NATIONAL UNIVERSITY OF SINGAPORE
INVENTION DISCLOSURE FORM

INSTRUCTIONS

1. Filling Out the Correct Forms

(a) If this is a software-based invention, complete instead a **Software Invention Disclosure** form.

(b) If this invention has a hardware component and a software component, then complete first this **Invention Disclosure Form** and questions 22-29 *only* in the **Software Invention Disclosure** form.

(c) If this is a reagent, complete instead a **Reagent Invention Disclosure** form.

(d) For all other intellectual property or inventions (hereinafter "**invention**"), please complete this Invention Disclosure Form

2. Things to Note

(a) This Invention Disclosure Form serves as an official notification to NUS via its Industry Liaison Office ("**ILO**") about the conception of any invention. Upon reception of the completed Invention Disclosure Form, ILO will conduct an evaluation of the invention disclosed and decide on the best course of action to commercialise this invention, which may, or may not include, filing a provisional patent application.

(b) Any public disclosure[1] of the invention prior to submission of this Invention Disclosure Form will jeopardise any potential application for patent protection. Submit this Invention Disclosure Form *well in advance* of a submission containing information on the invention to a conference or journal. Bear in mind the standard evaluation period of an Invention Disclosure is **90 days**.

(c) Only list as an inventor any individual who has conceived or contributed an essential element of the invention, either independently or jointly with others, during the evolution of the technology conception or reduction to practice (i.e., development of the invention).

3. Additional documents you will need to complete this form

a) Download Appendix 1 to understand what constitutes Public Disclosure at http://bit.ly/1rrVdUN.

b) Download Appendix 2 for more information on what constitutes Novelty & Unobviousness at http://bit.ly/1oYrbnJ.

c) Download Appendix 3 - TRL for HW and PMLH to assess the TRL of this invention at http://bit.ly/1w7o06I.

4. To Do

- Submit the following two documents to **ilobox3@nus.edu.sg** and cc your Head of Department and Dean for their information:
    1. One *MS Word* version of this invention disclosure.
    2. One *PDF* version of this invention disclosure **fully signed** by all NUS and non-NUS inventors.

- If in doubt about any information requested by this form, please contact: **ilobox3@nus.edu.sg**

[1] See Appendix 1.

INVENTION DISCLOSURE FORM

For ILO use only. ID:

1. Title of the Invention (What is it? Be clear and concise):

A method of producing value-added chemicals by using *Clostridium* and *Bacillus* co-cultures

I. GRANTS AND CONTRACTUAL OBLIGATIONS

Funding Agencies:

1. A*STAR
2. CERP (NRP)
3. CRP (NRF)
4. DSTA
5. DSO
6. EIRP (NRF)
7. GAMBIT
8. HEBREW
9. MDA
10. **MOE Tier 1**
11. MOE Tier 2
12. MOE Tier 3
13. NEA ETRP
14. NMRC
15. POC (NRF)
16. SEC
17. SJTU
18. SMa
19. SMART
20. Thermofisher
21. Other Agency

2. Grant	2.1 Additional source of Funding 1	2.2 Additional source of Funding 2
a. Funding source (from above):	1. Funding source (from above):	a. Funding source (from above)
a.1 If "Other Agency", please specify: Singapore Millennium Foundation (SMF)	a. If "Other Agency", please specify: MOE PhD scholarship	a.1 If "Other Agency", please specify: …
b. Grant Number[2]: R-279-000-516-592	b. Grant Number[3]: N/A	b. Grant Number[4]: …
c. Project title: Revive Acetone-Butanol-Ethanol (ABE) Fermentation through Metabolic Engineering of Clostridia	c. Project title: N/A	c. Project title: …

3. If the invention is the result of a signed research agreement, indicate:

a. Title:

b. ILO Reference Number: …

c. Collaborator(s)' organisation(s): …

II. PUBLIC DISCLOSURE[5]

4. Has this invention been publicly disclosed? If so, indicate when and where was this invention disclosed:	
5. If the invention has not been publicly disclosed, indicate when and where will it be disclosed, if at all:	

[2] This number can be found in the Grant's Letter of Award. This number is NOT the WBS number
[3] This number can be found in the Grant's Letter of Award. This number is NOT the WBS number
[4] This number can be found in the Grant's Letter of Award. This number is NOT the WBS number
[5] See Appendix 1

INVENTION DISCLOSURE FORM

III-A. NUS INVENTORS

Each undersigned Inventor acknowledges and agrees that if this Form is transmitted by facsimile transmission or other electronic means in portable document format ("**pdf**") to National University of Singapore which results in transmission of a facsimile of his signature as an inventor making the disclosures of the invention disclosed in the Form, such facsimile or other electronically-transmitted Form shall be for all purposes effective as if such inventor had delivered the original of this Form and the facsimile or electronic signature of the inventor shall be deemed to be his original signature for all purposes.

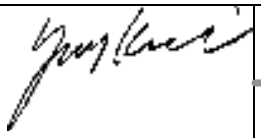
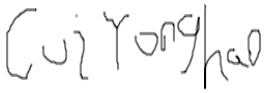
We/I hereby fully consent to NUS collecting, using and/or disclosing my/our personal data in any form and to disclose the same to third parties (including any third party located outside of Singapore) for the purpose of the registration, protection and management of the invention disclosed hereunder in compliance with the Singapore PDPA 2012. I represent and warrant that the personal data I have provided to NUS in this Form are true and accurate and that I am the user and/or subscriber of the telephone number submitted in this form.

I/We, (an) inventor(s) who is(are) employees of NUS, hereby declare to the best of my/our knowledge the information provided in this Form are true and correct. I/We hereby agree to assign all rights, title and interest to this invention to NUS and/or such third parties under the Grant or relevant contracting parties to the relevant research agreement giving rise to the invention, as the case may be, and agree to execute all documents as requested, assigning to NUS, and/or such third parties, our respective rights to this invention and in any patent application filed on this invention, and to cooperate with the NUS Industry Liaison Office ("**ILO**") in the protection of this invention. NUS will share any income derived from the invention with the inventor(s) who are NUS employees according to its I.P. Policy, as may be updated from time to time.

	Inventor 1	Inventor 2	Inventor 3	Inventor 4	Inventor 5
6. Family Name (as it appears in official document or passport)	Yang	Zhou	Cui		
7. Given Name (as it appears in official document or passport)	Kun-Lin	Kang	Yonghao		
8. Citizenship	Taiwanese	Chinese	Chinese		
9. Staff or Student No.	024801	53218	A0176533B		
10. Faculty & Department (to which this invention should be attributed to)	ChBE	ChBE	ChBE		
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12. Email (NUS and Personal account)	cheyk@nus.edu.sg	Kang.zhou@nus.edu.sg	e0225119@u.nus.edu		
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14. Intellectual Contribution/Income Split[7]	33.4%	33.3%	33.3%		

[6] All official documents will be sent to the mailing address indicated. For income distribution, please keep us updated of any changes to your mailing address.

[7] To fill out only if the inventive contribution is unequal among NUS inventors. If left blank, income (if any) will be distributed equally. The inventive contribution across NUS and non-NUS inventors should add up to 100%.

15. Signature					

III-B. NON-NUS INVENTORS

Each undersigned Inventor acknowledges and agrees that if this Form is transmitted by facsimile transmission or other electronic means in portable document format ("**pdf**") to National University of Singapore which results in transmission of a facsimile of his signature as an inventor making the disclosures of the invention disclosed in the Form, such facsimile or other electronically-transmitted Form shall be for all purposes effective as if such inventor had delivered the original of this Form and the facsimile or electronic signature of the inventor shall be deemed to be his original signature for all purposes.

We/I hereby fully consent to NUS collecting, using and/or disclosing my/our personal data in any form and to disclose the same to third parties (including any third party located outside of Singapore) for the purpose of the registration, protection and management of the invention disclosed hereunder in compliance with the Singapore PDPA 2012. I represent and warrant that the personal data I have provided to NUS in this form are true and accurate and that I am the user and/or subscriber of the telephone number submitted in this form.

I/We, (an) inventor(s) who is(are) not (an) NUS employee(s), hereby declare to the best of my/our knowledge the information provided in this Form are true and correct. I/We hereby agree to assign all rights, title and interest to this invention to NUS and/or such third parties under the Grant or relevant contracting parties to the relevant research agreement giving rise to the invention, as the case may be, and agree to execute all documents as requested, assigning to NUS and/or such third parties, our respective rights to this invention and in any patent application filed on this invention, and to cooperate with the NUS Industry Liaison Office ("**ILO**") in the protection of this invention. NUS will share any income derived from the invention with such third parties or contracting parties in accordance with the terms of the relevant Grant or research.

	Inventor 1	Inventor 2	Inventor 3	Inventor 4	Inventor 5
6. Family Name (as it appears in official document or passport)					
7. Given Name (as it appears in official document or passport)					
8. Citizenship					
9. Organization					
10. Contact Phone					
11. Contact Address					
12. Email (Organisation's and Personal account)					
13. Mailing Address[8]					
14. Intellectual Contribution/Income Split[9]					
15. Signature					

[8] See Footnote 6.

[9] To fill out only if the inventive contribution is unequal among non-NUS inventors. If left blank, income (if any) will be distributed equally. The inventive contribution across NUS and non-NUS inventors should add up to 100%.

IV. DETAILS OF THE INVENTION

16. **Overview** – Provide a summary or general description of the invention including its field of application:

Some strains from the genus of *Clostridium* can produce acetone-butanol-ethanol (ABE) during the anaerobic fermentation, which is known as ABE fermentation. However, because of the toxicity of solvents to the cells, the titers of solvents are relatively low. Besides, acetone, which accounts for 30% of the total solvents, is regarded as an undesired product in the current ABE fermentation, due to its poor fuel properties and corrosiveness to the rubber parts of the engines. These issues undermine the competitiveness of the traditional ABE fermentation and impede the further development of the process.

Herein, in this disclosure, we reported a method to produce more valuable products from ABE fermentation by introducing a second into the culture of *Clostridium.* By co-culturing *Clostridium* with an engineered *Bacillus* strain expressing a secondary alcohol dehydrogenase (CpSADH), the conventional anaerobic acetone-butanol (AB) fermentation could be shifted into the aerobic isopropanol-butanol (IB) fermentation with the production of 0.9 g/L of isopropanol and 4.8 g/L of butanol. In addition, the introduction of another *Clostridium* strain into the culture of an IBE-producing *Clostridium* helped to balance the acids/alcohols ratio, which resulted in the production of more esters in the subsequent lipase-mediated esterification stage.

The *Clostridium-Bacillus* co-culture strategy can be used for producing isopropanol and butanol without the need for costly and tedious anaerobic pretreatment. The *Clostridium-Clostridium* co-culture can be used for producing high value-added short chain fatty acid esters which can be used as flavor compounds aviation fuel constituent.

17. **Novelty & Unobviousness**[10] – List the features of this invention that make it a substantial and significant improvement, or the case of new and unexpected results, over existing technology (i.e., methods, devices and/or materials). Indicate what are the unique benefits or advantages these features provide.

Feature	Benefit/Advantage
The co-culture of *Clostridium* and *Bacillus* enables the fermentation to happen at the aerobic condition.	Traditional ABE fermentation can only be processed at the anaerobic condition which requires costly anaerobic pretreatments. By co-culturing *Bacillus* with *Clostridium*, this process can happen at the aerobic condition without the need for anaerobic pretreatments, which saves the cost.
The co-culture of *Clostridium* and *Bacillus* can convert acetone produced in ABE fermentation into isopropanol.	Acetone, which takes up 30% of the ABE products, is considered as an undesired product due to its corrosiveness to the rubber parts of the engines and its poor fuel properties. By co-culturing the *Bacillus* expressing a secondary alcohol dehydrogenase (CpSADH) with the ABE-producing *Clostridium*, the acetone can be reduced into isopropanol which can be used as detergent and fuel additive. Besides, compared with acetone, isopropanol can be further converted into esters such as isopropyl butyrate which is more valuable.
The co-culture of *Clostridium* and *Bacillus* avoids the engineering of *Clostridium*.	Although tremendous progress has been made in the field of *Clostridium* engineering, it is still relatively

[10] See Appendix 2.

	demanding to engineer *Clostridium* due to the requirement of the anaerobic environment. By engineering *Bacillus* which is easier to handle to express the target enzymes, the intended properties can be easily added without the need for *Clostridium* engineering.
The co-culture of two *Clostridium* strains produces isopropyl butyrate.	Acetone, which accounts for 30% of the solvents produced in ABE fermentation, is considered as an undesired product due to its poor fuel properties. Besides, it cannot be converted to esters during the lipase-mediated esterification process as butanol does. By introducing an IBE-producing strain *C. beijerinckii* BGS1, isopropanol, instead of acetone, can be produced. Further co-culturing *C. beijerinckii* BGS1 with *C. acetobutylicum* ATCC824 led to the conversion of isopropanol into isopropyl butyrate.
The co-culture of two *Clostridium* strains alleviates the addition of butyrate during production of esters.	Usually, exogenous butyrate needs to be added so that more esters can be produced from the culture of *Clostridium*. This increases the cost and makes the process less competitive. When *Clostridium beijerinckii* BGS1 and *Clostridium acetobutylicum* ATCC824 were co-cultured, more butyrate was produced from glucose, which finally resulted in the production of more esters during the esterification stage. This strategy avoids the addition of commercial butyrate.
The co-culture of two *Clostridium* strains improves the production of butanol.	The co-culture of *C. beijerinckii* BGS1 and *C. acetobutylicum* ATCC824 produced more butanol than the mono-culture of *C. beijerinckii* BGS1 and the mono-culture of *C. acetobutylicum* ATCC824.

18. **Limitations** – Describe the limitations, if any, of this invention in terms of, for instance, scalability, speed, power consumption, efficiency, use of exotic compounds, etc.

19. **Prior Art** – List any other existing technologies or literature that more closely resemble the features and/or functionalities of this invention. Indicate how this invention differentiates from existing technologies or literature listed.

A few studies have been reported to co-culture *Bacillus* with *Clostridium* to improve the production of butyrate, butanol or H_2 from substrates such as starch and glucose (Mi et al., 2018; Oliva-rodríguez et al., 2019; Tran et al., 2010; Wu et al., 2016). However, mostly of these studies only focused on using *Bacillus* as the oxygen consumer to provide respiratory protection so that *Clostridium* could grow. Besides, the *Bacillus* strains used in these studies are wild-type strains without being engineered. In our study, genetic engineering was applied to *Bacillus* so that more functions could be added. Instead of focusing on the improving the production of ABE or H_2 production, our study aims to convert the traditional ABE fermentation products into other high value-added chemicals.

Apart from the co-culture of *Bacillus* and *Clostridium*, the co-culture of two *Clostridium* strains has also been explored. These studies often focus on the expansion of the substrate spectrum by co-culturing two *Clostridium* strains. For example, Kiyoshi *et al.* managed to produce butanol from the alkali-pretreated rice straw by employing a co-culture system composed of *C. thermocellum* and *C. saccharoperbutylacetonicum* (Kiyoshi et

al., 2015). Li *et al.* utilized the co-culture of *C. thermocellum* and *C. thermosaccharolyticum* to produce hydrogen from the alkali pretreated cornstalk (Li et al., 2014). Besides, some studies tried to enhance butanol production by using the co-culture strategy. Li *et al*. reported that the production of butanol could be improved when an acidogenic species *C. tyrobutyricum* was co-cultured with a solventogenic species *C. beijerinckii*. Our study aims to better balance the acids/alcohols ratio by co-culturing the two *Clostridium* strains so that more esters (isopropyl butyrate, butyl butyrate, and butyl acetate) could be produced from the IBE fermentation.

20. **Commercial Applications** – List the critical commercial problems this invention solves:

1. Production of isopropanol and butanol under the aerobic condition: compared with conventional ABE fermentation that needs an obligate anaerobic condition, *Bacillus-Clostridium* can be co-cultured under an aerobic condition, by-passing the tedious anaerobic pretreatments including N_2 flushing and addition of reducing agents. Besides, acetone, which is considered as an undesired product due to its poor fuel properties and corrosiveness to the rubber parts of the engines, can be reduced into isopropanol which can be used as a detergent and fuel additive. In addition, the reduction of acetone into isopropanol also further facilitates the production of short chain fatty acid esters because isopropanol can be converted into esters such as isopropyl butyrate.

2. Production of isopropyl butyrate from IBE fermentation without the addition of exogenous butyrate: traditionally, production of short chain fatty acid esters from the culture of *Clostridium* usually focus on butyl butyrate, leading to the waste of acetone. In our *Clostridium-Clostridium* co-culture system, isopropanol, instead of acetone, was produced due to the existence of an IBE-producing strain. The isopropanol can be further converted into isopropyl butyrate by lipase. Besides, conventional production of short chain fatty acid esters requires the addition of butyrate so that the acids/alcohol ratio can be balanced, but that increases the cost. By having two *Clostridium* strains exhibiting different characteristics at pH 6, more butyrate could be produced. As a result, more esters can be produced without the need for adding butyrate.

21. **Commercial Interest** – Provide details of the commercial parties that may be interested in this technology:

Company	Contact Person	Email

22. **Plans** – List your plans regarding this invention (e.g., indicate whether someone is currently working on this invention and to what end, if additional research funds are being applied for, etc.).

23. **Technology Readiness Level** – What is the overall Technology Readiness Level (TRL) of this invention?[11]: 3

24. **Material** – If the invention uses material from another lab, company, or was purchased list the following, if applicable:

Material	Source	Material Transfer Agreement
Not applicable.		

25. **Technical Description** – Describe the technical details of this invention. You may include figures in this section. This section roughly corresponds to sections Methodology, Results, Analysis and Conclusions in a scientific paper:

As *Clostridium* is anaerobe which requires the obligate anaerobic condition to grow, anaerobic pretreatments including N_2 flushing and addition of reducing agents are necessary to perform the ABE fermentation. These anaerobic pretreatments are costly and tedious. Besides, acetone, which accounts for 30% of the solvents produced, is regarded as an undesired product due to its poor fuel properties and corrosiveness to the rubber parts of the engines Therefore, it would be better if the acetone could be converted into other useful chemicals

[11] See Appendix 3.

such as isopropanol. Compared with acetone, isopropanol can be used as fuel additive and detergent. Furthermore, isopropanol can also be converted into esters which are more valuable. For these reasons, we developed a *Bacillus*-*Clostridium* co-culture system to switch the anaerobic acetone-butanol (AB) fermentation into aerobic isopropanol-butanol (IB) fermentation. This new process omits the tedious and costly anaerobic pretreatment and enables the reduction of undesired acetone into isopropanol. Moreover, to further convert the butanol and isopropanol into short chain fatty acid esters, we also explored a *Clostridium*-*Clostridium* co-culture system which could enhance the production of short chain fatty acid esters with the addition of lipase.

