was designated as the start of the fermentation (0h). Cells were then cultured at 30 °C/225 rpm. Samples from the hexadecane layer and aqueous layer were taken after 24 h. The pH was not adjusted throughout the experiments. Experiments were done in triplicates.

As a control, MG1655DE3 strains hosting P_{T7} plasmids were cultivated in the same way, except that 0.1 mM IPTG was added at 0 h.

All experiments were done in triplicates.

For pH-dependent ester production experiments, YHECE1.11.4 expressing *ctfAB* and *vaat* under P_{adhE} was mono-cultured following the procedure above at starting pH of 5.0, 5.5, 6.0, and 6.5 respectively. The pH was not adjusted throughout the experiments.

1.2.5 Ester production by *E. coli*-*Clostridium* co-cultures

G117 was precultured in 30 mL of anaerobic DSMY containing 20 g/L glucose at 37 °C/225 rpm for 24 h. *E. coli* was precultured in LB with 100 µg/mL ampicillin at 37 °C/225 rpm overnight.

For MG1655DE3 hosting P_{T7} plasmids, precultured cells were cultured at 30 °C/225 rpm for 8 h after the addition of 0.1 mM IPTG. Then 10 mL or 30 mL of cell culture was centrifuged at 10,000 × g for 5 min. Subsequently, the cells were resuspended into 30 mL of aerobic DSMY with specific amount of sterile carbon source (initial OD: ~ 4). After 3 mL of precultured G117 was inoculated, the cells were transferred into a 160-mL serum bottle with butyl stopper. After 15 mL of hexadecane was added as extractant, cells were cultured at 30 °C/225 rpm. The pH was adjusted to 6.3 every 12 h. Samples from both aqueous phase and organic phase were taken every 24 h.

For P_{adhE} hosting MG1655DE3, precultured cells were inoculated into 30 mL of aerobic DSMY with specific amount of sterile carbon source at a starting OD₆₀₀ of 0.5. After 3 mL of precultured G117 was inoculated, the cells were transferred into a 160-mL serum bottle with butyl stopper. After 15 mL of hexadecane was added as extractant, cells were cultured at 30 °C/225 rpm. The pH was adjusted to 6.3 every 12 h. Samples from both aqueous phase and organic phase were taken every 24 h.

When 60 g/L microcrystalline cellulose (MCC, Y0002021, Sigma-Aldrich) was used as carbon source, 500 U of cellulase from *Aspergillus niger* (C1184, Sigma-Aldrich) was added into the cell culture together with sterile MCC at the beginning of the fermentation.

When 60 g/L pretreated rice straw was used as carbon source, 83.3 U/mL of cellulase from *Aspergillus niger* (C1184, Sigma-Aldrich) was added into the cell culture together with pretreated rice straw (sterilized) at the beginning of the fermentation.

All experiments were done in triplicates.

1.3 Bioreactor fermentation

For the biphasic extractive co-culture fermentations conducted in bioreactors, Applikon Bioreactors (MiniBioBundle, Applikon Biotechnology, The Netherlands) with 500 mL vessels were used. Precultured *E. coli* strain was used to inoculate 100 mL of aerobic DSMY at an initial OD₆₀₀ of 0.5, and 10 mL of precultured G117 was also inoculated.

5 Pretreated rice straw and cellulase from *A. niger* were added to 60 g/L and 83.3 U/mL respectively. Besides, 50 mL of hexadecane was added as the extractant. After the starting pH was adjusted to 6.5, the pH was allowed to drop to 5.8. Subsequently, pH was maintained at 5.8 till the end of the fermentation. Experiments were done in duplicates.

1.4 Anaerobic promoter screening

- 10 MG1655DE3 strains hosting plasmids with *ilov* gene and different promoters were precultured in LB medium with 100 µg/mL of ampicillin overnight. Then the cells were inoculated into 8 mL of aerobic DSMY with 20 g/L glucose in a 20-mL serum bottle. The initial OD₆₀₀ was 0.5. IPTG was added to reach a working concentration of 0.1 mM when P_{T7} was used. Samples were taken at 0h, 0.5h, and 6h for fluorescence measurement.
- 15 Experiments were done in triplicates.

For quantifying fluorescence signal, 100 µL of cell suspension was loaded into a well of a 96-well optical plate. Fluorescence was measured by a microplate reader (Infinite 200PRO [Tecan]) with excitation wavelength at 450 nm, emission wavelength at 495 nm, and gain at 65. Fluorescence of blank medium was regarded as background fluorescence.

20 1.5 Rice straw pretreatment

- Rice straw from India was used as model biomass in the study. The mean size of the rice straw is about 1 mm. It was stored in a dry cabinet before use. Fe-TAML catalyst was purchased from GreenOx Catalysts Inc., Pennsylvania. Hydrogen peroxide (20%) was purchased from Best Chemical Co (Singapore). All chemicals were used as received without
- 25 further purification. Deionized water was obtained from a Milli-Q system (U.S.A.).

