Optimization of an Economical Medium Composition For The Coculture of Clostridium Butyricum And Bacillus Coagulans

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Abstract

_Clostridium butyricum_ is a spore-forming probiotic existing in the intestines of humans and animals which can promote the enhancement of beneficial bacteria and maintain intestinal microecological balance. However, it is difficult to improve the production level of _C. butyricum_ by conventional fermentation process. In this study, a co-fermentation process of _C. butyricum_ DL-1 and _Bacillus coagulans_ ZC2-1 was established to improve the viable counts and spore yield of _C. butyricum_, and the formula of coculture medium was optimized by flask fermentation. The results showed that the optimum medium composition is bran 10 g/L, corn steep powder 15 g/L, peptone 15 g/L, K$_2$HPO$_4$ 1 g/L and MnSO$_4$ 0.5 g/L. Cultured stationarily in the optimal medium for 36 h, the number of viable bacteria of _C. butyricum_ DL-1 reached $1.5 \times 10^8$ CFU/mL and the spore forming rate was 92.6%. The results revealed an economical and effective medium composition for the coculture of _C. butyricum_ and _B. coagulans_. The co-fermentation process established in this study provides a new fermentation mode for the industrial production of other absolute anaerobic bacteria.

Key Points

1. A novel co-culture system was established.
2. Medium composition for the coculture of _C. butyricum_ and _B. coagulans_ was optimized.
3. The optimal medium cost is lower than what was reported.

Introduction

Probiotics can improve human health (Li et al. 2020a). They are effective in enhancing intestinal immunity (La Fata et al. 2018) and treating diseases such as dyslipidemia (Matey-Hernandez ML 2017), non-alcoholic high-fat diet-induced liver disease (Liu et al. 2017) and cancers (Jones et al. 2013). Increasing numbers of probiotics have been used as medicines and feed additives in recent years. Commonly used species include yeast (Nelson et al. 2020), _Bacillus_ (Mingmongkolchai and Panbangred 2018), _lactobacillus_ (Das et al. 2020) and _Bifidobacterium_ (Tian et al. 2020). _C. butyricum_ (Poolsawat et al. 2019) is a promising emerging member of them.

_C. butyricum_, a strictly anaerobic spore-forming probiotic (Li et al. 2020b), can regulate the imbalance of host intestinal flora and maintain microecological balance (Cassir et al. 2016; Kanai et al. 2015; Pan et al. 2019). It antagonizes pathogenic bacteria and promotes the proliferation of intestinal beneficial bacteria (Li et al. 2016; Li et al. 2019b). So it is widely used to improve human immunity and treat intestinal flora imbalance (Hai-dong Lia 2018). It is also used in animal husbandry to improve animal production performance (Khajeh Bami et al. 2020). With the complete ban of antibiotics as feed additives, _C. butyricum_ has a promising market prospect as an antibiotic substitute because of its marked animal health protection performance and excellent stress resistance (Yi et al. 2020). _C. butyricum_ and _B. coagulans_ can effectively inhibit _Helicobacter pylori_ with few adverse events (Zhang et al. 2020), and
have the potential to be used as alternative anti-Helicobacter pylori drug. They were also used in clinical
treatment for acute Enteritidis and Intestinal microbiologic disorder.

As an obligate anaerobe, *C. butyricum* need complex fermentation process to guarantee strict anaerobic
condition. Even cultured under absolute anaerobic condition, the viable counts and spore yield of *C. butyricum* was not as high as that of other probiotics. For example, the spore yield of *B. subtilis* reached 8.78×10^9 CFU/mL after the optimization of medium components and culture conditions (Posada-Uribe et al. 2015). The viable counts of *L. plantarum* and *L. paracasei* reached 2.77×10^9 and 2.78×10^9 CFU/g in anaerobic solid-state co-fermentation, respectively (Chen et al. 2020). The spore yield of *C. butyricum* is usually less than 1×10^9 CFU/mL in industrial production.

Solid-state co-fermentation of *C. butyricum* and other probiotics can effectively enhance *C. butyricum*
growth and sporulation (Su et al. 2018), suggesting that co-fermentation may be an effective alternative
for obligate anaerobes. Mixed fermentation creates a biological hybrid system (Englezos et al. 2019), in
which microorganisms synergistically metabolize and establish mutually beneficial symbiosis, providing
better fermentation condition than their purebred counterpart (Hamid et al. 2019). Co-fermentation are
widely used in food and feed industry. Dromedary yogurt was fermented with *L. bulgaricus* and
*Streptococcus thermophiles* to improve its nutrition, texture and syneresis (Jrad et al. 2020). *Rhizopus oligosporus* and *L. plantarum* co-fermentation is an effective method to increase the antioxidant potential
of Grass pea and flaxseed oil-cake (Stodolak et al. 2020).