Materials and methods

Media and culture conditions

A defined medium (termed as anaerobic medium in this work) was adopted (Xin et al., 2016). Glucose was supplemented upon inoculation. To prepare the aerobic medium, all ingredients were mixed in the same concentrations as the anaerobic medium except reductants and resazurin. The medium was not flushed with N_2 during the process.

For the anaerobic cultivation, the cells were inoculated into the anaerobic medium in the serum bottles with butyl stoppers and incubated at 37 ºC/225 rpm.

The aerobic co-culture in the open system was processed in the 50-mL conical tubes which contained the aerobic medium. After the caps of conical tubes were loosened, the cells were incubated at 37 ºC statically.

The aerobic co-culture in the closed system was done in the serum bottles which contained the aerobic medium. After the inoculum of the bacteria, the butyl stoppers were capped. The cells were incubated at 37 ºC/225 rpm.

The *Clostridium*-*Clostridium* co-culture was processed in the anaerobic medium in the serum bottles with butyl stoppers. After inoculation, the cells were incubated at 37 ºC/225 rpm. Samples were taken periodically.

Plasmid and strains

The *cpsadh* gene from *Candida parapsilosis* IFO 1396 was inserted into the pHT01 plasmid by using a GT-standard method, resulting in the pHT01-*cpsadh* plamsid (Ma et al., 2019). After that, the pHT01-*cpsadh* plasmid was used to transform *B. subtilis* 1A1, resulting in the BsADH2 strain. In comparison, the wild-type *B. subtilis* 1A1 strain was designated with Bs1A1.

C. beijerinckii G117 was used to be co-cultured with *B. subtilis* 1A1.

C. beijerinckii BGS1 and *C. acetobutylicum* ATCC824 were used in the *Clostridium*-*Clostridium* co-culture.

Results

Design of the *Clostridium-Bacillus* symbiotic system

An aerobic *B. subtilis* strain expressing a secondary alcohol dehydrogenase (CpSADH) was constructed and co-cultured with the anaerobic *C. beijerinckii* at aerobic condition. During the fermentation, *B. subtilis* grows first and consumes oxygen. Meanwhile, lactate is produced by *B. subtilis*. After oxygen is depleted, *C. berijerinckii* starts to grow and produces acetone, butanol, acetate, and butyrate. Lactate produced by *B. subtilis* is re-assimilated by *C. berijerinckii* to produce butanol and butyrate. At this stage, the acetone produced by *C. berijerinckii* is reduced into isopropanol by the CpSADH-expressing *B. subtilis.* By using acetone as the electron acceptor, the aerobic *B. subtilis* can maintain its active cellular metabolism at the anaerobic condition (**Figure 1**).

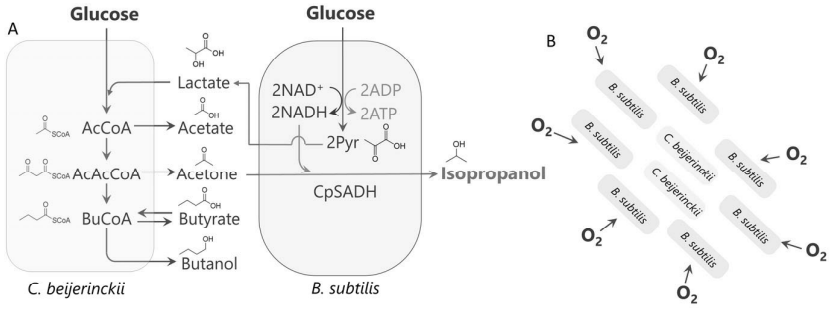


Figure 1 Design of the symbiotic system

Aerobic co-culture of *C. beijerinckii* and *B. subtilis*

After BsADH2 was constructed, it was co-cultured with *C. beijerinckii* G117 (CbG117) which can produce acetone and butanol at anaerobic condition. When the BsADH2 was co-cultured with CbG117 in the aerobic medium in the open system, 0.9 g/L of acetone, 4.9 g/L of butanol, and 0.2 g/L of isopropanol were produced (**Figure 2**). In contrast, no solvents or acids were produced when CbG117 was mono-cultured at the same condition (data now shown), suggesting that CbG117 cannot grow aerobically without *B. subtilis*. These results suggested that by introducing the CpSADH-expressing *B. sutbilis*, the original anaerobic AB fermentation could be shifted to the aerobic IB fermentation. This conversion avoided the tedious and costly anaerobic pretreatment including the N_2 flushing and the addition of reducing agents. Besides, the conversion of acetone into isopropanol also addressed the issue that acetone could not be utilized properly.

Aerobic co-culture of *C. beijerinckii* and *B. subtilis* in the aerobic medium in a closed system

Although solvents were successfully produced when BsADH2 and CbG117 were co-cultured, the titers of solvents especially the titer of isopropanol were relatively low, which could be due to the evaporation. Therefore, BsADH2 and CbG117 were further co-cultured in the aerobic medium in the serum bottle in order to avoid evaporation. As a result, the titer of isopropanol increased to 0.9 g/L after 72 hours' cultivation, although the titer of butanol remained unchanged (**Figure 2**). Nonetheless, the titer of acetone also increased to 1.7 g/L, resulting in an isopropanol/acetone ratio of 0.53. These results suggested that the low titer of isopropanol in the open-system aerobic co-culture was due to evaporation.

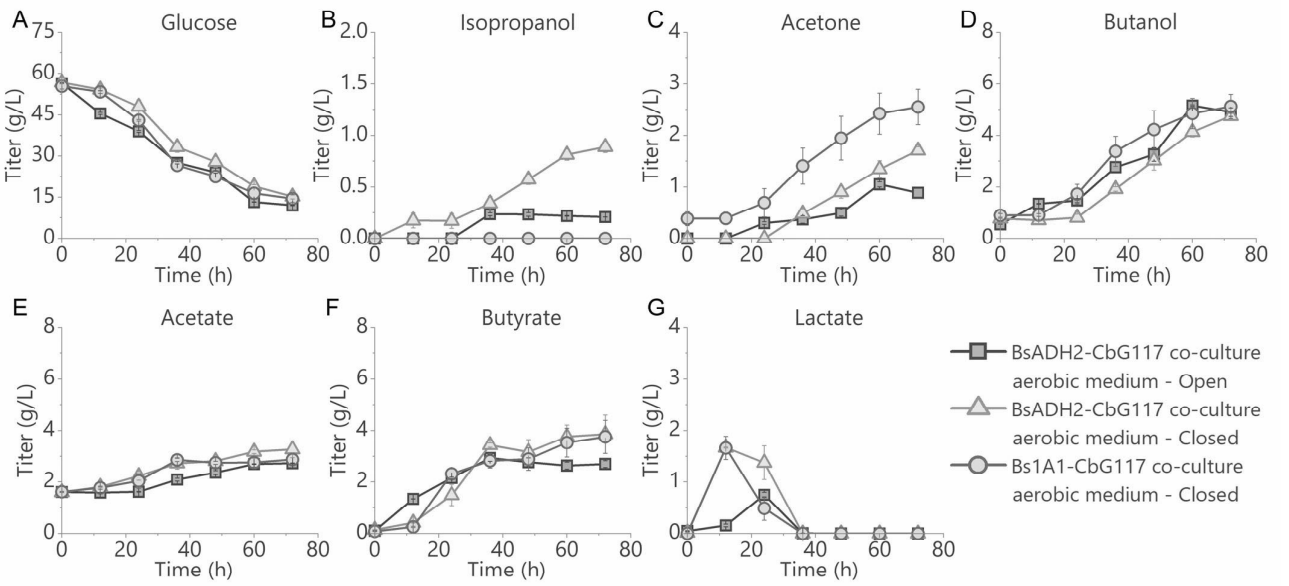


Figure 2 Co-culture of *C. beijerinckii* and *B. subtilis.* Co-culture of BsADH2 and CbG117 in the aerobic medium in the open system (blue square). Co-culture of BsADH2 and CbG117 in the aerobic medium in the closed system (yellow triangle). Co-culture of BsADH1 and CbG117 in the aerobic medium in the closed system (green circle).

Optimization of process parameters

To increase the isopropanol/acetone ratio and the titers of solvents, we further inoculated BsADH2 into the 24-hour anaerobic cell culture of CbG117.

When BsADH2 was added to the 24-hour anaerobic culture of CbG117, 1.6 g/L of isopropanol was produced within 12 hours (**Figure 3**). Further cultivating the cells for another 12 hours led to the production of another 0.7 g/L of isopropanol, resulting in an isopropanol/acetone ratio of 2. This is much higher than that of the open-system aerobic co-culture and the closed-system co-culture. In comparison, there was no isopropanol produced when Bs1A1 was added. Besides, the titer of butanol was also increased to 6.4 g/L after the optimization.

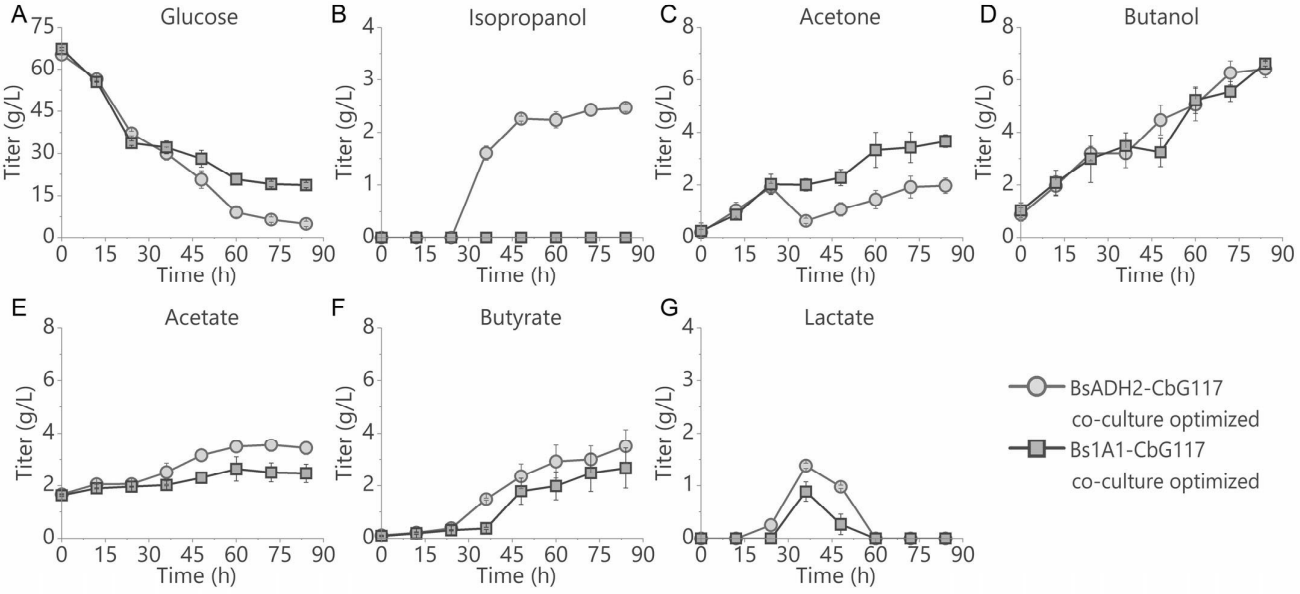


Figure 3 Optimization of the process parameters. Addition of BsADH2 (green circle) and Bs1A1 (blue square) into the 24-hour culture of CbG117.

Selection of lipases for producing short chain fatty acid esters

Although it has been reported that lipases can catalyze the esterification of products from ABE fermentation, those studies mainly focused on the production of butyl butyrate (Xin et al., 2016; Zhang et al., n.d.). In our study, as isopropanol should also be considered, a lipase that can convert isopropanol into corresponding esters is needed. Therefore, we first selected four lipases which have been reported to be able to catalyze the esterification reaction, including Lipase, immobilized on Immobead 150 from *Rhizopus oryzae* (termed as Lipase A in this study), Lipase from *Candida* sp. (recombinant, expressed in *Aspergillus niger*) (assigned as Lipase B in this study), Lipase from *Rhizopus oryzae* (powder, light brown, ≥30 U/mg) (termed as Lipase C in this study) and Lipase from *Candida rugosa* (Type VII, ≥700 unit/mg solid) (assigned as Lipase D in this study).

To evaluate their performance of converting isopropanol and butanol into esters, 2,000 U of each enzyme was added into 10 mL of the mixture containing 10 g/L of butanol, 5 g/L of isopropanol, 10 g/L of butyrate, and 10 g/L of acetate. Five milliliters of hexadecane were added as the extractant. After that, the reaction system was incubated at 37C ºC/225 rpm.

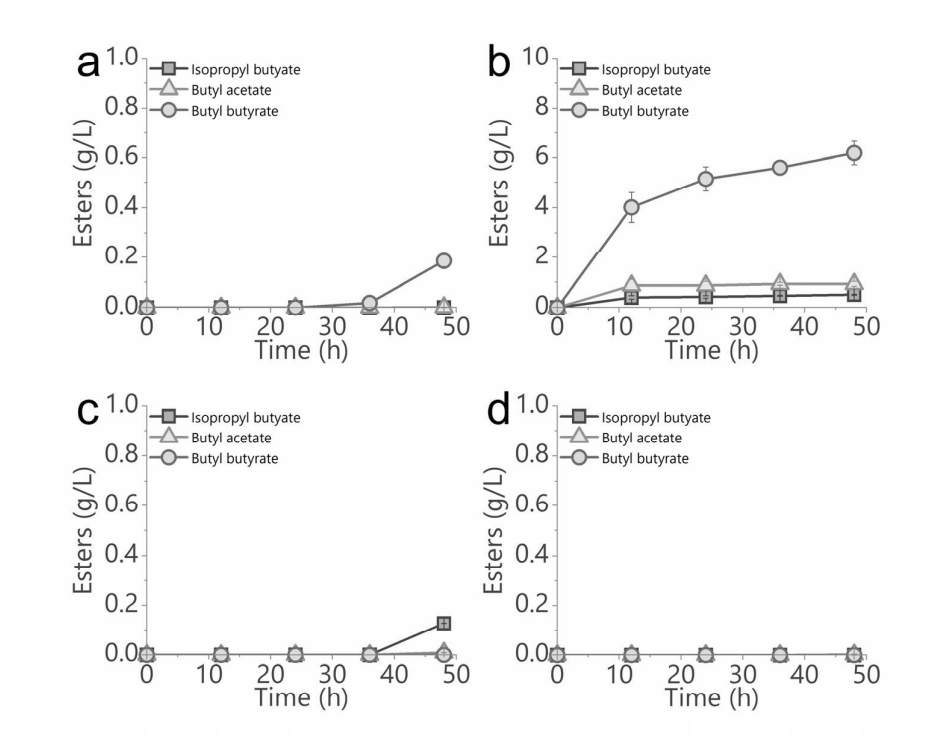


Figure 4 Esterification by using (a) Lipase A, (b) Lipase B, (c) Lipase C, and (D) Lipase D.

After 48 hours' reaction, Lipase B was found to be the only one that could catalyze the esterification of both isopropanol and butanol, with the production of 0.5 g/L of isopropyl butyrate, 0.9 g/L of butyl acetate, and 6.2 g/L of butyl butyrate (**Figure 4**). In comparison, only 0.2 g/L of butyl butyrate was produced after 48 hours' reaction when Lipase A was used. In the reaction with Lipase C added, 0.1 g/L of isopropyl butyrate was produced, without the production of any other esters. In terms of Lipase D, no esters were produced. As a result, Lipase B was selected as the lipase used in the following experiments.

Production of short chain fatty acid esters from the co-culture of *C. beijerinckii* BGS1 and *C. acetobutylicum* ATCC824

By using the *Bacillus-Clostridium* co-culture strategy, acetone was successfully reduced into isopropanol. However, the titer of isopropanol produced was relatively insufficient to produce esters compared to some

native IBE-producing *Clostridium*. Therefore, to achieve higher titer of esters and simplify the co-culture system, *C. beijerinckii* BGS1 was used as the isopropanol producer in the production of esters. As an IBE-producing strain, *C. beijerinckii* BGS1 was reported to be able to produced 10.21 g/L butanol and 3.41 g/L isopropanol (Zhang et al., 2018). However, it barely produces butyrate during the fermentation, which raises the requirement of supplementing exogenous butyrate during esterification. To alleviate the addition of butyrate, another strain *C. acetobutylicum* ATCC824 was introduced to be co-cultured with *C. beijerinckii* BGS1. At pH 6, *C. acetobutylicum* ATCC824 is in the acidogenic phase, with butyrate and acetate as the main products. *C. beijerinckii* BGS1 is solventogenic at the same pH, with isopropanol and butanol as the main products. Utilizing this difference, *C. beijerinckii* BGS1 and *C. acetobutylicum* ATCC824 were co-cultured anaerobically at 37 ºC/225 rpm, with pH maintained at 6.