- Ten grams of RS was added into 1% of NaOH solution to make the final concentration (50 g/L). The solution was placed inside a bioreactor (MiniBioBundle, Applikon Biotechnology, The Netherlands) with constant stirring of 1000 rpm at 50 °C. After 1 h of soaking, 1 mg/L Fe-TAML catalyst and 10 g/L H₂O₂ were added into the solution (the
- 30 concentrations are working concentrations). The pretreatment time was 3 h. After the pretreatment, the liquor was filtered, and all remaining solids were collected and dried at 65 °C for 10 h. Samples were autoclaved at 121 °C for 20 min and then store at room temperature before use.

1.6 Analytical techniques

1.6.1 Cell density measurement

Cell density was measured by monitoring the optical density (OD) at 600 nm with a visible spectrophotometer (Novaspec II, Pharmacia Biotech, Cambridge, England).

5 1.6.2 Metabolites quantification

Glucose, xylose, cellobiose, lactate, and succinate were quantified by High Performance Liquid Chromatography (HPLC) (Agilent Technologies, USA) equipped with a refractive index detector (RID) and variable wavelength detector (VWD) (210 nm). A Bio-Rad Aminex HPX-87H column (300 mm × 7.8 mm) was used. Five micromolar of sulfuric acid was
10 used as the mobile phase at the flow rate of 0.7 mL/min.

Acetate, butyrate, acetone, and butanol in the cell culture were quantified by gas chromatography (GC, model 7890A; Agilent Technologies, USA) equipped with a Durabond (DB)-WAXetr column (30m × 0.25mm × 0.25µm, J&W, USA) and flame ionization detector (FID). The oven temperature was initially maintained at 60 °C for 2 mins, increased at
15 15 °C/min to 230 °C, and held for 1.7 min. Helium was used as the carrier gas with a column flow rate of 1.5 mL/min (Chua et al., 2013). Samples were centrifuged at 10, 000 × g for 2 min and filtered. The supernatant of 475 µL was mixed with 25 µL of 2 M HCL before being quantified.

For the above metabolites, they were all quantified only from the aqueous samples
20 because their partition coefficients in the hexadecane/aqueous system are relatively low (Zhang et al., 2017).

Esters in the hexadecane phase were directly injected into GC-FID following the protocol above. Esters in the aqueous phase was neglected in this study because the partition coefficient of butyl butyrate in the hexadecane/aqueous system was reported to be
25 more than 300 (Van Den Berg *et al.*, 2013; Zhang et al., 2017). All esters were quantified from the hexadecane layer and the concentrations were divided by 2 (the volume of the aqueous phase was two times that of the hexadecane phase) so that the unit would be in gram of esters per liter of the aqueous phase.

1.6.3 Fluorescence measurement

30 For quantifying fluorescence signal, 100 µL of cell suspension was loaded into a well of 96-well optical plate. Fluorescence was measured by a microplate reader (Infinite 200PRO [Tecan]) with excitation wavelength at 450 nm, emission wavelength at 495 nm,

and gain at 65 to reach optimal results. Fluorescence of blank medium was regarded as background fluorescence. Specific fluorescence signal was obtained by the equation below:

$$\text{Specific fluorescence} = (\text{Fluorescence} - \text{Background fluorescence}) / \text{OD}_{600}$$

1.6.4 SDS-PAGE

- 5 One milliliter of cell culture was taken and centrifuged at $10,000 \times g$ for 2 min. Then the cell pellets were frozen at -20°C for 6 h. After that, 200 μL of B-PER Complete Reagent (Thermo Scientific) was added to resuspend the cells. After being incubated at room temperature for 15 min, the cell lysates were centrifuged at $16,000 \times g$ for 20 min. The supernatant was used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-
10 PAGE) based on the manufacturer's instruction.

1.6.5 Dynamic abundance measurement

- Live BacLight Bacterial Gram Stain Kit (L7005, ThermoFisher) was used to stain the *E. coli* and *C. beijerinckii* G117 in the co-culture. Fluorescence was measured by a microplate reader (Infinite 200PRO [Tecan]) with an excitation wavelength of 485 nm and
15 emission wavelengths of 530 nm (for green fluorescence of *E. coli*) and 620 nm (F_{620} , for red fluorescence of G117). The experiments were conducted following the manufacturer's protocol. Fluorescence signal at emission wavelengths of 530 nm and 620 nm were designated as F_{530} and F_{620} . The relative dynamic abundance of G117 and *E. coli* was calculated by F_{620} / F_{530} .

20 1.6.6 Dissolved oxygen measurement

Dissolved oxygen (DO) was measured by a DO probe (Applikon). The DO level at the beginning of the fermentation was defined calibrated as 100%.

Example 2: Establishing butyl butyrate production in *E. coli*-*Clostridium* co-culture

- To enable the production of butyl butyrate from butanol and butyrate in *E. coli*, at
25 least two enzymes are needed: a butyryl-CoA-generating enzyme that can convert butyrate into butyryl-CoA and one ester-producing enzyme that condenses butanol and butyryl-CoA into butyl butyrate (Figure 1).