In this study, a co-culture system of *C. butyricum* DL-1 and *B. coagulans* ZC2-1 were established. In the
co-fermentation system, the facultative anaerobic *B. coagulans* strain consumes oxygen in the culture
medium and provides anaerobic environment for the absolute anaerobic *C. butyricum* strain. The culture
medium composition of co-fermentation process was optimized so as to obtain high viable counts and
spore yield of *C. butyricum* at low medium cost. The co-fermentation process provided a new energy-
saving fermentation mode for other absolute anaerobic microbes.

**Materials And Methods**

**Inoculum**

*C. butyricum* DL-1 strain was provided by Jinbaihe biotechnology Co, Ltd in Tangyin Country, Anyang City,
Henan Province, China. Its 16S rRNA gene sequences have been deposited in GenBank with the accession
numbers of MW218001.

**Medias**

The broth media contained 10 g/L peptone, 3 g/L beef extract, and 5 g/L NaCl, with a pH value of 7.0 ±
0.1. Solid nutrient agar media composed of 10 g/L peptone, 3 g/L beef extract, 5 g/L NaCl, and 10 g/L
agar with a pH value of 7.0 ± 0.1. Solid acid-producing bacteria selection media contained 5 g/L glucose,
5 g/L peptone, 1 g/L yeast extract powder, 0.3 g/L CaCO_3_, and 10 g/L agar with a pH value of 7.0.
Proliferation medium of *C. butyricum* DL-1 was composed of 5 g/L glucose, 5 g/L sodium chloride, 3 g/L sodium acetate trihydrate, 10 g/L tryptone, 3 g/L yeast extract, 10 g/L beef extract, 1 g/L soluble starch, 0.5 g/L L-cysteine hydrochloride, pH 7.2. Proliferation medium of *B. coagulans* was composed of 10 g/L glucose, 5 g/L yeast extract, 10 g/L peptone, pH 6.8-7.0. Initial coculture media of *C. butyricum* DL-1 and the four *B. coagulans* strains was composed of 10 g/L glucose, 10 g/L tryptone, 5 g/L yeast extract, pH 6.8-7.0, all the mediums were sterilized at 121 °C for 20 min.

**B. coagulans** acclimation and screening

Inoculum samples were taken from the gut of health chicken and commercial kimchi. The mixtures of samples and 10 times volume of sterile water were shaken for 30 min, and then left to set for 10 min. The supernatants obtained were water bathed at 80 °C for 20 min and then inoculated to the broth medium and cultured at 200 rpm and 37°C for 24 h. Culture broth of appropriate concentration was spread on the solid acid-producing bacteria selection media and cultured at 37 °C stationarily for 48 h. The colonies with obvious calcium-dissolving circles were streaked on solid nutrient agar plates for further purification. The pure colonies were preserved on nutrient agar slants at 4°C for further study.

The screened strains were identified by morphological characterization combined with phylogenetic analysis. Microbial phenotypic characteristics were determined by observing colonial and mycelia morphology by naked eyes and an optical microscope, respectively. Phylogenetic analysis was based on their 16S ribosomal DNA sequences. the 16S rRNA gene sequence fragments were amplified with the primer pair of 27F (5’-AGAGTTTGATCCTGGCTCAG-3’) and 1492R (5’-GGTTACCTTGTTACGACTT-3’). The PCR protocol consisted of the following steps: 5 min at 94°C for the first denaturation step, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 2 min, and ended with a final extension step at 72°C for 7 min. The PCR reaction mixture (20 μL) consisted of an appropriate amount of DNA template (10-100 ng), 1.0 μL of Taq DNA polymerase (Beijing Com Win Biotech Co. Ltd., China), 0.5 μL of 10.0 μM each primer, and 8.5 μL of ddH₂O. The reaction mixture without template DNA was used as a negative control. The PCR product was verified using agarose gel electrophoresis and purified using the QIA Quick purification kit. Pure PCR products were sequenced by TsingKe Biological Technology Co. Ltd., Zhengzhou, China.