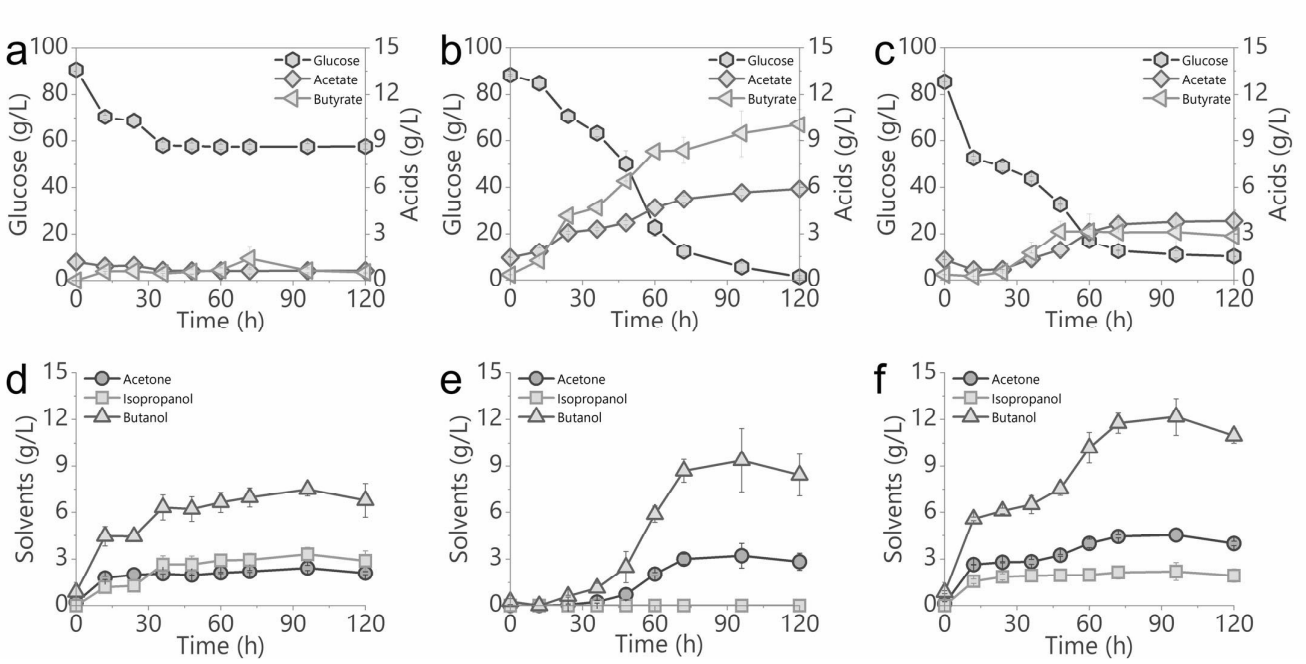


Figure 5 Profile of glucose and fermentative products. (a) profile of glucose and organic acids produced in the mono-culture of *C. beijerinckii* BGS1; (b) profile of glucose and organic acids produced in the mono-culture of *C. acetobutylicum* ATCC824; (c) profile of glucose and organic acids produced in the co-culture of *C. acetobutylicum* ATCC824 and *C. beijerinckii* BGS1; (d) profile of organic solvents produced in the mono-culture of *C. beijerinckii* BGS1; (e) profile of glucose and organic solvents produced in the mono-culture of *C. acetobutylicum* ATCC824; (f) profile of organic solvents produced in the co-culture of *C. acetobutylicum* ATCC824 and *C. beijerinckii* BGS1.

After 48 hours, 3.2 g/L of butyrate was produced by the co-culture system, along with 7.5 g/L of butanol. In comparison, only 0.6 g/L of butyrate was produced when *C. beijerinckii* BGS1 was mono-cultured. Further co-culturing the two strains for another 72 hours did not lead to the production of more butyrate. Noteworthily, the butanol titer increased to 11.8 g/L from the 48th hour to the 72nd hour, due to the existence of *C. acetobutylicum* ATCC824. These results suggested that the introduction of *C. acetobutylicum* ATCC824 helped to increase the titer of butyrate.

To further test the effect of the coculture strategy on the production of esters, cell culture of *C. beijerinckii* BGS1-*C. acetobutylicum* ATCC824 co-culture and *C. beijerinckii* BGS1 mono-culture incubated for 120 hours was used for esterification.

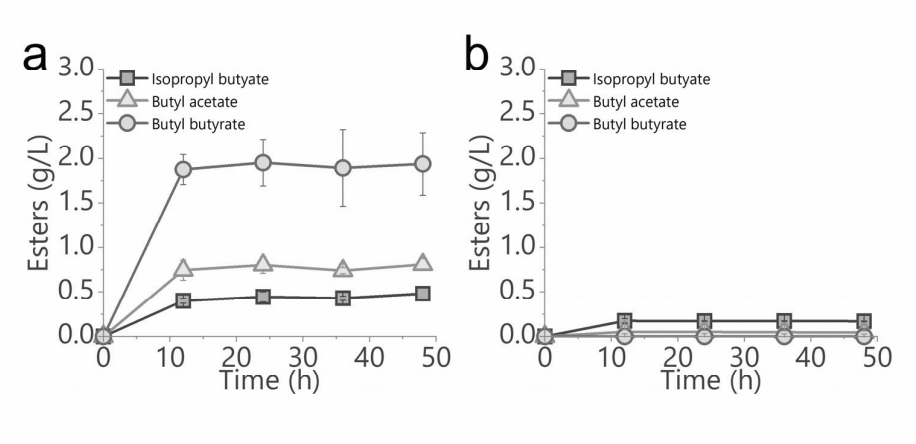


Figure 6 Production of esters from (a) co-culture of *C. beijerinckii* BGS1 and *C. acetobutylicum* ATCC824 and (b) mono-culture of *C. beijerinckii* BGS1.

After the addition of Lipase B, 0.5 g/L of isopropyl butyrate was produced from the co-culture system, along with 1.9 g/L of butyl butyrate. Besides, 0.8 g/L of butyl acetate was also produced, which was due to the production of acetate by *C. acetobutylicum* ATCC824. In comparison, there was only 0.2 g/L of isopropyl butyrate produced from the mono-culture of *C. beijerinckii* BGS1. No any other esters were produced. These results suggested that by co-culturing *C. acetobutylicum* ATCC824 and *C. beijerinckii* BGS1, the titers of ester could be improved.

Conclusion

In this work, *Clostridium* co-culture strategy was used to produce value-added chemicals. The co-culture of *Clostridium and Bacillus* successfully switched the anaerobic AB fermentation into the aerobic IB fermentation, with butanol and isopropanol as the main products. As the first study doing this, the co-culture of *Clostridium and Bacillus* alleviated the production of undesired acetone and avoided the costly anaerobic pretreatment. Moreover, to further convert the isopropanol and butanol into high value-added short chain fatty acid esters without the addition of butyrate, a *Clostridium*-*Clostridium* co-culture system was developed. Compared with the mono-culture of *C. beijerinckii* BGS1 which only led to the production of 0.2 g/L of isopropyl butyrate, the co-culture promoted the production of esters, resulting in the production of 0.5 g/L of isopropyl butyrate, 1.9 g/L of butyl butyrate, and 0.8 g/L of butyl acetate.

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Title of the invention:

A method of producing value-added chemicals by *Clostridium* and *Bacillus* co-cultures

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Aerobic acetone-butanol-isopropanol (ABI) fermentation through a co-culture of *Clostridium beijerinckii* G117 and recombinant *Bacillus subtilis* 1A1

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ABSTRACT

An engineered *B. subtilis* 1A1 strain (BsADH2) expressing a secondary alcohol dehydrogenase (CpSADH) was co-cultured with *C. beijerinckii* G117 under an aerobic condition. During the fermentation on glucose, *B. subtilis* BsADH2 depleted oxygen in culture media completely and created an anaerobic environment for *C. beijerinckii* G117, an obligate anaerobe, to grow. Meanwhile, lactate produced by *B. subtilis* BsADH2 was re-assimilated by *C. beijerinckii* G117. In return, acetone produced by *C. beijerinckii* G117 was reduced into isopropanol by *B. subtilis* BsADH2 via expressing the CpSADH, which helped maintain the redox balance of the engineered *B. subtilis*. In the symbiotic system consisting of two strains, 1.7 g/L of acetone, 4.8 g/L of butanol, and 0.9 g/L of isopropanol (with an isopropanol/acetone ratio of 0.53) was produced from 60 g/L of glucose. This symbiotic system also worked when oxygen was supplied to the culture, although less isopropanol was produced (0.9 g/L of acetone, 4.9 g/L of butanol, and 0.2 g/L of isopropanol). The isopropanol titer was increased substantially to 2.5 g/L when we increased the inoculum size of *B. subtilis* BsADH2 and optimized other process parameters. With the *Bacillus-Clostridium* co-culture, switching from the original acetone-butanol (AB) fermentation to an aerobic acetone-butanol-isopropanol (ABI) fermentation can be easily achieved without genetic engineering of *Clostridium*. This strategy of employing a recombinant *Bacillus* to co-culture with *Clostridium* should be potentially useful to modify traditional acetone-butanol-ethanol fermentation for the production of other value-added chemicals.

1. Introduction

Butanol is an important chemical that has been widely used as diluent and solvent for producing antibiotics, cosmetics, brake fluid, and hormones (Lee et al., 2008; Li et al., 2013). It has also gained more interest as a biofuel due to its superior performance over ethanol concerning energy content, corrosiveness, volatility, and hygroscopy (Lee et al. 2008, 2012; Wu et al., 2016). Although butanol has been historically produced from petrochemical processes, bio-butanol which can be produced by the genus of *Clostridium* through acetone-butanol-ethanol (ABE) fermentation has become an attractive and sustainable biofuel (Rude and Schirmer, 2009; Lee et al., 2012; Bankar et al., 2013; Li et al., 2013; Youn et al., 2016). However, conventional ABE fermentation is still facing several obstacles even though it has been used for large-scale butanol production in the past (Lee et al., 2012; Bankar et al., 2013). One of the problems is that acetone which accounts for 20–30% of ABE products is regarded as a by-product due to its poor fuel properties and corrosiveness to the rubber parts of the engines (Lee et al., 2012; Youn et al., 2016). Although efforts have been invested to eliminate the production of acetone in ABE fermentation by modifying the acetone synthetic pathway, butanol production usually decreased as well, accompanied by the accumulation of organic acids (Jiang et al., 2009; Sillers et al., 2009; Lehmann et al., 2012). An alternative strategy is to convert acetone into isopropanol which can be utilized as a fuel additive for preparing high-octane gasoline (Rassadin et al., 2006; Survase et al., 2011; Lee et al., 2012). By introducing a secondary alcohol dehydrogenase, acetone produced by *Clostridium* can be reduced into isopropanol without disrupting the acetone synthetic pathway (Dai et al., 2012; Lee et al., 2012). Meanwhile, isopropanol, together with butanol and ethanol produced in this process, can be directly used as biofuel (Lee et al., 2012). Besides, as

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an alcohol, isopropanol can also be easily converted into esters and recovered from the aqueous phase through liquid-liquid extraction. However, genetic engineering of *Clostridium* is relatively demanding due to the requirement of manipulating the cells under anaerobic conditions (Joseph et al., 2018). The nature of being an obligate anaerobe also requires a strict anaerobic condition during large scale fermentation, resulting in costly anaerobic pretreatment of the medium and equipment (Xin and He, 2013; Wu et al., 2016). A possible solution is biological oxygen depletion inspired by natural microorganism communities in which anaerobes often co-inhabit in aerobic environments together with aerobes (Wu et al., 2016; Mi et al., 2018). Among those genera which can provide respiratory protection for *Clostridium*, *Bacillus* is considered as one of the best candidates due to its fast growth and high tolerance to solvents (Dwidar et al., 2013). A variety of studies have attempted to co-culture *Clostridium* with *Bacillus* so that the ABE fermentation can take place under a condition without anaerobic pretreatment (Tran et al., 2010; Wu et al., 2016; Mi et al., 2018; Oliva-Rodríguez et al., 2019). However, due to the different oxygen requirements of *Bacillus* and *Clostridium*, *Bacillus* was often only used as the oxygen consumer, which limited further applications of these co-culture systems. Therefore, it would be interesting if a co-culture system can switch the anaerobic ABE fermentation into aerobic isopropanol-butanol fermentation and avoid genetic engineering of *Clostridium*.

In this study, we engineered *B. subtilis* 1A1 to express a secondary alcohol dehydrogenase from *Candida parapsilosis* IFO 1396 (CpSADH) and established a stable symbiotic system composed of this strain and a wild-type *C. beijerinckii* strain G117 that does not produce any ethanol. This co-culture successfully switched the anaerobic acetone-butanol (AB) fermentation into aerobic acetone-butanol-isopropanol (ABI) fermentation without needing any anaerobic pretreatment and reducing agents (Fig. 1).

2. Materials and methods

2.1. Bacterial strains and plasmids

All strains and plasmids used in this study are listed in Table 1.

2.2. Plasmid and strain construction

All primers and barcodes used in constructing the pHT01-*cpsadh* plasmid are listed in Table S2.

Table 1
Strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
C. beijerinckii G117	Wild type strain	Chua et al. (2013)
Bs1A1	Wild type *B. subtilis* 1A1	BGSC[a]
E. coli DH5α		NEB[b]
BsADH1	*B. subtilis* 1A1 Δ*ldh*::operonCm	This study
BsADH2	*B. subtilis* 1A1 harboring pHT01-*cpsadh*	This study
Plasmids		
pHT01	Shuttle vector; Ap^R, Cm^R [c]	MoBiTec
pHT01-***cpsadh***	pHT01 carrying *cpadh*, Ap^R and Cm^R	This study
pMB1-***ldh***::operonCm	pMB1 ori Ap^R carrying *ldh*::(*cpsadh* Cm^R)	This study

[a] Bacillus Genetic Stock Center.
[b] New England Biolabs.
[c] Ap^R, Cm^R indicate resistance to ampicillin and chloramphenicol.

The pHT01-*cpsadh* plasmid was constructed according to the GT standard (Ma et al., 2019). The *cpsadh* gene was amplified using P01 and P02 from a plasmid received as a gift. The sequence of *cpsadh* was included in the Supplementary note S1. Then the gene was barcoded with REC3-F and REC4-R. After another round of PCR by using P03 and P04, the fragment was digested by *Bam*HI and *Xba*I. Then the double-digested fragment was ligated with the pHT01 plasmid which was also digested by *Bam*HI and *Xba*I.

The plasmid pMB1-Δ*ldh*:operonCm was also constructed following the GT standard (Supplementary note S2 and Fig. S1).

Escherichia coli strain DH5α was used as the recipient in all cloning experiments. After the plasmids were confirmed by using Sanger sequencing, they were used to transform *B. subtilis* 1A1 following the manufacturer's instruction. The genome-edited *B. subtills* 1A1 was named as BsADH1.The pHT01-*cpsadh* harboring *B. subtills* 1A1 was designated as BsADH2.

2.3. Culture media, induction, and growth conditions

2.3.1. E. coli DH5α culture medium and growth conditions

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

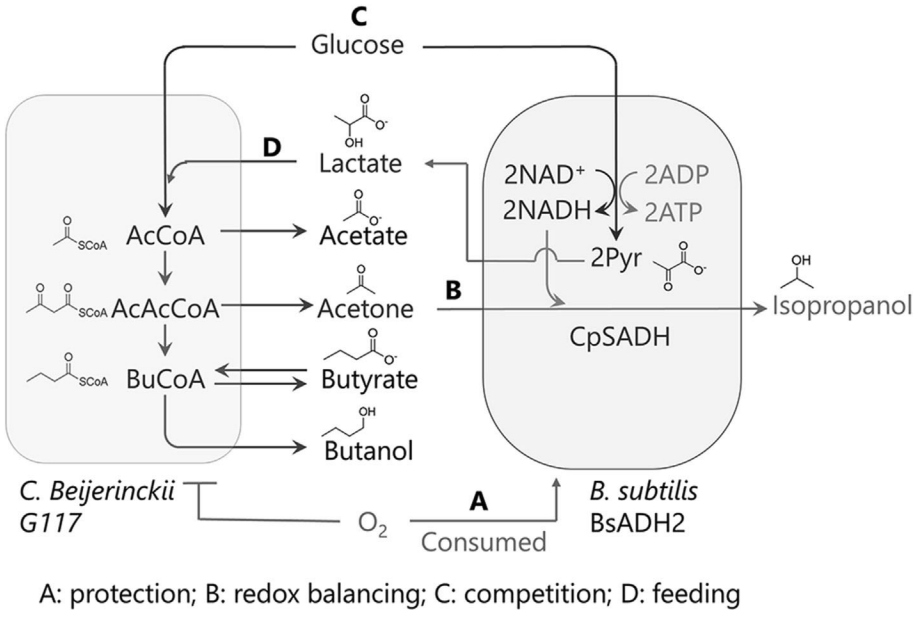


Fig. 1. Microbial interactions between the CpSADH-expressing *B. subtilis* (BsADH2) and *C. beijerinckii* G117 in the co-culture. (A) BsADH2 protects *C. beijerinckii* G117 from oxygen. (B) The expression of CpSADH enables BsADH2 to maintain its redox balance by reducing acetone produced by *C. beijerinckii* G117 into isopropanol. (C) BsADH2 and *C. beijerinckii* G117 compete for glucose which is a common carbon source for both strains. (D) lactate produced by BsADH2 was assimilated by *C. beijerinckii* G117. AcCoA: acetyl-CoA. AcAcCoA: acetoacetyl-CoA. BuCoA: butyryl-CoA. Pyr: pyruvate.

2.3.2. Induction of protein expression in BsADH2

Single colony of BsADH2 was picked from an agar plate with 5 μg/mL of chloramphenicol and inoculated into 10 mL of 2 × PY medium (20 g/L of peptone, 10 g/L of yeast extract, and 10 g/L of NaCl, pH 7) with 5 μg/mL of chloramphenicol and aerobically incubated at 37 °C overnight. Overnight grown cell culture was added into 30 mL of fresh 2 × PY medium with an initial cell density (OD_{600}) of 0.15. Then the cells were incubated at 37 °C/225 rpm. Isopropyl β-d-1-thiogalactopyranoside (IPTG, 1 mM) was added when OD_{600} reached 0.7 to 0.8 to induce protein expression. Then the cells were incubated at 37 °C/225 rpm unless otherwise stated.

2.3.3. C. beijerinckii G117 culture media and growth conditions

For the anaerobic culture of *C. beijerinckii* G117, a mineral salts medium (termed as anaerobic medium in this study) was adopted with modification (Xin et al., 2016). The medium contained 0.75 g/L of KH_2PO_4, 0.75 g/L of K_2HPO_4, and 5 g/L of yeast extract. Besides, 1 mL of the trace element solution, 1 mL of the Na_2SeO_3-Na_2WO_4 solution, and 10 mg of resazurin were added into 1 L of the medium. The trace lement solution contained 2.5 g/L of HCl, 1.5 g/L of $FeCl_24H_2O$, 0.19 g/L of $CoCl_26H_2O$, 0.1 g/L of $MnCl_2.4H_2O$, 0.07 g/L of $ZnCl_2$, 0.006 g/L of H_3BO_3, 0.036 g/L of $Na_2MoO_42H_2O$, 0.024 g/L of $NiCl_26H_2O$, and 0.002 g/L of $CuCl_22H_2O$. The Na_2SeO_3-Na_2WO_4 solution contained 0.006 g/L of $Na_2SeO_35H_2O$, 0.008 g/L of $Na_2WO_42H_2O$, and 0.5 g/L of NaOH. Then the medium was flushed with N_2 and boiled for 20 min. After it was cooled down to 25 °C, Na_2S, L-cysteine and dithiothreitol were added as reductants (their final concentrations were 0.2 mM, 0.2 mM and 0.5 mM, respectively). After that, CH_3COONH_4 and 2-(N-morpholino)ethanesulfonic (MES) were added to final concentrations of 2 g/L and 3.9 g/L respectively. After pH was adjusted to 7 using 100 g/L of NaOH, the medium was dispensed into serum bottles. Then the bottles were sealed with butyl stoppers and autoclaved at 121 °C for 20 min.