- As a proof of concept, the inventors first selected a gene from *E. coli* named *atoDA* that encoded a CoA transferase (CTF). This enzyme had been confirmed to convert butyrate
30 into butyryl-CoA efficiently at the expense of acetyl-CoA (Saini et al., 2015b). the inventors also chose an alcohol acyltransferase (ATF)-encoding gene that had been used for butyl butyrate production in *Clostridium* (gene name: *saat*; origin: strawberry) (Noh et al., 2018).

The inventors cultured the *E. coli* strain over-expressing *atoDA* and *saat* (YHECE1.1; promoter: T7) in a capped serum bottle containing an aqueous solution that mimicked the broth of an ongoing ABE fermentation (DSMY medium containing 20 g/L glucose, 3 g/L butanol, and 3 g/L butyrate; the medium did not undergo anaerobic treatment, termed as aerobic DSMY). The serum bottle also contained hexadecane as an organic extractant to recover any produced butyl butyrate. After being cultured for 24 h, YHECE1.1 produced 257 mg/L butyl butyrate (Figure 2A), while a negligible amount of butyl butyrate was made in control experiments where YHECE1.1 was replaced by a similar strain that only overexpressed *atoDA* or *saat*. The results confirmed that both CTF and ATF are needed for butyl butyrate production.

The inventors next co-cultured *E. coli* YHECE1.1 with a solventogenic *Clostridium beijerinckii* strain (G117) in the aerobic DSMY medium with 40 g/L glucose as the sole carbon source. In this co-culture, YHECE1.1 would grow first and consume oxygen in the peripheral environment. After oxygen was depleted, G117 should grow on glucose and produce butanol and butyrate. Meanwhile, YHECE1.1 should also grow on glucose and condense the produced butanol and butyrate into butyl butyrate, which could be in situ extracted by a hexadecane overlay (Figure 2C). In the co-culture, butanol and butyrate were successfully detected, suggesting that G117 could grow in the medium without any anaerobic treatment (Figures. 3B and C) – *E. coli* cannot produce butanol or butyrate. As G117 an obligate anaerobe and cannot grow in the aerobic medium, its growth in the co-culture should be attributed to the anaerobic environment created by YHECE1.1 (Cui et al., 2020a).

More importantly, the co-culture produced 156 mg/L butyl butyrate in 120 h (Figure 2B). This titer was almost three times the butyl butyrate titer achieved by another study in which a *saat*-expressing *Clostridium acetobutylicum* strain was used (Jenkins et al., 2013). When G117 was cultured alone under anaerobic condition, it produced a much smaller quantity of butyl butyrate (~40 mg/L in 120 h), possibly through the action of its native lipase or ATF (Xin et al., 2016). When G117 was co-cultured with the parent *E. coli* strain that did not overexpress *atoDA* and *saat* (MG1655DE3), the co-culture also produced ~40 mg/L butyl butyrate in 120 h, suggesting *atoDA* and *saat* were responsible for achieving the higher titer (156 mg/L). As expected, neither YHECE1.1 nor MG1655DE3 alone produced any butyl butyrate, further supporting that the higher butyl butyrate titer was the result of cooperation between YHECE1.1 and G117.

Example 3: Improved butyl butyrate production by pathway engineering

To improve the production of butyl butyrate, the inventors screened variants of the genes overexpressed in *E. coli*, expecting that the corresponding enzyme variants may have different product spectra and catalytic efficiency (Noh et al., 2018).

5 The inventors first focused on converting butyrate into its CoA thioester. *AtoDA*, the gene used in earlier part of this study, were compared with *ctfAB* and *ptb-buk* from *C. acetobutylicum*. Protein products of *atoDA* and *ctfAB* convert butyrate into butyryl-CoA directly by using acetyl-CoA as the CoA donor (Figure 4A). The enzymes encoded by *ptb* and *buk* do the same job in two steps: phosphorylation of butyrate by using ATP, followed by
10 condensation with free CoA (Yu et al. 2015). *E. coli* YHECE1.2 and YHECE1.5 were obtained by replacing *atoDA* in YHECE1.1 by *ctfAB* and *ptb-buk*, respectively (Figure 4B). These three strains were compared in mono-culture with the DSMY medium containing 20 g/L glucose, 3 g/L butanol, and 3 g/L butyrate. YHECE1.2 (*ctfAB*) produced much more butyl butyrate than YHECE1.1 (*atoDA*) (600 mg/L vs. 356 mg/L in 24 h, Figure 4B), possibly due
15 to that *ctfAB* are naturally used in *C. acetobutylicum* to re-assimilate butyrate in the solventogenic phase (Yu et al., 2011). *CtfAB* was used hereinafter.