Multiple sequence alignments were performed with ClustalW in MEGA 6, and phylogenetic trees were constructed from the evolutionary distance data calculated from Kimura's two-parameter model using the neighbor-joining method by MEGA 6. Bootstrap analyses were performed based on 1000 random resampling. Reference sequences were retrieved from GenBank with the accession numbers indicated in the trees.

**Selection of coculture system**

*B. coagulans* inoculum was cultured in proliferation medium at 37 °C and 180 rpm for 16 h. *C. butyricum* DL-1 inoculum was cultured at 37 °C stationarily for 16 h, with the shake flasks sealed with 8 layers of gauze and 2 layers of kraft paper. The co-fermentation condition was as follows: loading liquid
ratio was 40%, inoculation ratio of \textit{C. butyricum} DL-1 and \textit{B. coagulans} strains was 6% and 4%, respectively. The shake flask was sealed with 8 layers of gauze and 2 layers of kraft paper were cultured stationarily at 37 \textdegree C. After a 24 h culture, the viable counts and spore concentration were analyzed.

\textbf{Determination of total viable counts and spore yield of \textit{C. butyricum} DL-1}

After sequential tenfold dilution of cell suspensions of \textit{C. butyricum}, 100 \mu L of samples were spread on agar plates. The colonies formed after incubation at 37 \textdegree C for 16 h were counted and statistically analyzed. Viable counts were expressed as colony-forming units per milliliter (CFU/mL). Spore concentration was measured by the same method as that of viable counts except that the cell suspension was heated at 80 \textdegree C for 10 min in advance. Spore yield was calculated as the percentage of spore count to total viable cells of \textit{C. butyrium} DL-1. Three replicates were set for each dilution.

\textbf{Growth curve of strain \textit{C. butyricum} DL-1 and \textit{B. coagulans} ZC2-1}

Specific \textit{C. butyricum} DL-1 proliferation medium inoculated at a 4% inoculum size was cultured stationarily at 37 \textdegree C, with a 60% filling volume. \textit{OD}_{600} and pH of the culture broth were assayed every 4 h. Growth process of \textit{B. coagulans} ZC2-1 was monitored by the same protocol as that of \textit{C. butyricum} DL-1, except that the inoculated proliferation medium was cultured at 180 rpm, with a filling volume of 20%.

\textbf{Optimization of co-fermentation medium formula of strain \textit{C. butyricum} DL-1 and \textit{B. coagulans} ZC2-1}

Single-factor tests were used to study the effect of carbon source, nitrogen source, and inorganic salts on the viable counts and spore yield of \textit{C. butyricum} DL-1. The factors were studied successively, and the optimization results were used in subsequent experiment. Bran, corn starch, soluble starch, sucrose, lactose, maltose and glucose were used as alternative carbon source. Corn steep powder, soybean meal powder, peptone, beef extract, fishmeal, tryptone, yeast extract and yeast were used as alternative nitrogen source. NaCl, KCl, K$_2$HPO$_4$, CaCO$_3$, MnSO$_4$, MgSO$_4$, and Sodium acetate trihydrate \([C_2H_3NaO_2(H_2O)]\) were used as candidate inorganic salts. Viable counts and spore yield of \textit{C. butyricum} DL-1 were analyzed at regular intervals with the methods described above. Three parallel experiments were conducted for each experimental group.

Based on the results of single-factor tests, a L$_9$(3$^3$) orthogonal experiment was designed to optimize the concentration of carbon source and nitrogen source, and a L$_9$(3$^2$) orthogonal experiment was designed to study the effect of inorganic salts concentration on the viable counts and spore yield of \textit{C. butyricum} DL-1. The orthogonal experiment was designed by SPSS 20.0 software.

\textbf{Results}

\textbf{Isolation and Identification of \textit{B. coagulans} strains}
The strains were subjected to polyphasic taxonomic analyses based on their phenotypic characteristics and phylogenetic analysis (Fig. 1). Strain ZC2-1 form white opaque circular colonies with a white dot in the center (Fig. 1a), and its cells are short rod shape of 0.4-0.8 µm wide by 2.5-4.0 long (Fig. 1e). Strain ZA-1 and ZC-9 both from light milky white small round colonies with moist surface and viscous texture (Fig. 1b and Fig. 1d). There cells are of rod shape of 0.5-1.5 µm wide by 5.0–8.3 µm long (Fig. 1f) and 0.4–0.8 µm wide by 1.8–3.2 µm long (Fig. 1h), respectively. The colony of ZB-1 is white colony with irregular edge, rough surface, and viscous texture (Fig. 1c), and its cells are slender rods with the size of 0.4-0.8 µm wide by 1.8–3.2 µm long (Fig. 1g).