To prepare the medium without anaerobic pretreatment (named as aerobic medium in this study), all ingredients were mixed in the same concentrations as the anaerobic medium except the reductants and resazurin were omitted. The medium was not flushed with N_2 during the process. After pH of the medium was adjusted to 7 by 100 g/L of NaOH, the medium was filtered through polyethersulfone (PES) membrane whose pore size was 0.22 μm. Glucose was supplemented upon inoculation.

C. beijerinckii G117 from our laboratory spore stocks was precultured in 50 mL of the anaerobic medium supplemented with 60 g/L of glucose in a 160-mL serum bottle at 37 °C/225 rpm for 24 h. Then the precultured cells were inoculated into the fresh aerobic medium or anaerobic medium with 60 g/L of glucose (inoculum ratio = 10%) and incubated at 37 °C/225 rpm unless otherwise stated.

2.3.4. Anaerobic acetone reduction by BsADH2 mono-culture

BsADH2 was cultivated at 37 °C/225 rpm for 8 h after induction. Then the cell culture was centrifuged at 20,000 g for 10 min.

After the supernatant was discarded, the cell pellets were resuspended into 10 mL of the anaerobic medium with 60 g/L of glucose in a 20-mL serum bottle with a butyl stopper and incubated at 37 °C/225 rpm. Three grams per liter of acetone was added in the beginning where appropriate. In the control experiment, wild-type *B. subtilis* 1A1 (designated as Bs1A1) was cultivated under the same condition. Samples were taken periodically for analysis.

2.3.5. Co-culture of BsADH2 and C. beijerinckii G117

BsADH2 was cultivated at 37 °C/225 rpm for 8 h after the induction. After that, cell pellets collected from 10 mL of the cell culture were resuspended into 5 mL of the aerobic medium with 60 g/L of glucose in a 20-mL serum bottle with a butyl stopper. After being cultured in the anaerobic medium with 60 g/L of glucose at 37 °C/225 rpm for 24 h, 500 μL of the *C. beijerinckii* G117 cell culture was injected into the aerobic medium with BsADH2 above. Then the co-culture was processed at 37 °C/225 rpm. The pH of the aerobic medium was adjusted to 7 using 100 g/L NaOH at 0, 12th and 24th hour. Samples were taken periodically.

In the control experiment, Bs1A1 was firstly cultured following the same procedure with BsADH2 except that no IPTG was added. After that, Bs1A1 was co-cultured with *C. beijerinckii* G117 following the BsADH2-*C. beijerinckii* G117 co-culture protocol described above.

In the aerobic co-culture experiment in the open system, the co-culture was processed in a 50-mL conical tube with a loosened cap at 37 °C statically. Other procedures were the same as what was described above.

2.4. Determination of number of viable BsADH2 cells in the co-culture

Cell culture from different time points was taken. Then 100 μL of cell culture was centrifuged at 20,000 g for 2 min. After the supernatant was removed, the cell pellets were washed twice with 1 mL of sterile DI water. Subsequently, 100 μL of sterile deionized (DI) water was used to resuspend the cell pellets. Then the cell suspension was diluted with sterile DI water in series (10^2 folds, 10^4 folds, and 10^6 folds) to enable accurate counting of colonies. Finally, 50 μL of each suspension was plated on Lysogeny Broth (LB) agar plate and incubated at 37 °C overnight. The number of viable BsADH2 cells was determined by colony counting. Determination of viable Bs1A1 cell number was done following the same procedure as control.

2.5. Optimization of process parameters

C. beijerinckii G117 was cultured in 5 mL of the anaerobic medium with 60 g/L of glucose in a 20-mL serum bottle with a butyl stopper at 37 °C/225 rpm for 24 h. After that, cell pellets of the IPTG-induced BsADH2 from 10 mL of the cell culture was inoculated into the *C. beijerinckii* G117 culture. After the pH was adjusted to 7 using 100 g/L of NaOH, the cells were incubated at 37 °C/225 rpm. Samples were taken periodically. A control (Bs1A1) was also included.

2.6. Protein extraction and analysis

BsADH2 was cultivated at 37 °C/225 rpm for 8 h after induction. Then 10 mL of the cell culture was centrifuged at 20,000 g for 10 min. The supernatant was decanted. The cell pellets were frozen at −20 °C for 2 h. Subsequently, 1 mL 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 g for 20 min. The supernatant was used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) based on the manufacturer's instruction.

In the case of the BsADH1, the cells were cultured in 10 mL of the aerobic medium with 60 g/L of glucose in a 20-mL serum bottle with a butyl stopper. After being incubated at 37 °C for 8 h, the cell culture was centrifuged at 20,000 g for 10 min. Then the cell pellets were frozen at −20 °C for 2 h and lysed by B-PER. After the cells were lysed, the supernatant was used for SDS-PAGE. A control (Bs1A1) was also included.

2.7. Analytical techniques

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

Glucose, lactate, acetate, and butyrate were quantified by High Performance Liquid Chromatography (HPLC) (Agilent Technologies, USA) equipped with a refractive index detector (RID) and a 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 used as the mobile phase at the flow rate of 0.7 mL/min.

Butanol, acetone, ethanol, and isopropanol were quantified by gas chromatography (GC, model 7890A; Agilent Technologies, USA)

equipped with a Durabond (DB)-WAXetr column (30 m × 0.25 mm × 0.25 μm, J&W, USA) and a flame ionization detector (FID). The oven temperature was initially maintained at 60 °C for 2 min, increased at 15 °C/min to 230 °C, and held at 230 °C 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).

3. Results

3.1. Interaction between CpSADH-expressing B. subtilis and C. beijerinckii G117

When *B. subtilis* BsADH2 was co-cultured with *C. beijerinckii* G117 in the aerobic medium (as defined in Materials and Methods), BsADH2 grew first, which consumed oxygen in the medium. Meanwhile, lactate was produced by BsADH2 as a product. In this process, glucose was only consumed by BsADH2. After the oxygen was depleted in the peripheral environment, the anaerobic *C. beijerinckii* G117 started to grow under the protection of BsADH2. During the growth of *C. beijerinckii* G117, acetone, butanol, butyrate, and acetate were produced. Meanwhile, the lactate produced by BsADH2 was assimilated by *C. beijerinckii* G117. Besides, acetone was reduced into isopropanol by BsADH2 (Fig. 1). After the oxygen was depleted, glucose was consumed by both BsADH2 and *C. beijerinckii* G117, forming a competitive relationship between the two strains.

3.2. Expression of CpSADH in B. subtilis 1A1

CpSADH has been widely used in biocatalysis research (Liu and Li, 2019). To investigate the performance of CpSADH of reducing acetone into isopropanol, we constructed a replicative plasmid pHT01-*cpsadh* by cloning *cpsadh* into the pHT01, a commercial *B. subtilis* expression vector (Fig. 2A). Besides, an integrative vector pMB1-*ldh*:operonCm was also constructed as an alternative (Fig. S1). The gene that encodes lactate dehydrogenase (LDH) was chosen as the integration locus because *ldh* promoter was reported to be able to drive the expression of heterologous gene efficiently under non-aerated condition (Romero et al., 2007). We hypothesized that the CpSADH could be expressed by the native *ldh* promoter in the genome-edited strain (named as BsADH1) under the oxygen-limited condition during the co-culture process. These two plasmids were used to transform *B. subtilis* 1A1 separately to compare their performance. After successful insertion of *cpsadh* into *ldh* locus (validated by both colony PCR and Sanger sequencing of PCR products), BsADH1 was cultured in the aerobic medium for 8 h in a serum bottle with a butyl stopper to mimic the non-aerated condition that is typically used in *Clostridium* culture (Romero et al., 2007). The strain carrying the replicative plasmid (named as BsADH2) and the parental strain *B. subtilis* 1A1 (termed as Bs1A1, used as a control) were cultured in the same medium with IPTG supplemented when appropriate to induce the protein expression. After the protein expression, cells were harvested and a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted to assess the expression level of CpSADH. The lane of BsADH2 showed a thick band at 36 kDa (the molecular weight of CpSADH), which suggested the successful expression of CpSADH (Fig. 2B). By contrast, no target protein was produced by Bs1A1. Surprisingly, there was no target band when BsADH1 was used, indicating that CpSADH was not expressed even though the sequencing result proved that the integration was successful. This was probably due to the complicated working mechanism of the *ldh* promoter which was beyond the scope of this study. BsADH2 was employed for the rest of the study.

3.3. Anaerobic reduction of acetone by mono-culturing BsADH2

After the successful expression of CpSADH, the ability of CpSADH to convert acetone into isopropanol was tested under anaerobic condition. In this experiment, BsADH2 was cultured at 37 °C in the anaerobic medium supplemented with 60 g/L of glucose and 3 g/L of acetone which mimicked the acetone concentration produced by a typical *Clostridium* culture. Besides, BsADH2 was also cultured in the same medium without acetone as a control. Another control experiment with Bs1A1 was also included.

In the case of BsADH2 fed with acetone, 1.0 g/L of isopropanol was produced after 24 h with a productivity of 0.042 g/L/h, together with the production of 1.3 g/L of lactate from 1.6 g/L of consumed glucose (Fig. 3A, B, and D), indicating the successful conversion of some acetone into isopropanol under the anaerobic condition. In this process, BsADH2 converted glucose to lactate at anaerobic condition to generate ATP. Acetone should have served as electron acceptor to allow carbon metabolism to be active beyond the pyruvate branch point. Since 1.6 g/L

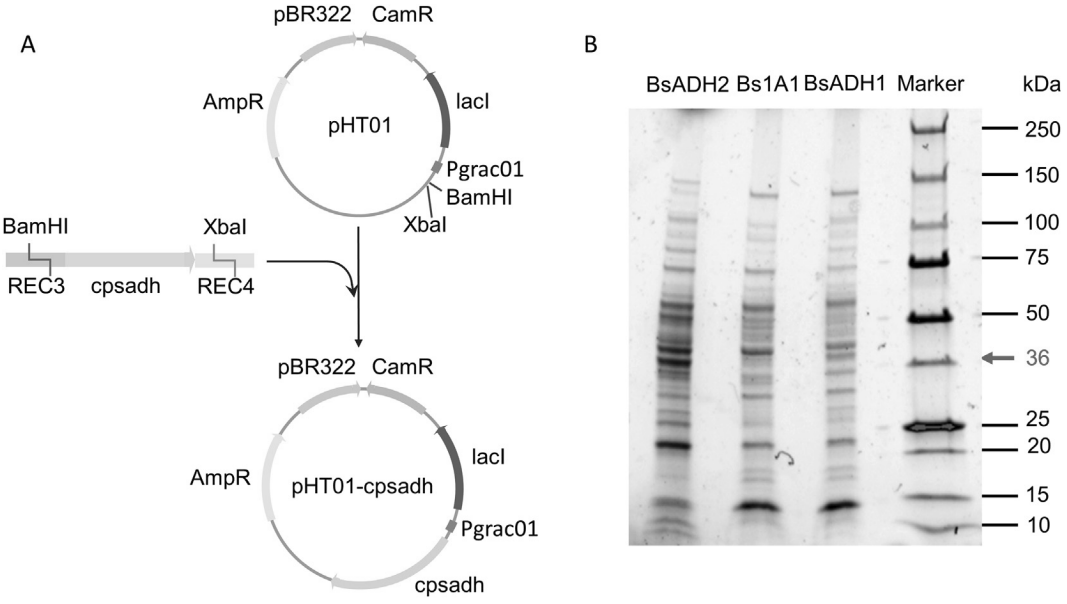


Fig. 2. Plasmid construction and expression of CpSADH. (A) Construction of the pHT01-*cpsadh* plasmid. After being barcoded with REC3-F and REC4-R, *cpsadh* was double-digested by *Bam*HI and *Xba*I and ligated with the pHT01 plasmid which was also digested by *Bam*HI and *Xba*I, yielding pHT01-*cpsadh*. (B) SDS-PAGE analysis of the CpSADH expression in BsADH2, Bs1A1 and BsADH1. A thick band at 36 kDa (molecular mass of CpSADH) was only seen in the lane of BsADH2.

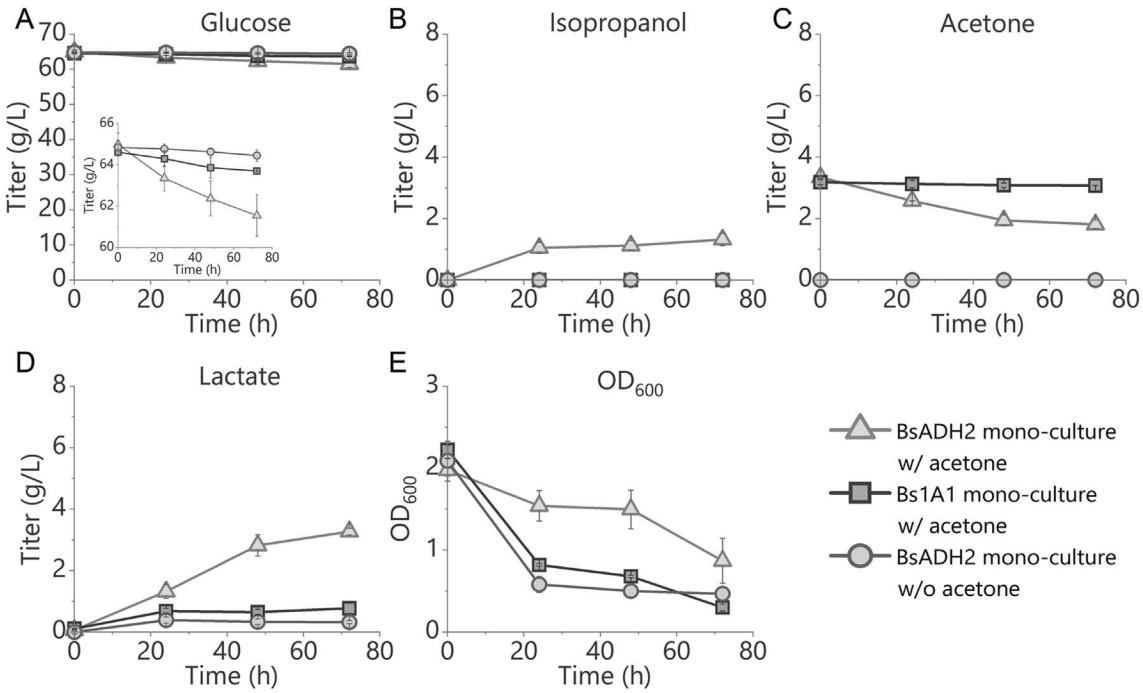


Fig. 3. Acetone reduction through the anaerobic mono-culture of BsADH2 and Bs1A1. Time profile of (A) glucose, (B) isopropanol, (C) acetone, (D) lactate, and (E) OD_{600} during the fermentation. Isopropanol was only produced when the *B. subtilis* strain expressed CpSADH and acetone was supplemented. Yellow triangles: BsADH2 anaerobically mono-cultured in the anaerobic medium supplemented with 60 g/L glucose and 3 g/L acetone. Blue squares: Bs1A1 anaerobically mono-cultured in the anaerobic medium supplemented with 60 g/L glucose and 3 g/L acetone. Green circles: BsADH2 anaerobically mono-cultured in the anaerobic medium supplemented with 60 g/L glucose only. Error bars indicate standard error (n = 3).

glucose could not provide all the NADH used for both lactate production and acetone reduction under the anaerobic condition, the needed electrons could be derived from further oxidation of glucose beyond pyruvate, which might occur at anaerobic condition in BsADH2 when acetone was used as the electron acceptor. Further cultivating the cells for another 48 h led to the production of another 0.3 g/L of isopropanol, resulting in total 1.3 g/L of isopropanol produced (Fig. 3B). Although the conversion rate was only 39.4% and there was still 1.8 g/L of acetone left in the medium (Fig. 3C), this result was comparable with some native isopropanol producers which achieved a conversion rate of 37.2% (Xin et al., 2017). As a comparison, no acetone was reduced when Bs1A1 was cultured anaerobically (Fig. 3C). Interestingly, the lactate titer obtained with Bs1A1 was 0.8 g/L, which was lower than what was produced by BsADH2 (3.3 g/L). This phenomenon may be due to that glucose uptake was enhanced when acetone was reduced into isopropanol by CpSADH, which was absent in Bs1A1. When BsADH2 was cultured in the same medium without acetone, only 0.3 g/L of lactate was produced, suggesting that expressing CpSADH alone was insufficient to trigger the phenotype. Finally, carbon recovery of BsADH2 mono-cultured with acetone, of Bs1A1 mono-cultured with acetone, and of BsADH2 mono-cultured without acetone reached 0.933, 0.736, and 0.865 respectively (Table S1 and Supplementary note S3).

Noteworthily, although the production of lactate kept increasing until the end of the culture, the production of isopropanol in the mono-culture of BsADH2 nearly stopped after 24 h (Fig. 3B and D). A possible reason is that the reduction of pyruvate to lactate and reduction of acetone to isopropanol both could accept electrons, the latter of which might not be needed by the cells after the production of lactate started.

B. subtilis was reported to be able to grow anaerobically in the presence of glucose/nitrite or glucose/pyruvate (Romero et al., 2007). In our experiment, Bs1A1 could only generate lactate to maintain the redox balance of glycolysis under the anaerobic condition when glucose was fed as the only carbon source. The lactate production cannot sink all the electrons liberated from decarboxylation of pyruvate through pyruvate dehydrogenase, so C2 metabolites may not be obtained anaerobically. We have observed that Bs1A1 became round and smaller under the anaerobic condition (Fig. S2), and the sharp decline of optical density from 2.2 to 0.7 after being cultured anaerobically for 48 h (Fig. 3E). As a comparison, the optical density of BsADH2 cultured in the medium with acetone decreased much slower from 2.0 to 1.5 within 48 h. By using acetone as the electron acceptor to sink the electrons, pyruvate in BsADH2 may be decarboxylated to produce the C2 metabolites under the anaerobic redox balance constraint, which led to a more active cellular metabolism when BsADH2 was cultured anaerobically. This hypothesis was also supported by the rapid decline of OD_{600} when BsADH2 was anaerobically cultured without acetone as the electron acceptor (Fig. 3E). Being able to reduce acetone anaerobically is vital in the co-culture process because *B. subtilis* needs to maintain an active metabolism for isopropanol production after the oxygen was depleted in the peripheral environment.

In an attempt to improve the production of isopropanol, a few inoculum sizes were tested, but the isopropanol production was not improved (Fig. S3A–B), suggesting that the rate-limiting factor was not the amount of CpSADH.