 Four acyltransferases (ATF) were screened for condensing butyryl-CoA with butanol (Figure 4C) (Horton and Bennett, 2006). *Saat* in YHECE1.5 was replaced by *atf1* (from *Saccharomyces cerevisiae*), *aat* (from apple), *vaat* (from strawberry), or *liaat* (from lavender,
20 the sequences of these genes are provided in Table 2). Under the same mono-culture condition as described above, only *vaat* outperformed *saat*, improving the butyl butyrate titer from 600 mg/L to 818 mg/L (Figure 4C). The butyl butyrate titer was more than 10 times what was produced in a previous study by an engineered *C. acetobutylicum* strain expressing the *saat* gene (Noh et al., 2018). Using *vaat* also resulted in the production of 14
25 mg/L butyl acetate which was not produced when *saat* was employed, which may be due to *vaat* having a higher binding affinity towards acetyl-CoA than *saat* (Layton and Trinh, 2016). The *atf1*-expressing strain produced 110 mg/L butyl acetate, whereas no butyl butyrate was produced. This was consistent with results from another study that *atf1* showed stronger preference for acetyl-CoA than *saat* and *vaat* (Horton and Bennett, 2006; Layton and Trinh,
30 2016). *Vaat* was used hereinafter.

Example 4: Application of anaerobic promoters

In the above examples, the inventors utilized T7 promoter in *E. coli*, which needed addition of isopropyl β -D-1-thiogalactopyranoside (IPTG). The practice incurred not only additional cost but also effort during the fermentation. To avoid using a chemical inducer and

enable the autonomous induction of gene expression, a few promoters were characterized that can be activated under an oxygen-limited condition (termed as anaerobic promoters). These promoters include P_{nar}, P_{adhE}, and P_{ldhA}. In addition, an auto-inducible promoter P_{thrC3} reported to induce protein expression at the early exponential phase of cell growth was also included (Anilionyte et al., 2018).

To characterize these promoters, a flavin-based fluorescent protein gene (*ilov*) was employed. As the protein product of *ilov* does not need oxygen for chromophore maturation and fluorescence emission, it was considered to be a suitable reporter (Mukherjee et al., 2013). *E. coli* strains were constructed to express *ilov* under P_{nar}, P_{adhE}, P_{ldhA}, P_{thrC3}, and P_{T7} (Figure 5B). These strains and the parent strain (MG1655DE3) were cultured in closed serum bottles containing the aerobic DSMY and 20 g/L glucose. With the cell growth, oxygen was consumed, activating the anaerobic promoters (Fig. 5A). The promoter strength was evaluated according to the specific fluorescence of their host strains. P_{adhE} showed the best performance after P_{T7}, among all the tested promoters (Fig. 5B). We then co-expressed *ctfAB* and *vaat* under P_{adhE} in *E. coli*, resulting in a new strain YHECE1.11.4. YHECE1.11.4 was used in the subsequent experiments to simplify the fermentation procedure – using P_{T7} requires manual addition of isopropyl β -D-1-thiogalactopyranoside (IPTG) during cell growth.

Example 5: Production of butyl butyrate from glucose

When YHECE1.11.4 was co-cultured with G117 in the aerobic DSMY medium containing 60 g/L glucose in a closed serum bottle, the dissolved oxygen (DO) level decreased from 100% to 0% within 30 min (Figure 6E). Butyl butyrate production started after 24 h, along with the production of butanol and butyrate. There was 581 mg/L butyl butyrate, 4.8 g/L butanol and 3.4 g/L butyrate produced after 120 h (Figure 6A and 6B). Besides, the production of 14.8 mg/L butyl acetate was also observed. Although the butyl butyrate titer (581 mg/L) was more than 10 times the highest butyl butyrate titer achieved previously without adding lipase and additional substrate, the product titer and yield still need further optimization.

Notably, the lactate concentration increased in the first 72 h and peaked at 3.8 g/L. After 72 h, the lactate concentration started to decrease at a constant rate until the end of fermentation (Figure 6C). We hypothesize that YHECE1.11.4 produced lactate as the main product during its anaerobic growth, while G117 assimilated lactate during fermentation to produce butanol or butyrate. The profile of lactate was consistent with the dynamic abundance of G117 and YHECE1.11.4 (Figure 6D). After G117 entered its exponential

phase, its lactate consumption rate exceeded the lactate production rate, leading to the observed peak in the lactate concentration profile.

Example 6: Production of butyl butyrate from lignocellulosic waste

5 The goal in this example was to produce butyl butyrate from cheaper substrates than glucose. As rice is the third most important grain crop worldwide, rice straw is an abundant lignocellulosic inedible waste (Binod et al., 2010). Moreover, rice straw has high cellulose and hemicellulose that can be hydrolyzed into fermentable sugars such as glucose and xylose (Table 5). Therefore, using inedible rice straw instead of starchy material for future energy needs to avoid food security issues has gained interest.