16S rRNA sequences of four *B. coagulans* ZC2-1, ZA-1, ZB-1, ZC-9 (shown in Supplementary Material) were deposited in GenBank with the accession numbers MW195020, MW504830, MW504831, MW504832. *B. coagulans* ZC2-1 is numbered 22951 in CGMCC.

The 16S rRNA sequence of ZC2-1 exhibited 99.93% identity with a *B. coagulans* strain (MT604689.1). The 16S rRNA sequence of ZA-1 revealed 100% identity with a *B. coagulans* strain (MT611810.1). The 16S rRNA sequence of ZB-1 showed 100% identity with a *B. coagulans* strain (MT611733.1). The 16S rRNA sequence of ZC-9 has 99.93% identity with a *B. coagulans* strain (MT626077.1). ZC2-1, ZA-1, ZB-1, ZC-9 was located in *B. coagulans* clade (Fig. 2), and their closest relative were *B. coagulans* strain KCCM203098 and *B. coagulans* strain E21.

**Establishment of coculture system**

The four *B. coagulans* strains ZC2-1, ZA-1, ZB-1, ZC-9 were co-fermented with *C. butyricum* DL-1, after 24 h culture, the *C. butyricum* spore concentration of four co-culture system were 5.5×10⁵, 4.5×10⁵, 4.8×10⁵, 5.1×10⁵ CFU/mL, respectively. The highest *C. butyricum* spore concentration was obtained in coculture system composed of *B. coagulans* ZC2-1 and *C. butyricum* DL-1. Therefore, this coculture system was studied further.

**The growth curve and pH curve of *C. butyricum* DL-1 and *B. coagulans* ZC2-1**

The lag period of *C. butyricum* DL-1 lasted only 4 h in the proliferation medium (Fig S1 in Supporting Information). Then the logarithmic phase began with the cell multiplying rapidly and pH dropping sharply. The stable period lasted from 12 h to 20 h, and fermentation process entered the decay period at 20 h with pH value increasing continuously.

*B. coagulans* ZC2-1 grew slowly in the proliferation medium in the lag period with pH decreasing slowly (Fig S2 in Supporting Information). From the 8th hour, the bacteria rapidly propagated into the logarithmic phase and reached the plateau at 16 h.

There is a markedly negative correlation between the changing trend of cell concentration and pH value. In the stable and decay period, bacterial growth was inhibited by low pH, low nutrients concentration and high harmful metabolites concentration caused by bacterial growth. The increasing pH value in the decay
period may be related to the autolysis of the bacteria cells. At late logarithmic phase, the bacterial cell concentration reached the highest, and the cells exhibited the highest viability and fertility in the same time. Therefore, the culture broth of *C. butyricum* DL-1 and *B. coagulans* ZC2-1 harvested after cultured for 16 h was used as inoculum for co-fermentation process.

**Optimization of mixed fermentation medium composition**

It was found that the concentration of *B. coagulans* cells in co-fermentation broth is far inferior to the culture result in purebred fermentation. Additionally, *B. coagulans* spore yield in co-fermentation process is almost negligible. Therefore, the concentration of *C. butyricum* viable bacteria and its spore yield was used as medium optimization criterion in this study.

**Effect of carbon source types on the viable counts and spore yield of *C. butyricum* DL-1**

When bran is used as carbon source, the number of viable bacteria and spores of *C. butyricum* in the culture broth reached the highest (Fig. 3). Besides provision carbon source, bran contains trace metal ions, various amino acids, vitamins and other grow factors which facilitate bacterial growth (Ritthibut et al. 2020). Bran served as much better fermentation performance than other carbon sources (Fig. 3), so it was used as the only carbon source in further study.

**Effect of nitrogen source sorts on the viable counts and spore yield of *C. butyricum***

When corn steep powder was used as nitrogen source, both the number of viable counts and spores of *C. butyricum* were the highest (Fig. S3), reaching $5.6 \times 10^7$ CFU/mL and $3.5 \times 10^7$ CFU/mL respectively. In order to further increase the viable counts and spore yield of *C. butyricum* DL-1, composite nitrogen source with more comprehensive nutrition was studied. The results are shown in Table 1:

The concentration of viable bacteria and spores of *C. butyricum* DL-1 of Group 2 was the highest (Table 1), reaching $7.1 \times 10^7$ CFU/mL and $5.8 \times 10^7$ CFU/mL respectively. The Group 1’s spore rate was the highest, but the number of viable counts and spore was lower than that of Group 2. With rich protein, amino acids, vitamins, minerals and trace grow factors, corn steep powder can provide comprehensive nutrient for the growth of microbes (Zeng et al. 2018). Peptone contains vitamins and other growth factors (Setiari et al. 2016). They are both good choices for bacterial nitrogen source.