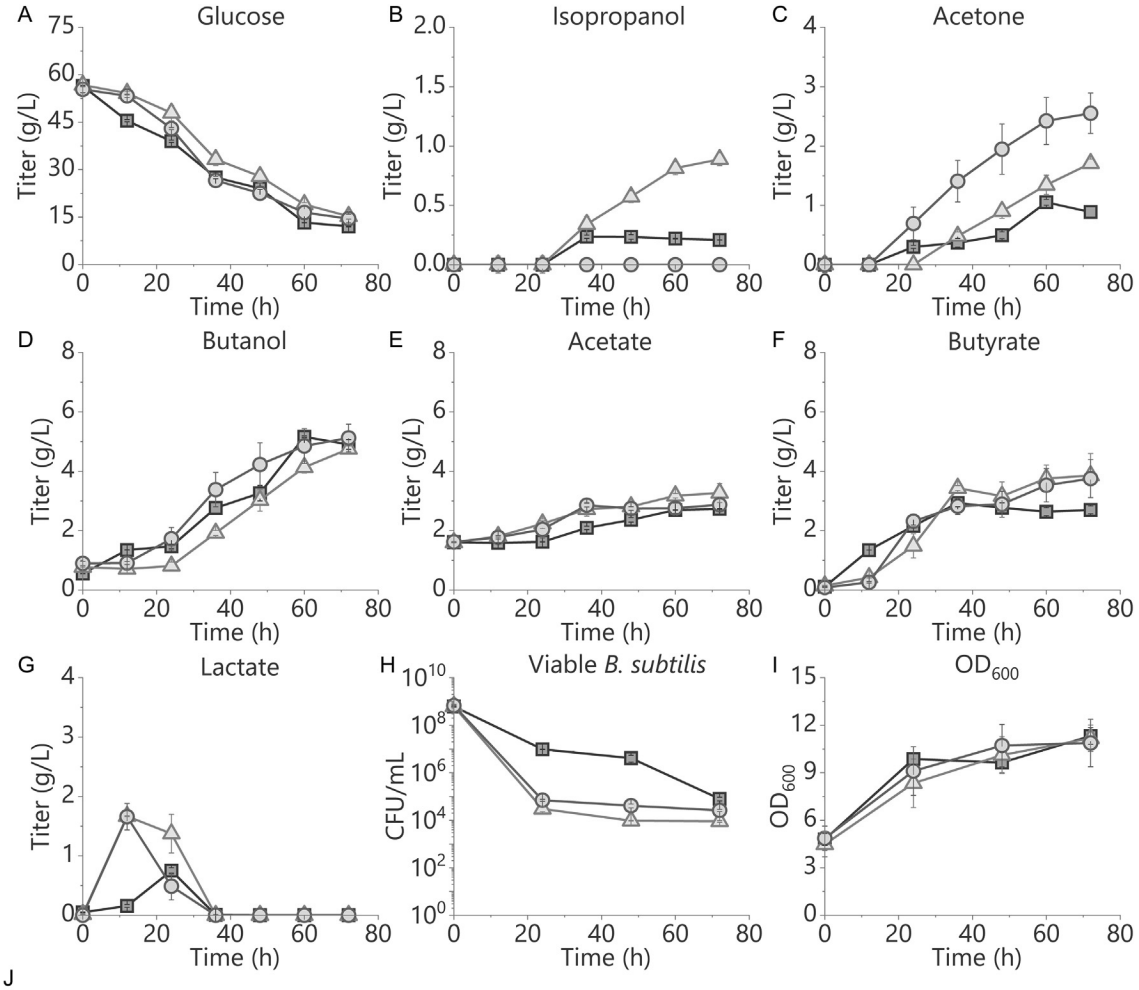
Culture temperature was reported to be critical in the expression of recombinant protein (Wang et al., 2009). Reducing growth temperature was used as a strategy to improve the expression of the recombinant protein by improving protein folding (Zhou et al., 2012; Mühlmann et al., 2017). Therefore, the cells were cultured at 25 °C, 30 °C, or 37 °C after induction to investigate the effect of temperature on the expression of CpSADH. However, induction at different temperatures did not substantially affect the anaerobic acetone reduction, further suggesting that the rate-limiting factor was not the activity of CpSADH (Fig. S3 C-D).

3.4. Switching AB to ABI fermentation by co-culturing C. beijerinckii G117 and BsADH2 in the aerobic medium in closed and open systems

After we confirmed that BsADH2 can reduce acetone into isopropanol

under the anaerobic condition, we further tested if the strain could reduce acetone produced by *Clostridium* when they were co-cultured. *C. beijerinckii* G117 was chosen as the *Clostridium* strain in the co-culture experiment because it does not produce ethanol, an undesired by-product (Chua et al., 2013). Besides, this strain can also tolerate 5 μg/mL of chloramphenicol, which was needed to maintain the replicative plasmid in BsADH2. In addition, *C. beijerinckii* G117 produces negligible amount of acids, which made pH adjustment easier during the fermentation (Chua et al., 2013).

To test if *C. beijerinckii* G117 can grow in the aerobic medium (which did not undergo any anaerobic treatment to remove oxygen) with the help of BsADH2, the two strains were inoculated into the aerobic medium at the beginning of the fermentation. As the induction of protein expression in BsADH2 in the co-culture system might fail due to the anaerobic condition (the promoter was only tested under the aerobic condition), we decided to inoculate high concentrations of induced and aerobically grown BsADH2 cells in the co-culture (initial cell density of BsADH2 = 4 based on OD_{600}). To alleviate the effect of lactate production



J

Yields relative to glucose consumption (mol/mol)	Acetate	Butyrate	Acetone	Isopropanol	Butanol	Alcohols	Acids	Total
BsADH2-CbG117 co-culture aerobic medium - Open	0.074	0.125	0.063	0.014	0.269	0.283	0.199	0.545
BsADH2-CbG117 co-culture aerobic medium - Closed	0.123	0.192	0.127	0.065	0.281	0.346	0.315	0.789
Bs1A1-CbG117 co-culture a -erobic medium - Closed	0.096	0.19	0.197	0	0.303	0.303	0.285	0.786

Fig. 4. Fermentation results of BsADH2-*C. beijerinckii* G117 and Bs1A1-*C. beijerinckii* G117 co-cultures. BsADH2 produced isopropanol when co-cultured with *C. beijerinckii* G117, even when oxygen continuously diffused to the system. A-I: Time profiles of (A) glucose, (B) isopropanol, (c) acetone, (D) butanol, (E) acetate, (F) butyrate, (G) lactate, (H) number of viable *B. subtilis* cells, and (I) OD_{600} during the co-cultures. J: yields of products. Yields were calculated based on obtained products (mol) per mole of consumed glucose. Alcohols: isopropanol and butanol. Acids: acetate and butyrate. Total: alcohols and acids. Blue squares: BsADH2-*C. beijerinckii* G117 co-culture in the aerobic medium supplemented with 60 g/L of glucose in 50-mL conical tubes (open system). Yellow triangles: BsADH2-*C. beijerinckii* G117 co-culture in the aerobic medium supplemented with 60 g/L of glucose in serum bottles (closed system). Green circles: Bs1A1-*C. beijerinckii* G117 co-culture in the aerobic medium supplemented with 60 g/L of glucose in serum bottles (closed system). Error bars indicate standard error (n = 3).

by BsADH2 on pH which might lead to slower growth of *C. beijerinckii* G117, pH was periodically adjusted back to 7 with 100 g/L of NaOH during the first 24 h (Chua et al., 2013). A control (*C. beijerinckii* G117-Bs1A1 co-culture) going through the same procedure was also included.

After the lag phase, *C. beijerinckii* G117 grew rapidly as evidenced by the production of butyrate in the co-culture with BsADH2 (Fig. 4F). After the brief acidogenic phase which produced acetate and butyrate, the cells entered the solventogenic phase from the 24th hour, when butanol and acetone were produced from glucose at 0.081 g/L/h and 0.047 g/L/h respectively (Fig. 4A, C, D, E and F). During this process, isopropanol was produced along with acetone. The isopropanol concentration stopped to increase after 72 h, and reached a final titer of 0.9 g/L, together with 4.8 g/L of butanol, 1.7 g/L of acetone, and 3.9 g/L of butyrate (Fig. 4B, C, D, and F). During the last 48 h, the productivity of isopropanol was 0.019 g/L/h which was 20% of that of butanol (Fig. 4B). Further cultivating the cells for another 48 h did not lead to an increase in the butanol and acetone concentrations (data not shown). Finally, the yield of alcohols (butanol and isopropanol) with respect to glucose achieved 0.346 (mol/mol), which was 14% higher than that from the Bs1A1-*C. beijerinckii* G117 co-culture, due to the reduction of acetone (Fig. 4J). The solvent production performance of the co-culture system was comparable with that of the *C. beijerinckii* G117 mono-culture which produced 5.7 g/L of butanol and 3.4 g/L of acetone under the anaerobic condition (Fig. S4A). As expected, no isopropanol was produced in the anaerobic mono-culture of *C. beijerinckii* G117 (Fig. S4A). Notably, less butyrate was produced in the anaerobic mono-culture of *C. beijerinckii* G117 than what was produced in the co-culture system, which may be due to the adjustment of pH during the co-culture process (Fig. S4B). In the case of Bs1A1 (control) co-cultured with *C. beijerinckii* G117, 5.1 g/L of butanol, 2.6 g/L of acetone, and 3.8 g/L of butyrate were produced (Fig. 4C, D, and F), without the production of isopropanol. This result demonstrated that Bs1A1 was not able to reduce the acetone produced by *C. beijerinckii* G117. When *C. beijerinckii* G117 was cultured in the aerobic medium without *B. subtilis* 1A1 strains (BsADH2 or Bs1A1), it neither produced organic acids/alcohols nor showed cell growth (Fig. S4C). This observation was consistent with the results of other studies (Wu et al., 2016). An interesting phenomenon is that when BsADH2 or Bs1A1 was co-cultured with *C. beijerinckii* G117, lactate was produced in the first 12 h. After reaching a plateau at 2 g/L at the 12th h, lactate was re-assimilated (Fig. 4G). In comparison to the anaerobic mono-culture of *B. subtilis* 1A1 (BsADH2 and Bs1A1) in which lactate concentration did not decrease, the re-assimilation observed in the co-culture should be attributed to *C. beijerinckii* G117 because some *C. beijerinckii* strains have been reported to utilize lactate to produce butanol and butyrate (Hou et al., 2017; Detman et al., 2019). The re-assimilation of lactate, which was the primary by-product of the *B. subtilis* cells, reduced the waste of carbon source. Besides, the lactate concentration in the Bs1A1-*C. beijerinckii* G117 co-culture decreased more rapidly than that in the BsADH2-*C. beijerinckii* G117 co-culture although the two systems shared similar *C. beijerinckii* G117 growth, suggesting that the BsADH2 may have produced more lactate during the fermentation, which was consistent with what was observed in the anaerobic mono-culture of BsADH2.

To better understand the interactions between the two strains in the co-culture, the number of viable *B. subtilis* (BsADH2 and Bs1A1) cells was determined by counting number of colonies that grew on the aerobically incubated LB agar plates—*C. beijerinckii* G117 cannot grow aerobically.

Although BsADH2-*C. beijerinckii* G117 co-culture produced isopropanol, it did not contain a larger number of viable *B. subtilis* cells than Bs1A1-*C. beijerinckii* G117 co-culture during the fermentation (Fig. 4H). This was probably due to the absence of acetone during the early stage of the fermentation. After the anaerobic environment was achieved, *C. beijerinckii* G117 experienced a lag phase during which acetone was not produced. However, oxygen was depleted rapidly by BsADH2, which led to the decline of viable BsADH2 cell number due to the lack of electron acceptor (acetone). This was also supported by the rapid decrease of OD_{600} when BsADH2 was anaerobically mono-cultured in the absence of acetone (Fig. 3E). As a result, only a part of BsADH2 population survived to produce isopropanol during the later stage of the co-culture, which might also contribute to the lower productivity of isopropanol in comparison with the anaerobic acetone reduction by BsADH2. By contrast, OD_{600} of the both Bs1A1-*C. beijerinckii* G117 and BsADH2-*C. beijerinckii* G117 co-culture increased over time, which should be due to the growth of *C. beijerinckii* G117 (Fig. 4I).

Conducting the experiments in the medium without any anaerobic pretreatment in serum bottles simplified the experimental procedure. However, the production of gas including H_2 and CO_2 by *C. beijerinckii* G117 in a closed system remained a problem. Gas needed to be released regularly to avoid pressure built-up inside the closed system. Therefore, it would be desirable if the co-culture could be maintained in an open system. To achieve this target, we further co-cultured the cells in a 50-mL conical tube whose cap was loosened. This condition was defined in this study as an aerobic condition because oxygen (air) continuously diffused into the system.

The aerobic co-culture of *C. beijerinckii* G117 and BsADH2 in the open system showed similar performance in some aspects as the co-culture in the aerobic medium in the serum bottle. After 72 h of cultivation, 4.9 g/L of butanol and 2.7 g/L of butyrate were produced (Fig. 4D and F). This result suggested that the dissolved oxygen level in the medium under this aerobic condition was zero or very close to zero, which was achieved by the action of BsADH2. Notably, only 0.9 g/L of acetone and 0.2 g/L isopropanol were obtained, leading to an isopropanol/acetone ratio of 0.22 which was less than half of that obtained from the co-culture in the closed system (Fig. 4B and C). This may be due to the evaporation of acetone and isopropanol which have lower boiling points than butanol. Besides, the respiration of BsADH2 in the open system may also compete with acetone reduction in oxidizing NADH. Additionally, the number of viable BsADH2 cells showed a slower decrease in the open system (Fig. 4H), suggesting that BsADH2 maintained a more active metabolism when oxygen was more accessible. On the other hand, BsADH2 still could protect *C. beijerinckii* G117 from oxygen, leading to the normal growth of *C. beijerinckii* G117 (Fig. 4I).

Compared with other studies only using *Bacillus* as an oxygen consumer, we successfully established a mutualistic relationship consisting of protection and feeding between *C. beijerinckii* and *B. subtilis* (Wu et al., 2016). *B. subtilis* (BsADH2) protected *C. beijerinckii* G117 from the exposure of O_2 and fed lactate to *C. beijerinckii* G117. In return, *C. beijerinckii* G117 produced acetone for the *B. subtilis* (BsADH2) to better maintain its redox balance. In addition, results from co-culture in the open system proved that the co-culture process we developed here showed the potential as a safer, easier, and more economical way to switch the anaerobic AB fermentation into the aerobic ABI fermentation which does not require the tedious and costly anaerobic pretreatment and troublesome gas-releasing practice.

3.5. Optimization of process parameters

As the titer of isopropanol produced in the co-culture system still needed to be improved, we further inoculated BsADH2 into the 24-h anaerobic cell culture of *C. beijerinckii* G117 so that there would be more acetone available when BsADH2 was added.

After the addition of BsADH2, 1.3 g/L of acetone was reduced into isopropanol within 12 h, and that was more than what was reduced in the aerobic co-culture system (Fig. 5C). Acetate and butyrate were also produced (Fig. 5E and F). Further cultivating the cells for another 12 h led to the production of another 0.7 g/L of isopropanol from glucose (Fig. 5A, B, C, and D). Afterward, no more isopropanol was produced. This is consistent with the result of the anaerobic acetone reduction experiment by BsADH2 mono-culture that isopropanol was only produced during the first 24 h. Overall, 6.4 g/L of butanol and 3.5 g/L of butyrate were produced after 72 h, which was comparable with what

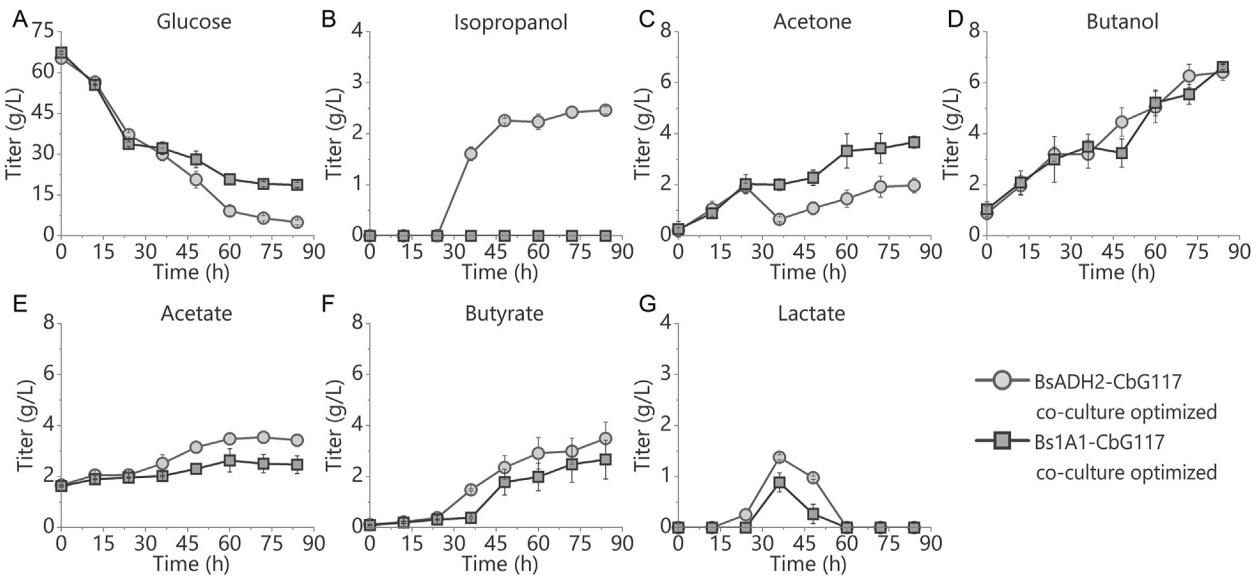


Fig. 5. Optimization of process parameters in the co-culture. The optimization was done by adding *B. subtilis* BsADH2 or Bs1A1 into the *C. beijerinckii* G117 culture that had been incubated for 24 h. The moment that *C. beijerinckii* G117 mono-culture started was designated as 0 h. Time profiles of (A) glucose, (B) isopropanol, (C) acetone, (D) butanol, (E) acetate, (F) butyrate, and (G) lactate during the fermentation are shown in the figure. Green circles: optimized BsADH2-*C. beijerinckii* G117 co-culture. Blue squares: optimized Bs1A1-*C. beijerinckii* G117 co-culture. Error bars indicate standard error (n = 3).

were produced by the anaerobic mono-culture of *C. beijerinckii* G117. Meanwhile, 2.0 g/L of acetone and 2.5 g/L of isopropanol were also obtained (Fig. 5B and C). In comparison, no isopropanol was produced when Bs1A1 was added into the *C. beijerinckii* G117 culture, with similar titers of butanol and butyrate (Fig. 5B, D, and F).