10 The inventors first tested if the co-culture could utilize hemicellulose and cellulose. As G117 can directly ferment xylan by secreting xylanase, the inventors first chose xylan as the hemicellulose to be used in the co-culture (Figure 7A) (Yan et al., 2016; Ng et al., 2015). When 60 g/L xylan (from beechwood, X4252) was used as carbon source, 103 mg/L butyl butyrate was produced within 120 h without the addition of any enzyme (Figure 7C). Xylose
15 accumulation during the fermentation was observed, suggesting that G117 secreted xylanase very efficiently (Figure 7B and D). In addition, 4.9 g/L butyrate was produced as the main product, which was much higher than the concentration of butanol (1.1 g/L) (Figure 7D). The high butyrate/butanol ratio was because xylan triggered the low expression level of *ctfAB* which is responsible for acid re-assimilation in G117 (Yan et al., 2016). As the
20 production of butyl butyrate from xylan did not involve the addition of commercial xylanase, this process was actually the consolidated bioprocessing (CBP) of xylan because it combined the enzyme production, substrate hydrolysis, and fermentation into one single step (Cui et al., 2020).

When cellulose was used as carbon source, cellulase had to be added because
25 neither G117 nor YHECE1.11.4 could utilize cellulose directly. With the help of cellulase, the co-culture produced 231 mg/L butyl butyrate from 60 g/L microcrystalline cellulose (MCC), along with 5.9 g/L butanol and 2.8 g/L butyrate (Figure 7F and G). As G117 can produce glucosidase to hydrolyze cellobiose into glucose, cellobiose liberated from cellulose by cellulase was also used for butyl butyrate production (Figure 8B).

30 Example 7: Production of butyl butyrate from rice straw

The co-culture system was tested with rice straw which is a raw biomass. One concern with rice straw and other lignocellulosic biomass is that the lignin content which mainly consists of monomers such as coniferyl alcohol, p-coumaryl, and sinapyl alcohol which protect cellulose and hemicellulose against enzymatic hydrolysis. Therefore,

pretreatment is needed to remove lignin from the rice straw to enable more access of enzyme to the cellulose and hemicellulose. A pretreatment method named catalytic alkaline peroxide (cAHP) that incorporates alkaline hydrogen peroxide and Fe-TAML catalyst was employed to depolymerize lignin biopolymers. Phenolic components in lignin were oxidized by hydrogen peroxide and an iron-complex catalyst under an alkaline condition according to method described in Example 1.5.

Table 5 Composition of rice straw used in this study

Component	Percentage (%)
Glucan	32.9
Xylan	15.9
Arabinan	2.4

When 60 g/L pretreated rice straw was used as the carbon source with 83.3 U/mL of exogenously added *Aspergillus niger* (C1184, Sigma-Aldrich) cellulase, 465 mg/L butyl butyrate was produced by the co-culture of YHECE1.11.4 and G117 within 120 h in serum bottles (Fig. 10A). Further co-culturing the cells for another 48 h led to the production of another 69 mg/L butyl butyrate, bringing the total titer to 534 mg/L. This titer was comparable to the butyl butyrate titer achieved using purified glucose, suggesting that the pretreatment step did not affect the microbial growth. 10.2 g/L butanol and 2.3 g/L butyrate were also produced, representing the highest butanol titer among all co-culture experiments in this study (Fig. 10B). This butanol titer was also comparable to many other anaerobic *Clostridium* mono-cultures (Al-Shorgani et al., 2018; Chua et al., 2013; Xin et al., 2014).

Example 8: Production of butyl butyrate by *E. coli* is sensitive to pH.

A higher pH (within the range of 5.0-6.5) during the fermentation period led to the production of more butyl butyrate by *E. coli* (Figure 10H), while G117 produced most butanol within the pH range of 5.5 to 6.0 (Chua et al., 2013). In the serum bottle experiments described above, pH was manually adjusted twice a day to 6.3. To test if a better controlled pH would lead to higher ester titer, a biphasic extractive co-culture fermentation was set up in a bioreactor with pH maintained at 5.8 (Figure 10G). The same rice straw-based medium was used. Co-culturing the cells in the bioreactors led to the production of 1,007 mg/L butyl butyrate within 168 h (Figure 10D), which was almost two times the butyl butyrate titer produced in serum bottles, suggesting the importance of the pH control. Besides, 109 mg/L butyl acetate, 1.5 g/L butyrate, and 8.0 g/L butanol were also produced (Figure 10E). Further co-culturing the cells for another 72 h resulted in the production of another 273 mg/L butyl butyrate, bringing the final butyl butyrate titer up to 1,280 mg/L. To our knowledge, this is the highest butyl butyrate titer achieved without using lipase. Notably, a decrease in butanol and

acetone concentrations was observed during the late stage of the fermentation, which could be due to evaporation (Figure 10E and 10F) because the bioreactor was not airtight (unlike the serum bottles).

To avoid the addition of cellulase, the *E. coli* strain was engineered to secrete
5 cellulase. Although the secretion was successful under an aerobic condition in LB medium, no progress was made in the aerobic DSMY medium (Figure 9A and B). Nevertheless, due to the ability of G117 to secrete hydrolyzing enzymes such as xylanase (Chua et al., 2013; Yan et al., 2016), the co-culture could successfully produce butyl butyrate from xylan and cellobiose without the addition of exogenous hydrolyzing enzymes, although the titer was
10 lower than what was achieved from the rice straw when cellulase was added (Figures 7 and 8).