Effect of the carbon and nitrogen source concentration on the viable counts and spore yield of *C. butyricum* DL-1.

A $L_9(3^3)$ orthogonal table (Table 2) was designed to optimize the concentration of carbon source and nitrogen source: bran (A), peptone (B) and corn steep powder (C) concentration were set as factors in this orthogonal test. The results were analyzed as shown in Table 3.

According to the range analysis results (Table 3), the three factors had the similar influence on the viable counts and spore yield of *C. butyricum* DL-1. The order of importance was corn steep powder
concentration>peptone concentration >bran concentration. The optimum contents of carbon source and nitrogen source were determined as 10 g/L bran, 15 g/L peptone, and 15 g/L corn steep powder. The verification results conducted under the optimal condition combination were as follows: the viable counts and spore yield of *C. butyricum* reached 8.8×10⁷ and 7.6×10⁷ CFU/mL, respectively. The results were better than all those shown in the orthogonal table, further verified the conclusion drawn by the orthogonal experiment.

**Effect of inorganic salts on the viable counts and spore yield of *C. butyricum* DL-1**

Inorganic salts play an important role in the growth of microorganisms, since many metal ions in them serve as cofactors of metabolic enzymes. It could be seen from Fig. 4 that K₂HPO₄ was the top factor in promoting *C. butyricum*’s growth and sporulation. Sodium acetate trihydrate, MnSO₄, and MgSO₄ also exhibited marked enhancement effect, so the combination of K₂HPO₄ and these the three inorganic salts was further studied. The results shown that the optimal inorganic salts combination for coculture of *C. butyricum* DL-1 and *B. coagulans* ZC2-1 was K₂HPO₄ and MnSO₄ (Table S1 in Supporting Information), with the viable counts and spores rate reaching 1.04×10⁸ CFU/mL and 91.3%, respectively. The concentration of K₂HPO₄ and MnSO₄ was optimized by a L₉(3²) orthogonal experiment (Table S2 and Table S3 in Supporting Information). With K₂HPO₄ (A) and MnSO₄ (B) content as factors.

According to the range analysis results (Table S3 in Supporting Information), K₂HPO₄ is the most important influence factor for the viable bacteria and spore rate of *C. butyricum* DL-1. The optimum inorganic salt combination was 1 g/L K₂HPO₄ and 0.5 g/L MnSO₄. Under this condition, the viable counts and spore rate of *C. butyricum* DL-1 reached 1.3×10⁸ CFU/mL and 92.3%, respectively.

**Fermentation result in the optimal medium**

Different from the growth curves in proliferation medium, *B. coagulans* ZC2-1 started growth earlier than *C. butyricum* DL-1. At 0-12 h, *B. coagulans* ZC2-1 used the nutrients in medium to multiply preferentially (Fig. 5), and consumed the dissolved oxygen in the medium in the same time, providing an anaerobic environment for *C. butyricum*. Consequently, *C. butyricum* DL-1 entered the logarithmic phase at 12 h after a long lag phase and began to spore at 20 h. At 36 h, the viable counts and spores yield of *C. butyricum* DL-1 both reached the peak value of 1.5×10⁸ CFU/mL and 1.4×10⁸ CFU/mL respectively. Therefore, 36 h was the high time to stop the mixed fermentation process.

**Discussion**

Although antibiotics has made significant contributions in improving human and animal health. Their abuse may lead to environmental antibiotic residues (L.Silvia Munoz-Price et al. 2016). Residual antibiotics could interfere natural beneficial gut flora and improve pathogenic bacterial antibiotic tolerance (Chen et al. 2019). It could also lead to the spread of antibiotic resistance genes (ARGs) in environment and cause serious health problems by changing human and animals' gut microbiota
structure (Ben et al. 2019; Duan et al. 2020). Therefore, the development of ecofriendly disease preventative approaches will be beneficial to the health management in animals farming (Alagawany et al. 2018). Compared with antibiotics, probiotic preparations are non-resistant, health and nutritious. Therefore, they are considered to be the most promising antibiotic substitutes (Ouwehand et al. 2016). Besides feed supplement, probiotics are also used in medicines and healthcare products. Probiotic preparation on the market includes bacteria, fungus and yeast. The commonly used probiotics are the strains of *B. subtilis*, *Lactobacillus* sp, *Bifidobacterium* sp, and *Streptococcus* sp. As an emerging spore forming probiotic, *C. butyricum* only has a small market share yet. So it is of great interest to research simple and cost-effective fermentation technology so as to increase its production and application.