4. Discussion and conclusions

The symbiotic system demonstrated in this study is a new approach to improve the *Clostridium* fermentation by introducing an aerobe that can be easily genetically modified. As a proof-of-concept, the co-culture system successfully shifted the anaerobic AB fermentation into the aerobic ABI fermentation, although the acetone produced in the process was not completed reduced into isopropanol. Neither the conversion rate of acetone nor the titer of isopropanol was improved after a few attempts were made to optimize the parameters of the process (Fig. S3). Therefore, improvement of the enzymatic activity is needed and may be achieved by either engineering the CpSADH or evaluating more secondary alcohol dehydrogenases (SADH). Besides, more strains from the genus of *Clostridium* may also be tested in order to improve the titer of products.

In the future, further modifying *B. subtilis* will enable the symbiotic system to have the potential for many other applications. For example, there are many studies trying to produce short-chain esters from ABE fermentation by either expressing an acyl acetyltransferase in *Clostridium* or adding commercial lipases into the medium (Zhang et al., 2017; Noh et al., 2018). Metabolic engineering of *Clostridium* to express acyltransferase led to the production of only 50 mg/L of butyl butyrate, which proved the concept and opened the door for further optimization works (Noh et al., 2018). Although the utilization of lipases resulted in the production of 34.7 g/L of butyl butyrate by employing an acidogenic *C. tyrobutyricum* strain with the supplementation of butanol, adding commercial lipase and butanol would incur high cost during commercialization (Zhang et al., 2017). Therefore, it would be desired if esters could be produced by co-culturing a *Clostridium* strain with a lipase- and SADH-expressing *B. subtilis*. In this case, *B. subtilis* would provide respiratory protection for *Clostridium* to initiate the ABE fermentation. Meanwhile, *B. subtilis* would maintain its active cellular metabolism to produce lipase by reducing acetone into isopropanol. As a result, isopropanol, butanol, butyrate, and acetate can be further converted into more valuable esters by the lipase. These esters can be easily extracted *in situ* by the utilization of extractant. In addition, further introducing the ability of secreting cellulase, xylanase, and amylase to *B. subtilis* may also enable the expansion of the substrate spectrum of the co-culture system and contribute to the consolidated bioprocessing. Since *B. subtilis* genetic engineering is much easier and faster than the *Clostridium* counterpart, the aforementioned applications with the co-culture would be interesting alternatives to the conventional *Clostridium* mono-culture approach.

Using the co-culture system may face some obstacles. One of them is the usage of the IPTG inducible promoters and antibiotics, which restricts the large-scale application of the process. This could be overcome by utilizing auto-inducible promoters and genome integration vectors. Besides, in order to improve the performance of the co-culture system and enable a better interaction between the two species, several strategies can be explored. For example, controlling oxygen transfer rate (OTR) can be utilized as a strategy to control the microbial interactions in the co-culture system. Due to the different oxygen requirements of the two species, maintaining different oxygen level through the control of OTR can have an impact on the ratio of *B. subtilis* to *Clostridium*. As an important factor, the population ratio of the two specie will further affect the performance of the co-culture system.

As the first co-culture study of an engineered *B. subtilis* strain with *Clostridium*, we successfully switched anaerobic AB fermentation into aerobic ABI fermentation. This study highlighted the potential of using easy-to-engineer *B. subtilis* to functionalize fermentation processes that involve *Clostridium* species. We believe it will inspire more and better co-culture systems involving *Clostridium* species in the future.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Yonghao Cui: Conceptualization, Methodology, Validation, Writing - original draft, Investigation. **Jianzhong He:** Resources, Writing - review & editing. **Kun-Lin Yang:** Conceptualization, Methodology, Writing - review & editing, Supervision. **Kang Zhou:** Conceptualization, Methodology, Writing - review & editing, Resources, Supervision.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mec.2020.e00137.

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Supplementary Materials

Journal: Metabolic Engineering Communications

Title: Aerobic acetone-butanol-isopropanol (ABI) fermentation through a co-culture of *Clostridium beijerinckii* G117 and recombinant *Bacillus subtilis* 1A1

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Supplementary note S1

Sequence of *cpsadh*

5’-

ATGTCAATCCCGTCGTCCCAATACGGCTTTGTCTTCAATAAACAGTCTGGTCTGAAT
CTGCGTAATGATCTGCCGGTCCATAAACCGAAAGCCGGCCAGCTGCTGCTGAAAG
TCGATGCAGTGGGTCTGTGTCATTCTGACCTGCACGTGATTTATGAAGGCCTGGAT
TGCGGTGACAACTACGTTATGGGCCATGAAATTGCAGGTACCGTTGCAGCAGTCG
GTGATGACGTCATCAACTATAAAGTGGGTGATCGTGTGGCGTGTGTTGGCCCGAA
TGGTTGCGGCGGTTGTAAATACTGCCGCGGCGCTATCGATAACGTGTGCAAAAAT
GCGTTTGGTGATTGGTTCGGCCTGGGTTATGACGGCGGTTATCAGCAATACCTGC
TGGTTACCCGTCCGCGCAACCTGAGCCGTATTCCGGATAATGTCTCTGCTGACGT
GGCAGCTGCGAGTACCGATGCGGTCCTGACGCCGTATCACGCCATCAAAATGGCA
CAGGTTTCACCGACCTCGAACATTCTGCTGATCGGTGCCGGCGGTCTGGGCGGTA
ATGCAATTCAAGTGGCCAAAGCATTTGGTGCGAAAGTTACGGTCCTGGATAAGAAA
AAAGAAGCGCGTGATCAGGCGAAAAAACTGGGCGCTGATGCGGTTTATGAAACCC
TGCCGGAAAGCATTTCTCCGGGTAGTTTTTCCGCCTGTTTTGATTTCGTGTCAGTTC
AGGCAACGTTCGACGTTTGCCAAAAATACGTCGAACCGAAAGGCGTTATCATGCC
GGTCGGTCTGGGTGCTCCGAACCTGTCGTTTAATCTGGGTGATCTGGCGCTGCGT
GAAATTCGCATCCTGGGCAGCTTCTGGGGTACCACGAACGACCTGGATGACGTGC
TGAAACTGGTTTCCGAAGGCAAAGTGAAACCGGTGGTTCGTTCAGCCAAACTGAAA
GAACTGCCGGAATATATCGAAAAACTGCGTAACAATGCATACGAAGGTCGCGTCGT
CTTTAACCCGTAA -3’

Supplementary note S2

Construction of Integrative Vector pMB1-Δ*ldh*::operonCm

In order to construct the integrative vector, a GT standard plasmid construction method was adopted (Ma et al., 2019). The chloramphenicol resistance encoding gene was amplified from pHT01 plasmid by using primers PS01 and PS02. The homologous arms *ldh*-HF and *ldh*-HT was amplified from *B. subtilis* genome by using primers PS03 and PS04, PS05 and PS06 respectively. Notably, an RBS barcode was used between *ldh*-HF and *cpsadh*. An I-CeuI barcode was used between *ldh*-HT and AmpR gene so that

the plasmid could be linearized before transformed into *B. subtilis* in order to improve the integration efficiency. Barcodes N21, N22, 5UTR3 and N31 were used between CamR and *ldh*-HT, AmpR and pMB1, pMB1 and *ldh*-HF, *cpsadh* and CamR. All the barcodes and primers used in this section can be found in the paper published by Ma et al., 2019 unless otherwise stated.

Supplementary note S3

Carbon recovery calculation of *B. subtilis* anaerobic mono-culture

Assumptions

1. Cell population did not increase.
2. Isopropanol was all reduced from acetone.

Consumed carbons from glucose (mol/L):

$$\Delta C_{glucose} = \frac{\Delta m_{glucose}}{180} \times 6$$

Recovered carbons from lactate (mol/L):

$$\Delta C_{lactate} = \frac{\Delta m_{lactate}}{90} \times 3$$

Carbon recovery for glucose:

$$CR_{glucose} = \frac{\Delta C_{lactate}}{\Delta C_{glucose}}$$

Table S1 Carbon recovery calculation

	BsADH2 w/ acetone	Bs1A1 w/ acetone	BsADH2 w/o acetone
$\Delta m_{glucose}$	3.44	0.91	0.37
$\Delta C_{glucose}$	0.114667	0.030333	0.012333
$\Delta m_{lactate}$	3.21	0.67	0.32
$\Delta C_{lactate}$	0.107	0.022333	0.010667
$CR_{glucose}$	0.933	0.736	0.865

Table S2 Primers and barcodes used in constructing the pHT01-cpsadh plasmid

Primer or barcode	Sequence	Source or reference
Primer		
P01	5'-G*TCAATCCCGTCGTCCCAATA-3'	This study
P02	5'-A*CGGGTTAAAGACGACGCGAC-3'	This study
P03	5'-TCCCAATTAAAGGAGGAAGGA-3'	This study
P04	5'-CTGCCCCGGGGACGTC-3'	This study
Barcode		
REC3-F	5'-TCCCAATTAAAGGAGGAAGGATCCAT*G-3'	Ma et al., 2019
REC4-R	5'-T*GATCTAGAGTCGACGTCCCCGGGGCAG-3'	Ma et al., 2019

Figures

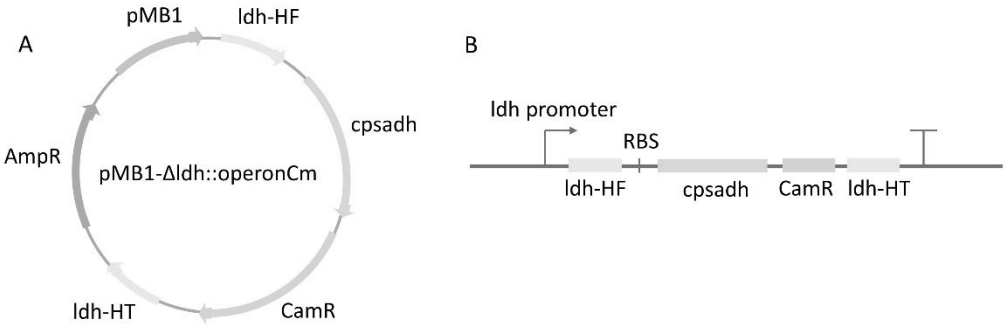


Fig. S1 (A) Plasmid construction of pMB1-Δ*ldh*::operonCm and (B) illustration of the integration process.

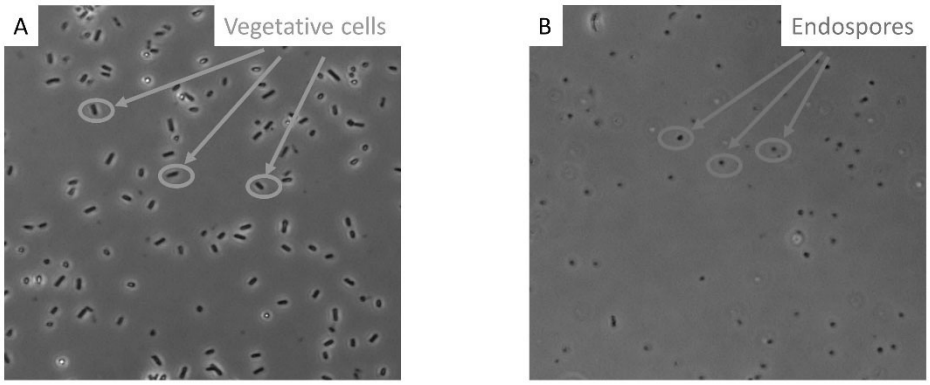


Fig. S2 Microscopic images of cells from the 48 hours' anaerobic mono-culture supplemented with 60 g/L of glucose and 3 g/L acetone of (A) BsADH2 and (B) Bs1A1, which were taken by using a phase-contrast microscope with 40 × magnification. The cells in the yellow circles are vegetative cells. The cells in the green circles are endospores.

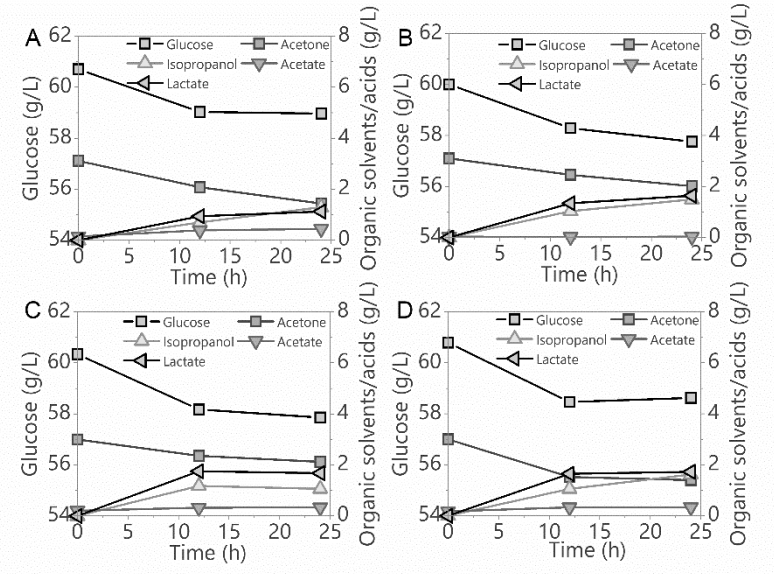


Fig. S3 Profiles of anaerobic mono-culture of BsADH2 in the anaerobic medium with 60 g/L of glucose and 3 g/l of acetone. (A) Cells were induced at 37 ºC for 8 hours before

inoculation. Cell pellets from 15 mL of induced cell culture was inoculated into 5 mL of the anaerobic medium. (B) Cells were induced at 37 ºC for 8 hours before inoculation. Cell pellets from 20 mL of induced cell culture was inoculated into 5 mL of the anaerobic medium. (C) Cells were induced at 25 ºC overnight before inoculation. Cell pellets from 10 mL of induced cell culture was inoculated into 5 mL of the anaerobic medium. (D) Cells were induced at 30 ºC overnight before inoculation. Cell pellets from 10 mL of induced cell culture was inoculated into 5 mL of the anaerobic medium.

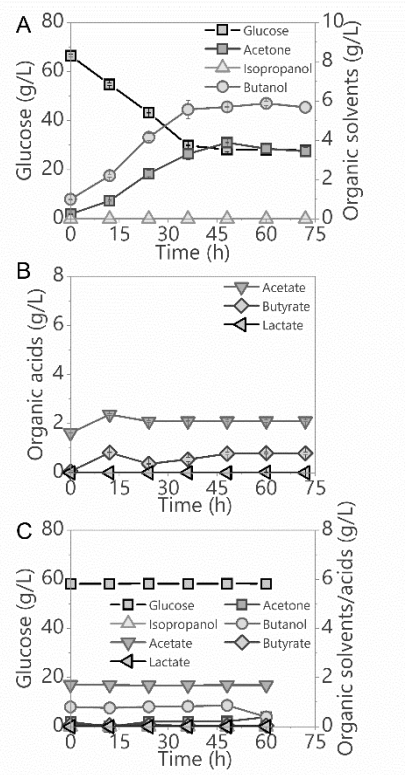


Fig. S4 Batch fermentation profiles of *C. beijerinckii* G117. (A) Glucose and organic solvents concentration of anaerobic mono-culture of *C. beijerinckii* G117. (B) Organic acids concentration of anaerobic mono-culture of *C. beijerinckii* G117. (C) Glucose, organic solvents and acids concentration of *C. beijerinckii* G117 mono-culture in the aerobic medium.

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BIOENERGY/BIOFUELS/BIOCHEMICALS - ORIGINAL PAPER

Production of isopropyl and butyl esters by *Clostridium* mono-culture and co-culture

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Abstract
Production of esters from the acetone-butanol-ethanol (ABE) fermentation by *Clostridium* often focuses on butyl butyrate, leaving acetone as an undesired product. Addition of butyrate is also often needed because ABE fermentation does not produce enough butyrate. Here we addressed the problems using *Clostridium beijerinckii* BGS1 that preferred to produce isopropanol instead of acetone, and co-culturing it with *Clostridium tyrobutyricum* ATCC 25,755 that produced butyrate. Unlike acetone, isopropanol could be converted into ester using lipase and acids. *C. tyrobutyricum* ATCC 25,755 produced acids at pH 6, while *C. beijerinckii* BGS1 produced mainly solvents at the same pH. When the two strains were co-cultured, more butyrate was produced, leading to a higher titer of esters than the mono-culture of *C. beijerinckii* BGS1. As the first study reporting the production of isopropyl butyrate from the *Clostridium* fermentation, this study highlighted the potential use of lipase and co-culture strategy in ester production.

Keywords Isopropyl-butyrate · Lipase · Co-culture · *Clostridium* · Metabolic engineering

Introduction

As flavor and fragrance compounds that are widely exploited in food, cosmetic and pharmaceutical industries [1], short-chain fatty acid esters will have an estimated global market exceeding US$2 billion by 2022 [2]. Conventionally, Fischer esterification was used for producing esters [3]. However, Fischer esterification requires high temperature and the use of concentrated sulfuric acid, so it is energy intensive and requires corrosion-resistant equipment [3, 4]. An alternative way of producing the esters is enzyme-catalyzed esterification. An ester can be produced using an acyl-transferase (ATF), which transfers an acyl group to an alcohol. ATF has been successfully used in *Escherichia coli* to produce high titer of esters (up to 17.2 g/L of isobutyl acetate) [5], but such success was only achieved with esters that cover a limited number of acyl groups [1, 6]. For example, only 50.07 mg/L of ester was produced when the acyl group was butyryl group [7]. As a comparison, lipase can efficiently catalyze the esterification reaction between a broad range of alcohols and carboxylic acids including butanol and butyrate [8, 9]. Among these lipases, *Candida antarctica* lipase B showed a wide substrate spectrum [8]. It was used to produce 17.4 g/L of butyl butyrate using fermentation products of *Clostridium tyrobutyricum* strain ATCC 25,755 [3].

Solventogenic *Clostridium* usually possesses an active acetone-butanol-ethanol (ABE)-producing pathway. During the fermentation, carboxylic acids (acetate and butyrate) and alcohols (butanol and ethanol) are co-produced, along with the production of acetone. This feature enables *Clostridium* to be a good platform for short-chain fatty acid esters production.