References

- Anilionyte, O., Liang, H., Ma, X., Yang, L., Zhou, K., 2018. Short, auto-inducible promoters for
15 well-controlled protein expression in *Escherichia coli*. *Appl Microbiol Biotechnol*. 102, 7007-7015.
- Binod, P., et al. (2010) Bioethanol production from rice straw: an overview. *Bioresource technology* 101, 4767-4774.
- Brault, G., et al. (2014) Short-chain flavor ester synthesis in organic media by an *E. coli* whole-
20 cell biocatalyst expressing a newly characterized heterologous lipase. *PLoS One* 9, e91872.
- Chua, T.K., et al. (2013) Characterization of a butanol–acetone-producing *Clostridium* strain and identification of its solventogenic genes. *Bioresource technology* 135, 372-378.
- Cui, Y., et al. (2020a) Aerobic acetone-butanol-isopropanol (ABI) fermentation through a co-
culture of *Clostridium beijerinckii* G117 and recombinant *Bacillus subtilis* 1A1. *Metabolic Engineering Communications*, e00137.
- Cui, Y., et al. (2020b) Production of isopropyl and butyl esters by *Clostridium* mono-culture and
co-culture. *Journal of Industrial Microbiology & Biotechnology* 47, 543-550.
- Cui, Y., et al. (2020c) Using Co-Culture to Functionalize *Clostridium* Fermentation. *Trends in Biotechnology* doi.org/10.1016/j.tibtech.2020.11.016.
- 30 Dürre, P. (2011) Fermentative production of butanol—the academic perspective. *Current opinion in biotechnology* 22, 331-336.
- Ebrahimi, E., et al. (2020) Efficient butanol production under aerobic conditions by coculture of *Clostridium acetobutylicum* and *Nesterenkonia* sp. strain F. *Biotechnology and Bioengineering* 117, 392-405.
- 35 Horton, C.E. and Bennett, G.N. (2006) Ester production in *E. coli* and *C. acetobutylicum*. *Enzyme and microbial technology* 38, 937-943.

- Jenkins, R.W., *et al.* (2013) Potential renewable oxygenated biofuels for the aviation and road transport sectors. *Fuel* 103, 593-599.
- Layton, D.S. and Trinh, C.T. (2016) Expanding the modular ester fermentative pathways for combinatorial biosynthesis of esters from volatile organic acids. *Biotechnology and bioengineering* 113, 1764-1776.
- 5 Lee, S.Y., *et al.* (2008) Fermentative butanol production by Clostridia. *Biotechnology and bioengineering* 101, 209-228.
- Li, Q., *et al.* (2010) Enhancing clostridial acetone-butanol-ethanol (ABE) production and improving fuel properties of ABE-enriched biodiesel by extractive fermentation with biodiesel.
- 10 *Applied biochemistry and biotechnology* 162, 2381-2386.
- Li, J., *et al.* (2014) Efficient production of acetone-butanol-ethanol (ABE) from cassava by a fermentation-pervaporation coupled process. *Bioresource technology* 169, 251-257.
- Lin, P.P., *et al.* (2015) Consolidated bioprocessing of cellulose to isobutanol using Clostridium thermocellum. *Metabolic engineering* 31, 44-52.
- 15 Mahapatra, M.K. and Kumar, A. (2017) A short review on biobutanol, a second generation biofuel production from lignocellulosic biomass. *J Clean Energy Technol* 5, 27-30.
- Mukherjee, A., Walker, J., Weyant, K. B., Schroeder, C. M., 2013. Characterization of flavin-based fluorescent proteins: an emerging class of fluorescent reporters. *PloS one*. 8, e64753.
- Ng, C.H., *et al.* (2015) Purification and characterization of a GH11 xylanase from biobutanol-producing Clostridium beijerinckii G117. *Applied biochemistry and biotechnology* 175, 2832-2844.
- 20 Noh, H.J., *et al.* (2018) Metabolic engineering of Clostridium acetobutylicum for the production of butyl butyrate. *Applied microbiology and biotechnology* 102, 8319-8327.
- Noh, H.J., *et al.* (2019) Microbial production of butyl butyrate, a flavor and fragrance compound.
- 25 *Applied microbiology and biotechnology* 103, 2079-2086.
- Oudshoorn, A., *et al.* (2009) Assessment of options for selective 1-butanol recovery from aqueous solution. *Industrial & Engineering Chemistry Research* 48, 7325-7336.
- Qureshi, N., *et al.* (2005) Energy-efficient recovery of butanol from model solutions and fermentation broth by adsorption. *Bioprocess and biosystems engineering* 27, 215-222.
- 30 Sreekumar, S., *et al.* (2015) Production of an acetone-butanol-ethanol mixture from Clostridium acetobutylicum and its conversion to high-value biofuels. *Nature protocols* 10, 528-537.
- Saini, J.K., *et al.* (2015) Lignocellulosic agriculture wastes as biomass feedstocks for second-generation bioethanol production: concepts and recent developments. *3 Biotech* 5, 337-353.
- Saini, M., *et al.* (2015) Potential production platform of n-butanol in Escherichia coli. *Metabolic engineering* 27, 76-82.
- 35 Schiel-Bengelsdorf, B., *et al.* (2013) Butanol fermentation. *Environmental technology* 34, 1691-1710.