Scientists have made great effort to improve the viable counts and spore yield of *C. butyricum* strains. Kong et al. (QING KONG 2004) increased *C. butyricum’s* viable counts by optimizing the medium composition. Li et al. (Li et al. 2020b) reported that pH value was a crucial factor for the spore formation of *C. butyricum*. Down-regulation pH value from 6.5 to 5.5 during the fermentation process promoted *C. butyricum’s* sporulation rate to 90%. For most *C. butyricum*, it is obligatory to provide anaerobic agents or nitrogen gas protection to create an anaerobic fermentation environment, which increase the production equipment investment and operation cost greatly. As an alternative, we established a coculture technology of *C. butyricum* DL-1 and *B. coagulans* ZC2-1. In the co-fermentation system, the facultative anaerobic *B. coagulans* strain consumes oxygen in the culture medium and provides anaerobic environment for the strict anaerobic *C. butyricum* strain.

Fermentation medium component such as carbon sources, nitrogen sources, inorganic salts and growth factors are important factors affecting microbial growth. Therefore, we optimized the culture medium composition of the co-fermentation technology so as to obtain high viable counts and spore yield of *C. butyricum* at low medium cost. Despite the differences among the medium compositions and their concentrations, the cost of reported media for *C. butyricum* cultivation are quite high (He et al. 2017; Li et al. 2019a). In this study, a low-cost medium formula for the co-fermentation of *C. butyricum* DL-1 and *B. coagulans* ZC2-1 was designed. Readily available inexpensive food industry byproducts were used as the main raw material so as to further decrease the cost.

The cost of our medium and the reported medium was compared in Table 4. As shown in Table 4, a 64.6% reduction in culture medium cost is achieved compared with Qing Kong’ medium.

Co-fermentation together with facultative anaerobic strains provide an effective solution for absolute anaerobic bacteria production. Microbial co-fermentation is widely used in the production of animal feed (da Silva Brito et al. 2020), protein (Jia et al. 2018), drugs (Pettit 2009), foods (Capece et al. 2018), biological control (Ma et al. 2020), and environmental management (Chen et al. 2019). B_{12} was produced by mixed fermentation of *Propionibacterium freudenreichii* and *L. brevis* (Xie et al. 2019); Dairy products (Kongo et al. 2006) and lactic acid (Zhang and Vadlani 2015) was produced by co-fermentation of *B. animalis* and *L. acidophilus*. Cong Y et al. (Cong et al. 2019) used the mixed fermentation broth of *R. nigricans* and *Trichoderma pseudokoningii* to control cucumber wilt, and proved that the combined
fermentation of the two strains has a synergistic effect on the control of *Fusarium oxysporum*. Based on the characteristics of *C. butyricum* and *B. coagulans*, a green and energy-saving co-fermentation process was established in this study. Without the need of anaerobic environment, *C. butyricum* co-fermentation process was simplified, and the production cost was reduced greatly.

In the mixed culture of multiple strains, the interaction among the strains should be explored based on their growth characteristics. Viable counts, spore transformation rate, fermentation period and other factors should be weighed in the establishment of fermentation process to obtain the maximum benefits. Furthermore, it is necessary to further optimization of the co-fermentation process of *C. butyricum* and *B. coagulans* so as to provide theoretical basis and technical guidance for the production of *C. butyricum*.

In this study, the effect of carbon source, nitrogen source and inorganic salts were explored. The co-fermentation medium of *C. butyricum* DL-1 and *B. coagulans* ZC2-1 was optimized as 10 g/L bran, 15 g/L corn steep powder, 15 g/L peptone, 1 g/L K$_2$HPO$_4$, and 0.5 g/L MnSO$_4$ at pH 7.0. Using the optimized medium formula, the concentration of viable bacteria and spore forming rate of *C. butyricum* DL-1 reached 1.5×10$^8$ CFU/mL and 92.6% after a 36 h static culture at 37 °C. Besides, economic assessment demonstrated the great potential of the medium for *C. butyricum* large-scale production. The mixed fermentation of microorganisms with different characteristics can solve some problems impossible for purebred fermentation. The co-fermentation process established in this study provides an effective alternative for the industrial production of other absolute anaerobic bacteria.