However, two main challenges emerged when ABE-producing *Clostridium* strains were used to produce esters. The first one is that acetone cannot be used as a reactant in an esterification reaction. Accounting for 30% of the products, acetone

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is often regarded as an undesired fuel product because of its corrosiveness to the rubber parts in the engines and its poor fuel properties [10]. Some *Clostridium* strains can produce isopropanol instead of acetone due to the involvement of secondary alcohol dehydrogenase. The related fermentation process is also known as isopropanol-butanol-ethanol (IBE) fermentation. Compared with acetone, isopropanol can theoretically be converted into esters such as isopropyl butyrate. Among the IBE-producing strains, *Clostridium beijerinckii* BGS1 is one of the best strains, which can produce up to 10.2 g/L of butanol and 3.4 g/L of isopropanol [11]. In this study, it was used to produce butanol and isopropanol using glucose as a substrate.

Another hurdle of producing esters using *Clostridium* fermentation products is the need of adding butyrate, if the strain was solventogenic. As a major donor of the acid component in the esterification process, a stoichiometric amount of butyrate is needed to completely utilize all the alcohols, but the amount usually cannot be provided by typical solventogenic *Clostridium* strains [4]. To avoid adding exogenous butyrate, another strain may be introduced in a co-culture to overproduce butyrate. As an acidogenic strain, *C. tyrobutyricum* ATCC 25,755 produces butyrate as the main product when pH is 6, at which *C. beijerinckii* BGS1 produces alcohols. Therefore, these two strains were co-cultured in this study to balance the acid-to-alcohol ratio.

Material and methods

Strains and lipases

Clostridium beijerinckii BGS1 used in this study was from a previous study [11]. *Clostridium tyrobutyricum* ATCC 25,755 was acquired from Deutsche Sammlung von Mikroorganisem und Zellkulturen (DSMZ) (Braunschweig, Germany).

Lipases used in this study include lipase immobilized on Immobead 150 from *Rhizopus oryzae* (assigned as Lipase A in this study); lipase from *Candida* sp. (recombinant, expressed in *Aspergillus niger*, assigned as Lipase B in this study); lipase from *Rhizopus oryzae* (powder, light brown, ≥ 30 U/mg, assigned as Lipase C in this study); lipase from *Candida rugosa* (Type VII, ≥ 700 unit/mg solid, assigned as Lipase D in this study). All the lipases used in this study were purchased from Sigma-Aldrich PTE LTD.

Medium and culture conditions

Medium

A mineral salts medium was used in this study [4]. The medium contained 0.75 g/L K_2HPO_4, 0.75 g/L KH_2PO_4, 5 g/L yeast extract. In addition, 1 mL of trace element solution, 1 mL of Na_2SeO_3–Na_2WO_4 solution, and 10 mg of resazurin were added into 1 L of the medium. The composition of the trace element solution and Na_2SeO_3–Na_2WO_4 were attached in the Supplementary Information. After that, the medium was boiled for 20 min under the flushing of N_2. Then it was cooled down to 25 °C. Subsequently, Na_2S, L-cysteine, and DL-dithiothreitol were added to a final concentration of 0.2, 0.2, and 0.5 mM, respectively. After that, CH_3COONH_4 and 2-(N-morpholino)ethanesulfonic acid were added with final concentrations of 2.0 g/L and 3.9 g/L, respectively. Then pH was adjusted to 7 with 100 g/L NaOH. After that, the medium was dispensed into serum bottles with butyl stoppers. Subsequently, the serum bottles were sealed with aluminum seal crimp caps. Finally, the dispensed medium was autoclaved at 121 °C for 20 min and cooled down to 25 °C. The medium was stored at room temperature before it was used.

Culture conditions of *C. beijerinckii* BGS1 monoculture

C. beijerinckii BGS1 from laboratory spore stocks was precultured in 50 mL of mineral salts medium supplemented with 90 g/L of glucose in a 160-mL serum bottle at 37 °C/225 rpm for 24 h. Then 5 mL of the preculture was inoculated into 45 mL of the defined mineral salts medium with 90 g/L of glucose in a 160-mL serum bottle and cultured at 37 °C/225 rpm. The pH was allowed to decrease to 6 with the growth of the cells. After that, pH was adjusted to 6 with 100 g/L NaOH every 12 h. Samples were taken periodically.

Culture conditions of *C. beijerinckii* BGS1-*C. tyrobutyricum* ATCC 25,755 co-culture

C. beijerinckii BGS1 from laboratory spore stocks was precultured in 50 mL of mineral salts medium supplemented with 90 g/L of glucose in a 160-mL serum bottle at 37 °C/225 rpm for 24 h.

C. tyrobutyricum ATCC 25,755 from laboratory spore stock was precultured in 50 mL of mineral salts medium supplemented with 90 g/L of glucose in a 160-mL serum bottle at 37 °C/225 rpm for 24 h.

Five milliliters of *C. beijerinckii* BGS1 preculture was inoculated into 45 mL of the mineral salts medium with 90 g/L of glucose in a 160-mL serum bottle and cultured at 37 °C/225 rpm. Then 0.5 mL of the *C. tyrobutyricum* ATCC 25,755 preculture was inoculated. The pH was allowed to decrease to 6 with the growth of the cells. After that, pH was adjusted to 6 with 100 g/L NaOH every 12 h. Samples were taken periodically.

Lipase screening

Ten milliliters of an aqueous solution composed of 10 g/L butanol, 5 g/L isopropanol, 10 g/L butyric acid, and 10 g/L acetic acid were prepared and added into a 20-mL serum bottle with a butyl stopper. After 250 U of each lipase was added into different bottles, 5 mL of hexadecane was added as the extractant. The bottles were incubated at 37 °C/225 rpm. Samples were taken regularly.

Production of esters at different pH values

Butanol, isopropanol, butyric acid, and acetic acid were added into a 20-mL serum bottle with a butyl stopper in a final concentration of 10 g/L, 5 g/L, 10 g/L, and 10 g/L, respectively to form a 10-mL aqueous solution. After pH was adjusted to 3, 4, 5, and 6 with 100 g/L NaOH, 250 U of Lipase B was added. Five milliliters of hexadecane was added as extractant. The pH was monitored throughout the experiment so that it was constant.

Esterification from *C. beijerinckii* BGS1 mono-culture and *C. beijerinckii* BGS1-*C. tyrobutyricum* ATCC 25,755 co-culture

After being cultured for 120 h, 10 mL of the cell culture from *C. beijerinckii* BGS1 mono-culture or *C. beijerinckii* BGS1-*C. tyrobutyricum* ATCC 25,755 co-culture was transferred into a 20-mL serum bottle with a butyl stopper. Then, sodium butyrate was added where appropriate. After pH was adjusted to 3 to 4 with 100 g/L H_2SO_4, 250 U of the Lipase B was added. Finally, 5 mL of hexadecane was added as extractant. The reaction was allowed to happen at 37 °C/225 rpm. Samples were taken periodically.

Analytical methods

Butanol, acetone, isopropanol, and esters were quantified by gas chromatography (GC, model 7890A; Agilent Technologies, USA) equipped with a Durabond (DB)-WAXetr column (30 m × 0.25 mm × 0.25 μm, J&W, USA) and flame ionization detector (FID). The oven temperature was initially maintained at 60 °C for 2 min, increased at 15 °C/min to 230 °C, and held for 1.7 min. Helium was used as carrier gas with a flow rate of 1.5 mL/min [4].

Glucose, acetate, and butyrate were measured by high-performance liquid chromatography (HPLC, Agilent Technologies, USA) equipped with a refractive index detector (RID) and variable wavelength detector (VWD) (210 nm). The column used was Bio-Rad Aminex HPX-87H column (300 mm × 7.8 mm). The mobile phase was 5 mM sulfuric acid and the flow rate was 0.7 mL/min.

As the partition coefficient of butyl butyrate in the hexadecane/aqueous system was reported to be more than 300 [3, 12], the esters in the aqueous phase were neglected in this study. All esters were quantified from the hexadecane layer and the concentrations were divided by two (the volume of the aqueous phase was two times that of the hexadecane phase) so that the unit would be in (g of esters)/(L of aqueous phase). For other metabolites such as glucose, acids, and alcohols, they were all quantified from the aqueous samples because their partition coefficients in the hexadecane/aqueous system are relatively low (0.027 for butyrate, 0.043 for butanol) [3].

Results and discussion

Mechanism of the reaction and selection of lipases

Adding lipase to the culture medium of *Clostridium* fermentation can condense the acids and alcohols in the medium into esters, which can be extracted by an organic phase if it is present (Fig. 1). This process will drive the esterification reaction forward until an equilibrium is achieved.

Organic extractants such as kerosene, Bio-OSR, and hexadecane have been used for the extraction of butyl-butyrate from the aqueous phase [4]. Considering the partition coefficient, biocompatibility, and availability [3], hexadecane was chosen as the extractant in this study.

To identify a suitable lipase in this application, four commercially available lipases—lipase from *Rhizopus oryzae* (immobilized on Immobead 150, termed as Lipase A in this study), lipase from *Candida* sp. (recombinant, expressed in *Aspergillus niger*, termed as Lipase B in this study), Lipase from *Rhizopus oryzae* (powder, light brown, ≥ 30 U/mg, termed as Lipase C in this study), and lipase from *Candida rugosa* (Type VII, ≥ 700 unit/mg solid, termed as Lipase D in this study)—were evaluated using the mixture of 10 g/L butanol, 5 g/L isopropanol, 10 g/L butyric acid, and 10 g/L acetic acid as substrates.

Among the four lipases, Lipase B was the best in terms of ester productivity and titer. After 12 h, 0.4 g/L of isopropyl butyrate was produced, along with 0.9 g/L of butyl acetate and 4.0 g/L of butyl butyrate (Fig. 2b). Further allowing the reaction to proceed for another 36 h led to the production of another 0.1 g/L of isopropyl butyrate, 0.1 g/L of butyl acetate, and 2.2 g/L of butyl butyrate. As a comparison, no isopropyl butyrate was produced when other lipases were used. When Lipase A was added, 0.7 g/L of butyl butyrate was produced within 48 h (Fig. 2a). In the case of Lipase C and D, 0.1 g/L and 0.06 g/L of butyl butyrate were produced respectively as the only product within 48 h (**F**ig. 2c and

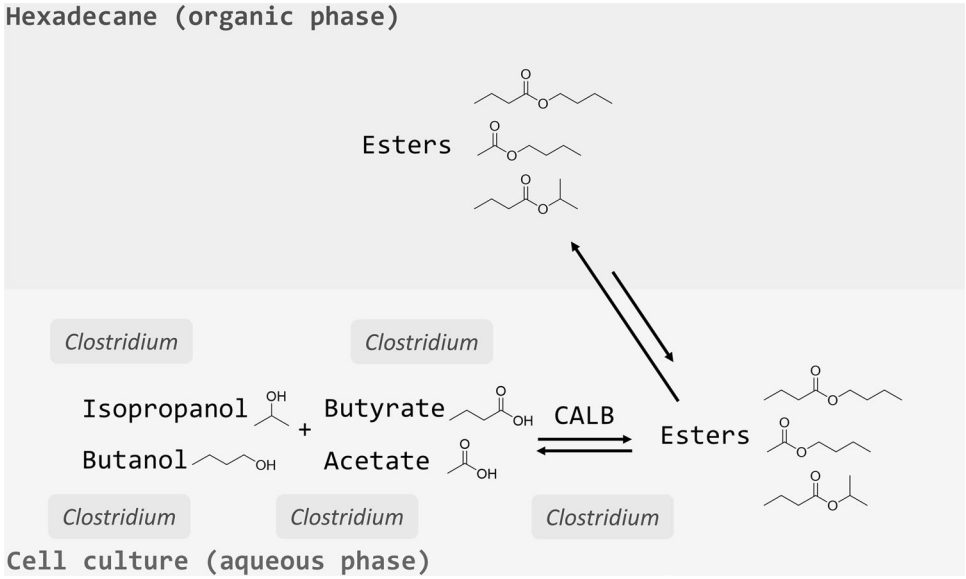


Fig. 1 Mechanism of the biphasic esterification. CALB: *Candida antarctica* lipase B

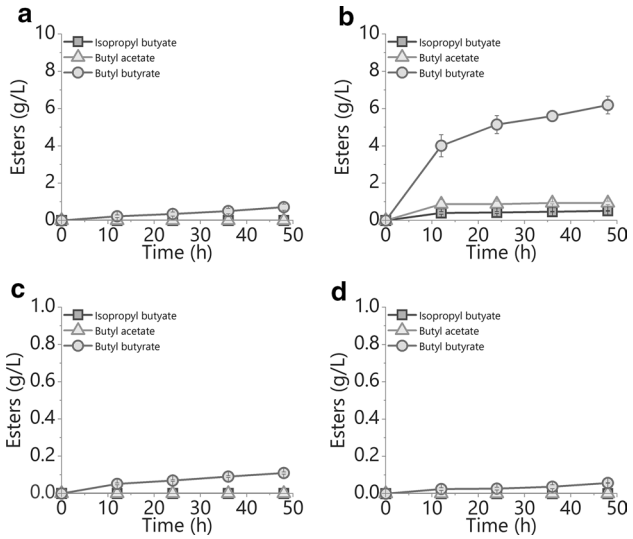


Fig. 2 Selection of lipases. Esterification of butanol, isopropanol, butyric acid, and acetic acid using four lipases. **a** Lipase A (lipase, immobilized on Immobead 150 from *Rhizopus oryzae*). **b** Lipase B (lipase from *Candida* sp.[recombinant, expressed in *Aspergillus niger*]). **c** Lipase C (lipase from *Rhizopus oryzae* [powder, light brown, ≥ 30 U/mg]). **d** Lipase D (lipase from *Candida rugosa* [Type VII, ≥ 700 unit/mg solid]). Error bars, mean ± S.E. in all graphs (some error bars are smaller than the plot symbols). There are three replicates for each data point

d). Lipase B was selected in the subsequent esterification experiments based on the results obtained.

Effect of pH during the esterification stage

As an important factor in the esterification process, pH was empirically optimized. The pKa of butyric acid at room temperature is 4.82, while the pKa of acetic acid is 4.76. Lower pH will lead to more undissociated acids in the reaction system, which promotes the production of esters (only undissociated acids can participate in esterification reaction). However, lipase could be denatured if the pH is too low, leading to poor ester production [13].

Lipases were added into the mixture of 10 g/L butanol, 5 g/L isopropanol, 10 g/L butyric acid, and 10 g/L acetic acid with different pH. During the fermentation of *C. beijerinckii* BGS1, pH usually decreases from 7 to 6 due to the production of butyrate and acetate. After that, pH remains at around 6 till the end of the fermentation. Therefore, pH 6 was initially selected for producing esters from the acid/alcohol mixture. However, almost no esters were produced when pH 6 was used during the esterification stage, suggesting that lower pH was needed to facilitate the esterification (Fig. 3a). Failing to produce esters at pH 6 suggests that esters cannot be produced using this lipase during the fermentation. When pH was decreased to 5, isopropyl butyrate was produced at a titer of 0.2 g/L, accompanied by the production of 0.3 g/L of butyl acetate and 2.4 g/L of butyl butyrate within 12 h (Fig. 3a), confirming that a lower pH can promote the production of esters. We further decreased pH to 4, which led to the production of more than doubled amount of esters (0.5 g/L of isopropyl butyrate, 0.7 g/L of butyl acetate, and 5.3 g/L of butyl butyrate) within 12 h. No substantial improvement was achieved in terms of ester titer when pH was further decreased to 3, suggesting that a pH of 3–4 was good for producing esters. Therefore, pH was maintained at 3–4 in the following esterification reactions. Noteworthily, equilibrium was achieved after 12 h in all the cases (Fig. S2), suggesting the high efficiency of Lipase B in catalyzing the esterification reactions.

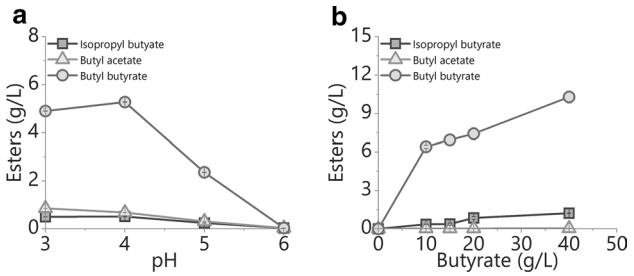


Fig. 3 **a** Effect of pH on the production of esters. Lipase B was used. A mixture of 10 g/L butanol, 5 g/L isopropanol, 10 g/L butyric acid, and 10 g/L acetic acid were used as substrate. The reaction time was 12 h. **b** Effect of exogenously added butyrate on the production of esters. Lipase B was used. The 120-h cell culture of *C. beijerinckii* BGS1 was used as substrate. The reaction time was 12 h. Error bars, mean ± S.E. in all graphs (some error bars are smaller than the plot symbols). There are three replicates for each data point

Effect of exogenously added butyrate

During the fermentation of *C. beijerinckii* BGS1, 6.8 g/L of butanol and 2.9 g/L of isopropanol were produced. However, only 0.8 g/L of butyrate was produced, which was much lower than the stoichiometric quantity that is needed to completely utilize the alcohols (Fig. 4a and c). As a result, butyrate needed to be supplemented exogenously so that a better acid/alcohol ratio could be achieved [4]. In theory, a high concentration of butyrate can also drive the equilibrium of the reaction to the ester side. However, ester production might be inhibited if the butyrate concentration was too high due to the enzyme inactivation or inhibitory action of the substrate [14]. To optimize butyrate concentration, sodium butyrate was added into the 120-h culture of *C. beijerinckii* BGS1 to achieve the following final concentration of butyrate (not considering the butyrate produced by the cells): 10.0 g/L, 15.0 g/L, 20.0 g/L, and 40.0 g/L.