- Seo, S.O., *et al.* (2017) Characterization of a *Clostridium beijerinckii* spo0A mutant and its application for butyl butyrate production. *Biotechnology and bioengineering* 114, 106-112.
- Van Den Berg, C., *et al.* (2013) Simultaneous clostridial fermentation, lipase-catalyzed esterification, and ester extraction to enrich diesel with butyl butyrate. *Biotechnology and*
5 *bioengineering* 110, 137-142.
- Wu, P., *et al.* (2016) Butanol production under microaerobic conditions with a symbiotic system of *Clostridium acetobutylicum* and *Bacillus cereus*. *Microbial cell factories* 15, 1-11.
- Xin, F., *et al.* (2016) Strategies for production of butanol and butyl-butyrate through lipase-catalyzed esterification. *Bioresource technology* 202, 214-219.
- 10 Xin, F., *et al.* (2018) Recent advances on conversion and co-production of acetone-butanol-ethanol into high value-added bioproducts. *Critical reviews in biotechnology* 38, 529-540.
- Xin, F., *et al.* (2019) Bioprocessing Butanol into More Valuable Butyl Butyrate. *Trends in biotechnology* 37, 923-926.
- Yan, Y., *et al.* (2016) Direct conversion of xylan to butanol by a wild-type *Clostridium* species
15 strain G117. *Biotechnology and bioengineering* 113, 1702-1710.
- Yen, H.-W. and Wang, Y.-C. (2013) The enhancement of butanol production by in situ butanol removal using biodiesel extraction in the fermentation of ABE (acetone–butanol–ethanol). *Bioresource technology* 145, 224-228.
- Zhang, Z.T., *et al.* (2017) In situ esterification and extractive fermentation for butyl butyrate
20 production with *Clostridium tyrobutyricum*. *Biotechnology and Bioengineering* 114, 1428-1437.
- Zhang, C., *et al.* (2018) Characterization and genome analysis of a butanol–isopropanol-producing *Clostridium beijerinckii* strain BGS1. *Biotechnology for biofuels* 11, 1-11.

CLAIMS

1. A composition for the production of butyl butyrate, comprising an acetone-butanol-ethanol (ABE)-producing *Clostridium* strain and a genetically engineered *E. coli* strain, wherein said genetically engineered *E. coli* strain has been transformed by at least one polynucleotide molecule;
5 the at least one polynucleotide molecule comprising a CoA transferase (CTF) gene and an alcohol acyltransferase (ATF) gene operably linked to at least one promoter.
2. The composition of claim 1, wherein the *Clostridium* strain is *C. beijerinckii* G117.
3. The composition of claim 1 or 2, wherein the CoA transferase (CTF) gene is selected from the group comprising *atoDA* and *ctfAB* and the alcohol acyltransferase (ATF) gene is selected from the group comprising *aaat* (from apple), *saat* (from strawberry), *liaat* (from lavender) and *vaat* (from strawberry).
10
4. The composition of any one of claims 1 to 3, wherein the CTF gene is *ctfAB* and the ATF gene is *vaat*.
5. The composition of any one of claims 1 to 4, wherein the CTF gene is substituted by a butyrate kinase (*buk*) gene and a phosphate butyryltransferase (*ptb*) gene.
15
6. The composition of any one of claims 1 to 5, wherein:
 - the *atoDA*; *atoD* and *atoA* genes encode a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 11 and 13, respectively, or a functional sequence variant thereof; and/or
20
 - the *ctfAB*; *ctfA* and *ctfB* genes encode a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 15 and 17, respectively, or a functional sequence variant thereof; and/or
 - the *aaat* gene encodes a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 7, or a functional sequence variant thereof; and/or
25
 - the *saat* gene encodes a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 1, or a functional sequence variant thereof; and/or
 - the *liaat* gene encodes a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 5, or a functional sequence variant thereof; and/or
 - the *vaat* gene encodes a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 3, or a functional sequence variant thereof; and/or
30

the *buk* gene encodes a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 21, or a functional sequence variant thereof; and/or

the *ptb* gene encodes a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 19, or a functional sequence variant thereof.