**Declarations**

**Ethics approval and consent to participate**: not applicable.

**Consent for publication**: Written informed consent for publication was obtained from all participants.

**Availability of data and materials**: All data generated or analysed during this study are included in this published article and its supplementary information files.

**Competing interests**: To the best of our knowledge, the named authors have no conflict of interest, financial or otherwise.

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**Author contributions**: Yonghong Li: Investigation, Validation, Methodology, Resources, Supervision. Yun Wang: Project administration, Writing-Original draft, Data curation. Yingying Liu: Prepared experiments and isolated strain, Project administration, Data curation, Writing-Reviewing and Editing. Xuan Li: Data curation, Writing-Reviewing and Editing. Keke Li and Lifei Feng: Writing-Reviewing and Editing.

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Tables

Table 1 The optimized results of compound nitrogen source
| Nitrogen source | *C. butyricum* viable counts ($\times 10^7$CFU/mL) | *C. butyricum* spores ($\times 10^7$CFU/mL) | Spore rate (%) |
|----------------|---------------------------------|---------------------------------|----------------|
| Group1         | 4.8                             | 4.1                             | 85.4           |
| Group2         | 7.1                             | 5.8                             | 81.7           |
| Group3         | 3.1                             | 2.4                             | 77.4           |
| Group4         | 4.0                             | 2.2                             | 55.0           |
| Group5         | 5.0                             | 1.7                             | 34.0           |
| Group6         | 1.8                             | 0.9                             | 50.0           |
| Group7         | 4.5                             | 2.1                             | 46.7           |
| Group8         | 4.2                             | 2.7                             | 64.3           |
| Group9         | 2.3                             | 1.3                             | 56.5           |
| contrast       | 5.6                             | 3.5                             | 62.5           |

Group1 to 9 indicate the combination of corn steep powder with soybean meal powder, peptone, beef paste, fishmeal, tryptone, yeast extract, yeast, NH$_4$Cl and (NH$_4$)$_2$SO$_4$ respectively. The ratio of corn steep powder to other nitrogen sources is 1:1, and the total nitrogen source content is 15 g/L. 15 g/L corn steep powder was used as control.

**Table 2** Factors and levels of orthogonal experiment design.

| Level | Factor          |
|-------|-----------------|
|       | A: Bran(g/L)    | B: Peptone(g/L) | C: Corn steep powder(g/L) |
| 1     | 10              | 5               | 5                           |
| 2     | 15              | 10              | 10                          |
| 3     | 20              | 15              | 15                          |

**Table 3** The orthogonal experiment design table ($L_9(3^3)$) and results analysis.
| Level | Bran (g/L) | Peptone (g/L) | Corn steep powder (g/L) | C. butyricum viable counts (×10^7 CFU/mL) | C. butyricum spores (×10^7 CFU/mL) |
|-------|------------|---------------|-------------------------|------------------------------------------|----------------------------------|
| 1     | A3         | B3            | C1                      | 5.8                                      | 4.4                              |
| 2     | A1         | B2            | C3                      | 8.5                                      | 7.4                              |
| 3     | A3         | B1            | C3                      | 6.1                                      | 5.2                              |
| 4     | A1         | B3            | C2                      | 7.4                                      | 5.7                              |
| 5     | A2         | B3            | C3                      | 8.2                                      | 6.1                              |
| 6     | A3         | B2            | C2                      | 5.6                                      | 3.9                              |
| 7     | A2         | B2            | C1                      | 6.5                                      | 3.5                              |
| 8     | A2         | B1            | C2                      | 3.1                                      | 2.7                              |
| 9     | A1         | B1            | C1                      | 5.8                                      | 3.5                              |
| K_{1d} | 7.23       | 5.00         | 6.03                    |                                          |                                  |
| K_{2d} | 5.93       | 6.87         | 5.37                    |                                          |                                  |
| K_{3d} | 5.83       | 7.13         | 7.60                    |                                          |                                  |
| R_D   | 1.40       | 2.13         | 2.23                    |                                          |                                  |
| K_{1e} | 4.10       | 4.93         | 4.10                    |                                          |                                  |
| K_{2e} | 4.50       | 5.40         | 6.23                    |                                          |                                  |
| K_{3e} | 1.43       | 1.60         | 2.43                    |                                          |                                  |

K value stands for the mean value, for example, K_{1d}, K_{2d} and K_{3d} correspond to A are the mean values of factor A, at level 1, level 2, and level 3, respectively, and so on. R stand for range value, correspond to the different between the highest k value and the lowest k value. English letter D and E in rang analysis results stand for the results of C. butyricum DL-1 viable counts and spore yields, respectively.