Supplementing 10 g/L of butyrate led to the production of 0.4 g/L of isopropyl butyrate within 12 h, along with the production of 0.1 g/L of butyl acetate and 6.4 g/L of butyl butyrate (Fig. 3b). As a comparison, no esters were produced without the addition of butyrate. This is consistent with the results from other studies that adding exogenous butyrate was necessary to produce esters from the fermentation product of solventogenic *Clostridium* [4]. To increase the titers of esters, more butyrate was added. Increasing the butyrate concentration to 40.0 g/L led to the production of 1.3 g/L of isopropyl butyrate and 11.0 g/L of butyl butyrate. At this condition, 83% of the produced butanol and 21% of the produced isopropanol were converted into esters. The titer of butyl butyrate showed a slower increase when butyrate concentration increased from 10 g/L to 40 g/L, indicating that the titers of esters might be mainly limited by the

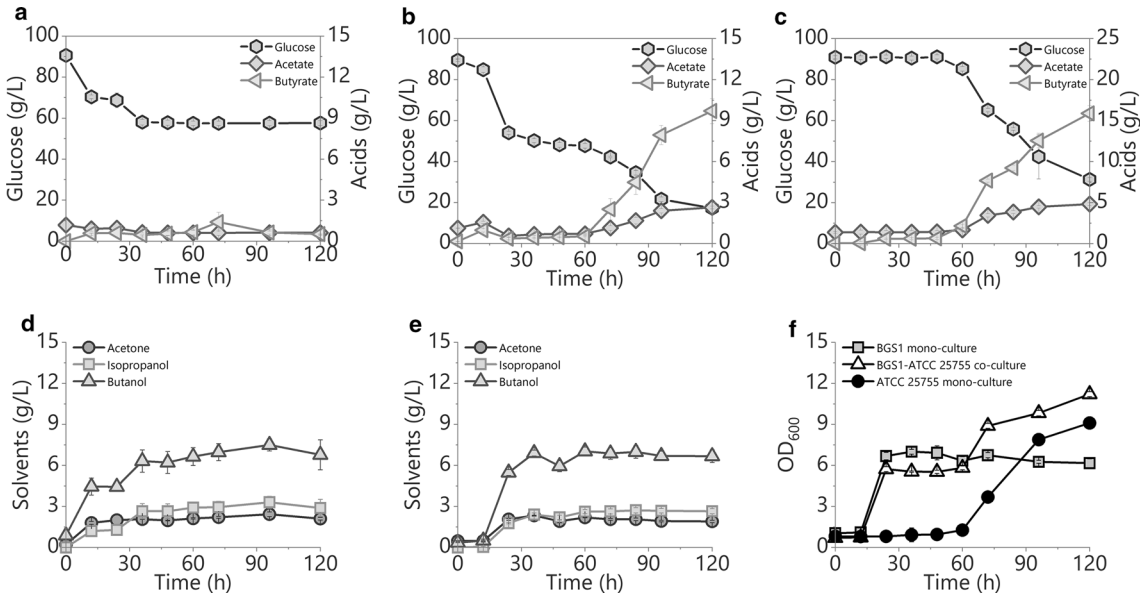


Fig. 4 Using *C. tyrobutyricum* ATCC 25,755 to provide butyrate in a co-culture for ester production. **a** Time profile of glucose and organic acids concentration in the mono-culture of *C. beijerinckii* BGS1. **b** Time profile of glucose and organic acids concentration in the co-culture of *C. beijerinckii* BGS1 and *C. tyrobutyricum* ATCC 25,755. **c** Time profile of glucose and organic acids concentration in the mono-culture of *C. tyrobutyricum* ATCC 25,755. **d** Time profile of organic solvents concentration in the mono-culture of *C. beijerinckii* BGS1. **e** Time profile of organic solvents concentration in the co-culture of *C. beijerinckii* BGS1 and *C. tyrobutyricum* ATCC 25,755. **f** Time profile of OD_{600} during the *C. beijerinckii* BGS1 mono-culture, *C. beijerinckii* BGS1-*C. tyrobutyricum* ATCC 25,755 co-culture, and *C. tyrobutyricum* ATCC 25,755 mono-culture error bars, mean ± S.E. in all graphs (some error bars are smaller than the plot symbols). There are three replicates for each data point

concentration of alcohols when the concentration of butyrate exceeded 10 g/L.

As far as we know, this is the first study reporting the production of both isopropyl butyrate and butyl butyrate from the fermentation product of *Clostridium*. Compared with those experiments leaving acetone wasted after the esterification of butanol, this study partly solved the problem by utilizing the IBE-producing *Clostridium*.

Co-culture of *C. beijerinckii* BGS1 and *C. tyrobutyricum* ATCC 25,755

Although adding butyrate exogenously increased the titers of esters, the operation increases the complexity of the fermentation process and incurs higher substrate cost.

As an acidogenic strain, *C. tyrobutyricum* ATCC 25,755 is well known for its good ability of producing butyrate [15]. In our experiments, 15.9 g/L of butyrate was produced by *C. tyrobutyricum* ATCC 25,755 mono-culture within 120 h, without the production of solvents (Fig. 4c). As a comparison, *C. beijerinckii* BGS1 produces mainly solvents at pH 6 based on our experiments (Fig. 4a, d). Therefore, we adopted a co-culture strategy that utilized the different characteristics of these two strains at pH 6 to produce the solvents and more butyrate, so that the titers of esters could be increased during the esterification stage without adding butyrate.

When *C. beijerinckii* BGS1 was co-cultured with *C. tyrobutyricum* ATCC 25,755, 6.9 g/L of butanol and 2.4 g/L of isopropanol were produced within 36 h (Fig. 4e). After a 36-h lag, butyrate started to be produced with a productivity of 0.15 g/L/h, leading to continuous consumption of glucose. Finally, 9.7 g/L of butyrate was produced by the co-culture system (Fig. 4b). A discrepancy of butyrate titer with the *C. tyrobutyricum* ATCC 25,755 mono-culture was observed, which was probably due to the toxicity of solvents produced by *C. beijerinckii* BGS1 (Fig. 4c). On the contrary, a negligible amount of butyrate was produced by the mono-culture of *C. beijerinckii* BGS1 (Fig. 4a). Compared with the co-culture, mono-culture of *C. beijerinckii* BGS1 led to a similar production of butanol and isopropanol (6.8 g/L and 2.9 g/L, respectively) (Fig. 4d). Considering that there were two different strains from two different species in this co-culture system and the solvent tolerance of the two species might differ, the difference in the fermentation performance between the co-culture and mono-culture might be explained by solvent inhibition or toxicity. While the amount of solvents produced by *C. beijerinckii* BGS1 was inhibitory/toxic to *C. beijerinckii* BGS1 itself, *C. tyrobutyricum* ATCC 25,755 might be able to tolerate the solvents and hence grew in the presence of those solvents, leading to continued consumption of glucose. This statement is supported by the results from another study which proved that *C. tyrobutyricum* can tolerate up to 2.0% (v/v) butanol while *C. beijerinckii* was completely inhibited by 2.0% (v/v) butanol [16]. The production of butanol and isopropanol in the first 36 h should be from *C. beijerinckii* BGS1 in the co-culture, because *C. tyrobutyricum* ATCC 25,755 was not known to produce the solvents. From the 60th hour, *C. tyrobutyricum* ATCC 25,755 started to grow, leading to the production of butyrate. This statement was supported by the profile of OD_{600} (Fig. 4f). Cell density of the *C. beijerinckii* BGS1 stopped increasing after 24 h in the mono-culture. However, the cell density in the co-culture started to increase again from the 60th hour, along with the rapid production of butyrate, which is a typical feature of *C. tyrobutyricum* (Fig. 4c). As a more direct measurement of cell growth of these two strains, expressing an anaerobic fluorescent protein in either *C. tyrobutyricum* ATCC 25,755 or *C. beijerinckii* BGS1 and differentiating them under fluorescence microscope might be helpful in the future. In addition, metabolic engineering of *C. tyrobutyricum* ATCC 25,755 and *C. beijerinckii* BGS1 may also help to further convert the remained acetone into isopropanol. This might be achieved by expressing a secondary alcohol dehydrogenase in either of the strains, which has been proved to be feasible in *C. acetobutylicum* [17].

These results suggested that by co-culturing *C. beijerinckii* BGS1 and *C. tyrobutyricum* ATCC 25,755, more butyrate could be produced without sacrificing the production of alcohols.

Following the co-culture, we further conducted the esterification reaction using the fermentation product from *C. beijerinckii* BGS1 mono-culture and *C. beijerinckii* BGS1-*C. tyrobutyricum* ATCC 25,755 co-culture.

During the esterification stage, 5.1 g/L of butyl butyrate was produced within 12 h from the fermentation product of the co-culture (Fig. 5a). Besides, 0.2 g/L of butyl acetate and 0.2 g/L of isopropyl butyrate was also produced. The titers of esters were similar to those from the experiment in which 10 g/L of butyrate was added into the fermentation product of *C. beijerinckii* BGS1. As a comparison, there was 0.5 g/L of butyl butyrate produced as the sole product from the fermentation product of the *C. beijerinckii* BGS1 mono-culture (Fig. 5b). These results suggested that the introduction of the acidogenic *C. tyrobutyricum* ATCC 25,755 did help improve the production of esters due to the production of more butyrate.

To the best of our knowledge, this is the first study reporting utilizing the *Clostridium-Clostridium* co-culture strategy to improve the production of esters (butyl butyrate, butyl acetate, and isopropyl butyrate). By adopting this strategy, esters could be produced in descent titers without the addition of exogenous butyrate, representing a more cost-effective way to produce esters from the fermentation product of *Clostridium*. Compared with the strategy in which butyrate-producing strain and alcohol-producing strain were

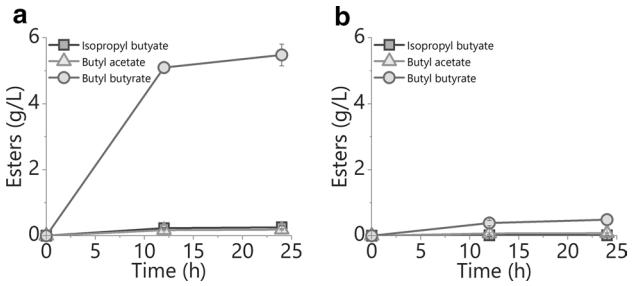


Fig. 5 Profile of esters from **a** co-culture of *C. beijerinckii* BGS1 and *C. tyrobutyricum* ATCC 25,755 and **b** mono-culture of *C. beijerinckii* BGS1. Error bars, mean ± S.E. in all graphs (some error bars are smaller than the plot symbols). There are three replicates for each data point

cultured separately and then mixed to produce esters, this co-culture strategy might be more advantageous because dilution of the reactants could be avoided, which may lead to high ester titers (Fig. 3b). However, given that commercial lipase was used in this study and its cost could be a concern (Supplementary note S1), further investigation on alleviating or avoiding the usage of commercial lipase is needed. This might be achieved by expressing lipase or alcohol acyltransferase in *Clostridium* in the future [4, 6].

Conclusions

C. beijerinckii BGS1 was used for producing esters with the help of *Candida antarctica* lipase B. When 50 g/L of sodium butyrate was added into the 120-h culture of *C. beijerinckii* BGS1 at pH 3, 1.3 g/L of isopropyl butyrate was produced with 11.0 g/L of butyl butyrate. Besides, 0.2 g/L of isopropyl butyrate and 5.1 g/L of butyl butyrate were produced when *C. tyrobutyricum* ATCC 25,755 was co-cultured with *C. beijerinckii* BGS1. As the first study reporting the production of isopropyl butyrate from the IBE fermentation and co-culture of the two *Clostridium* strains, this study highlighted the potential use of lipase and co-culture strategy in ester production.

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Author contributions KZ, KLY, and YC contributed to the study conception and design. Material preparation, data collection and analysis were performed by YC. JH contributed the strains and analytical tools. The first draft of the manuscript was written by YC and KZ and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest We declare that there is no conflict of interest.

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

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1. A composition or combination for the production of butanol and isopropanol, comprising an acetone-butanol-ethanol (ABE)-producing *Clostridium* strain and a genetically engineered *B. subtilis* strain, wherein said genetically engineered *B. subtilis* strain has been transformed by at least one polynucleotide molecule;

   the at least one polynucleotide molecule comprising a secondary alcohol dehydrogenase gene operably linked to at least one promoter.

2. The composition or combination of 1, wherein the *Clostridium* strain is *C. beijerinckii* G117.

3. The composition or combination of 1 or 2, wherein the secondary alcohol dehydrogenase gene is *cpsadh*.

4. The composition or combination of any one of 1 to 3, wherein the secondary alcohol dehydrogenase gene comprises a polynucleotide 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;

   5’-
   ATGTCAATCCCGTCGTCCCAATACGGCTTTGTCTTCAATAAACAGTCTGGTCTGAATC
   TGCGTAATGATCTGCCGGTCCATAAACCGAAAGCCGGCCAGCTGCTGCTGAAAGTC
   GATGCAGTGGGTCTGTGTCATTCTGACCTGCACGTGATTTATGAAGGCCTGGATTGC
   GGTGACAACTACGTTATGGGCCATGAAATTGCAGGTACCGTTGCAGCAGTCGGTGA
   TGACGTCATCAACTATAAAGTGGGTGATCGTGTGGCGTGTGTTGGCCCGAATGGTT
   GCGGCGGTTGTAAATACTGCCGCGGCGCTATCGATAACGTGTGCAAAAATGCGTTT
   GGTGATTGGTTCGGCCTGGGTTATGACGGCGGTTATCAGCAATACCTGCTGGTTAC
   CCGTCCGCGCAACCTGAGCCGTATTCCGGATAATGTCTCTGCTGACGTGGCAGCTG
   CGAGTACCGATGCGGTCCTGACGCCGTATCACGCCATCAAAATGGCACAGGTTTCA
   CCGACCTCGAACATTCTGCTGATCGGTGCCGGCGGTCTGGGCGGTAATGCAATTCA
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   GTGATCAGGCGAAAAAACTGGGCGCTGATGCGGTTTATGAAACCCTGCCGGAAAGC
   ATTTCTCCGGGTAGTTTTTCCGCCTGTTTTGATTTCGTGTCAGTTCAGGCAACGTTCG
   ACGTTTGCCAAAAATACGTCGAACCGAAAGGCGTTATCATGCCGGTCGGTCTGGGT
   GCTCCGAACCTGTCGTTTAATCTGGGTGATCTGGCGCTGCGTGAAATTCGCATCCTG
   GGCAGCTTCTGGGGTACCACGAACGACCTGGATGACGTGCTGAAACTGGTTTCCGA
   AGGCAAAGTGAAACCGGTGGTTCGTTCAGCCAAACTGAAAGAACTGCCGGAATATAT
   CGAAAAACTGCGTAACAATGCATACGAAGGTCGCGTCGTCTTTAACCCGTAA -3’.

5. A composition or combination for the production of isopropyl butyrate and/or butyl acetate and/or butyl butyrate, comprising a first isopropanol-producing *Clostridium* strain and a second butyrate-producing *Clostridium* strain.

6. The composition or combination of 5, wherein the first *Clostridium* strain is *Clostridium beijerinckii* BGS1 and the second *Clostridium* strain is *Clostridium acetobutylicum* ATCC824 or *Clostridium tyrobutyricum*.

7. A method of producing butanol and isopropanol in a co-culture, comprising the steps:

a) co-culturing an acetone-butanol-ethanol (ABE)-producing *Clostridium* strain with a genetically engineered *B. subtilis* strain in medium under conditions for conventional ABE fermentation with exception that no anaerobic treatment is used, and

b) supplementing the medium with glucose,

wherein said genetically engineered *B. subtilis* strain has been transformed by at least one polynucleotide molecule;

the at least one polynucleotide molecule comprising a secondary alcohol dehydrogenase gene operably linked to at least one promoter,

wherein the co-cultured *Clostridium* and *B. subtilis* are capable of increased production of butanol and/or isopropanol compared to a co-culture with a non-transformed *B. subtilis* cell.

8. The method of claim 7, wherein the secondary alcohol dehydrogenase gene is *cpsadh*.

9. The method of claim 8, wherein the secondary alcohol dehydrogenase gene comprises a polynucleotide 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;

5’-
ATGTCAATCCCGTCGTCCCAATACGGCTTTGTCTTCAATAAACAGTCTGGTCTGAATC
TGCGTAATGATCTGCCGGTCCATAAACCGAAAGCCGGCCAGCTGCTGCTGAAAGTC
GATGCAGTGGGTCTGTGTCATTCTGACCTGCACGTGATTTATGAAGGCCTGGATTGC
GGTGACAACTACGTTATGGGCCATGAAATTGCAGGTACCGTTGCAGCAGTCGGTGA
TGACGTCATCAACTATAAAGTGGGTGATCGTGTGGCGTGTGTTGGCCCGAATGGTT
GCGGCGGTTGTAAATACTGCCGCGGCGCTATCGATAACGTGTGCAAAAATGCGTTT
GGTGATTGGTTCGGCCTGGGTTATGACGGCGGTTATCAGCAATACCTGCTGGTTAC

CCGTCCGCGCAACCTGAGCCGTATTCCGGATAATGTCTCTGCTGACGTGGCAGCTG
CGAGTACCGATGCGGTCCTGACGCCGTATCACGCCATCAAAATGGCACAGGTTTCA
CCGACCTCGAACATTCTGCTGATCGGTGCCGGCGGTCTGGGCGGTAATGCAATTCA
AGTGGCCAAAGCATTTGGTGCGAAAGTTACGGTCCTGGATAAGAAAAAAGAAGCGC
GTGATCAGGCGAAAAAACTGGGCGCTGATGCGGTTTATGAAACCCTGCCGGAAAGC
ATTTCTCCGGGTAGTTTTTCCGCCTGTTTTGATTTCGTGTCAGTTCAGGCAACGTTCG
ACGTTTGCCAAAAATACGTCGAACCGAAAGGCGTTATCATGCCGGTCGGTCTGGGT
GCTCCGAACCTGTCGTTTAATCTGGGTGATCTGGCGCTGCGTGAAATTCGCATCCTG
GGCAGCTTCTGGGGTACCACGAACGACCTGGATGACGTGCTGAAACTGGTTTCCGA
AGGCAAAGTGAAACCGGTGGTTCGTTCAGCCAAACTGAAAGAACTGCCGGAATATAT
CGAAAAACTGCGTAACAATGCATACGAAGGTCGCGTCGTCTTTAACCCGTAA -3'.

10. The method of any one of 7 to 9, wherein the *Clostridium* strain is *C. beijerinckii* G117.

11. The method of any one of 7 to 10, wherein 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.

12. The method of any one of 7 to 11, wherein the genetically engineered *B. subtilis* strain is added to an anaerobic culture of Clostridium after a period of at least 8 hr, at least 16 hr, preferably at least 24 hr and co-cultured for a further period.

13. The method of any one of 7 to 12, further comprising isolating said butanol and/or isopropanol.

14. A method of producing butyrate, isopropanol and butanol in a co-culture, comprising:

    a) co-culturing a first isopropanol/butanol-producing *Clostridium* strain with a second butyrate-producing *Clostridium* strain in medium supplemented with glucose, at a pH of about 6.

15. The method of 14, wherein said first isopropanol/butanol-producing *Clostridium* strain is *Clostridium beijerinckii* BGS1 and said second butyrate-producing *Clostridium* strain is *Clostridium tyrobutyricum* ATCC 25,755 or *Clostridium acetobutylicum* ATCC824.

16. The method of 14 or 15, wherein at least one of said Clostridium strains has been engineered to produce lipase.

17. The method of 14 or 15, further comprising;

    b) adding lipase to the medium to convert alcohols in the medium to esters.

18. The method of 17, wherein esters produced in step b) include isopropyl butyrate, butyl butyrate and butyl acetate.

19. The method of any one of 16 to 18, further comprising isolating said esters.