5 7. The composition of claim 6, wherein:

the *atoDA*; *atoD* and *atoA* genes comprise a nucleic acid sequence having at least 80% sequence identity, due to the degeneracy of the genetic code, to the polynucleotide sequence set forth in SEQ ID NO: 12 and 14, respectively; and/or

10 the *ctfAB*; *ctfA* and *ctfB* genes comprise a nucleic acid sequence having at least 80% sequence identity, due to the degeneracy of the genetic code, to the polynucleotide sequence set forth in SEQ ID NO: 16 and 18, respectively; and/or

the *aaat* gene comprises a nucleic acid sequence having at least 80% sequence identity, due to the degeneracy of the genetic code, to the polynucleotide sequence set forth in SEQ ID NO: 8; and/or

15 the *saat* gene comprises a nucleic acid sequence having at least 80% sequence identity, due to the degeneracy of the genetic code, to the polynucleotide sequence set forth in SEQ ID NO: 2; and/or

20 the *liaat* gene comprises a nucleic acid sequence having at least 80% sequence identity, due to the degeneracy of the genetic code, to the polynucleotide sequence set forth in SEQ ID NO: 6; and/or

the *vaat* gene comprises a nucleic acid sequence having at least 80% sequence identity, due to the degeneracy of the genetic code, to the polynucleotide sequence set forth in SEQ ID NO: 4; and/or

25 the *buk* gene comprises a nucleic acid sequence having at least 80% sequence identity, due to the degeneracy of the genetic code, to the polynucleotide sequence set forth in SEQ ID NO: 22; and/or

the *ptb* gene comprises a nucleic acid sequence having at least 80% sequence identity, due to the degeneracy of the genetic code, to the polynucleotide sequence set forth in SEQ ID NO: 20.

30 8. The composition of any one of claims 1 to 7, wherein the promoter is a strong promoter such as P_T7, or an anaerobic promoter, such as P_adhE.

9. The composition of any one of claims 1 to 7, wherein the at least one polynucleotide molecule further comprises a cellulase gene.
10. A method of producing butyl butyrate in a co-culture, comprising the steps:
- 5 a) co-culturing a composition of any one of claims 1 to 9 in medium under conditions for conventional ABE fermentation with exception that no anaerobic treatment is used, and
- b) supplementing the medium with a carbon source selected from the group comprising glucose, xylan, cellulose and lignocellulosic biomass,
- wherein the co-cultured *Clostridium* and *E. coli* are capable of increased production of
- 10 butyl butyrate compared to a co-culture with non-transformed *E. coli*.
11. The method of claim 10, wherein the lignocellulosic biomass is rice straw or corn stover.
12. The method of claim 10 or 11, wherein:
- i) the medium is supplemented with cellulase; or
- ii) the *E. coli* has been engineered to express cellulase,
- 15 when cellulose and/or lignocellulosic biomass are the carbon source.
13. The method of claim 12, wherein said lignocellulosic biomass has been pretreated to remove lignin in order to increase access of enzyme to cellulose and hemicellulose.
14. The method of any one of claims 10 to 13, wherein:
- a) the medium is supplemented with glucose at a concentration of at least 6 g/L, at
- 20 least 30 g/L, at least 60 g/L, at least 90 g/L or in the range from 30 g/L to 90 g/L, preferably in the range 50 g/L to 70 g/L; and/or
- b) the co-culturing step a) is performed with a starting pH of about 6.5, then the pH is allowed to drop to about 5.8 where it is maintained for the duration of the fermentation; and/or
- 25 c) the method further comprises isolating said butyl butyrate.
15. A composition for the production of butyl acetate, comprising an acetone-butanol-ethanol (ABE)-producing *Clostridium* strain and a genetically engineered *E. coli* strain, wherein said genetically engineered *E. coli* strain has been transformed by at least one polynucleotide molecule;

the at least one polynucleotide molecule comprising a CoA transferase (CTF) gene and an alcohol acyltransferase (ATF) gene operably linked to at least one promoter,

wherein the ATF gene is *atf1* (from *Saccharomyces cerevisiae*).

16. The composition of claim 15 or 16, wherein

- 5 a) the *Clostridium* strain is *C. beijerinckii* G117, and/or
- b) the *atf1* gene encodes a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 9, or a functional sequence variant thereof.

17. The composition of claim 15 or 16, wherein the CoA transferase (CTF) gene is selected from the group comprising *atoDA* and *ctfAB*.

10 18. The composition of claim 17, wherein:

 the *atoDA*; *atoD* and *atoA* genes encode a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 11 and 13, respectively, or a functional sequence variant thereof; and/or

 the *ctfAB*; *ctfA* and *ctfB* genes encode a polypeptide comprising the amino acid
15 sequence set forth in SEQ ID NO: 15 and 17, respectively, or a functional sequence variant thereof.

19. A method of producing butyl acetate in a co-culture, comprising the steps:

 a) co-culturing a composition of any one of claims 15 to 18 in medium under conditions for conventional ABE fermentation with exception that no anaerobic treatment is used,
20 and

 b) supplementing the medium with a carbon source selected from the group comprising glucose, xylan, cellulose and lignocellulosic biomass,

wherein the co-cultured *Clostridium* and *E. coli* are capable of increased production of butyl acetate compared to a co-culture with a non-transformed *E. coli* cell.

25 20. The method of claim 19, wherein the lignocellulosic biomass is rice straw or corn stover.