**Table 4** Cost comparison of our medium and reported medium
| Input               | Price (USD/Kg\(^a\)) | Reported Medium\(^Q\) (g/L\(^b\)) | (USD/L\(^c\)) | Newly designed medium (g/L) | (USD/L) |
|---------------------|-----------------------|-----------------------------------|----------------|--------------------------|---------|
| Glucose             | 249                   | 2.44                              | 0.608          | -                        | -       |
| Yeast extract       | 224                   | 2.08                              | 0.466          | -                        | -       |
| Tryptone            | 448                   | 1                                 | 0.045          | -                        | -       |
| (NH\(_4\))\(_2\)SO\(_4\) | 175                  | 0.1                               | 0.018          | -                        | -       |
| NaHCO\(_3\)        | 142                   | 0.1                               | 0.014          | -                        | -       |
| MnSO\(_4\)\(H\(_2\)O\) | 151                  | 0.02                              | 0.003          | 0.5                      | 0.0755  |
| MgSO\(_4\)\(7H\(_2\)O\) | 75                   | 0.02                              | 0.0015         | -                        | -       |
| CaCl\(_2\)         | 161                   | 0.002                             | 0.0003         | -                        | -       |
| Bran                | 0.255                 | -                                 | 10             | 0.00255                  |         |
| Corn steep powder   | 0.413                 | -                                 | 15             | 0.006195                 |         |
| Peptone             | 0.295                 | -                                 | 15             | 0.004425                 |         |
| K\(_2\)HPO\(_4\)   | 320                   | -                                 | 1              | 0.32                     |         |
| **Total (USD/L)**   | **1.1558**            | **0.40867**                       |                |                          |         |

\(^a\): Cost of each component was calculated based on Sigma-Aldrich prices (accessed on December 2020)

\(^b\): Amount of component (g) per liter of medium.

\(^c\): Cost of component per liter of medium.

\(Q\): Formula reported by Qing Kong et al

**Figures**
Figure 1

Colonial morphology and microscopic structure of the strain ZC2-1, ZA-1, ZB-1 and ZC-9. (a), (b), (c), (d) colonial morphology of ZC2-1, ZA-1, ZB-1 and ZC-9. (e), (f), (g), (h) microscopic structure of ZC2-1, ZA-1, ZB-1 and ZC-9 observed by microscope with 1000 folds amplification.

|        | Bacillus coagulans strain 2720 (MT611733.1) | Bacillus coagulans strain 1906 (MT626077.1) | Bacillus coagulans strain 2839 (MT611810.1) | Bacillus coagulans strain 2190 (MT604689.1) | Bacillus coagulans strain E21 (KX986311.1) | Bacillus coagulans strain KCCM203098 (MF992239.1) | ZC-9 (MW504832.1) | ZB-1 (MW504831.1) | ZC2-1 (MW195020.1) | ZA-1 (MW504830.1) | Bacillus licheniformis (LT669761.1) | Bacillus paralicheniformis strain KJ-16 (KY694465.1) | Bacillus subtilis strain H10-5-5 (EU882846.1) | Bacillus tequilensis strain Lmb056 (KT986121.1) | Bacillus amylotiquefaciens strain C3 (HQ668178.1) | Bacillus amylotiquefaciens strain HS8 (GU323369.1) | Bacillus amyloiquesfaciens strain SXA001 (KY271752.1) | Bacillus velezensis strain A2 (MG727659.1) | Bacillus licheniformis (HE586589.1) |
|--------|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|-----------------|-----------------|-----------------|-----------------|--------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|
| 100    |                                             |                                             |                                             |                                             |                                             |                                             |                 |                 |                 |                 |                                |                                              |                                              |                                             |                                             |                                              |                                             |                                             |                                              |                                             |                                              |
Figure 2
Phylogenetic trees constructed by the Neighbor-Joining approach. The GenBank accession numbers of the strains are shown in the parentheses.

Figure 3
The effect of carbon source types on the viable counts and spore yield of C. butyricum.
Figure 4

The effect of inorganic salts on C. butyricum's viable counts and spore yield. The concentration of NaCl and KCl was 5 g/L, the concentration of K2HPO4 and CaCO3 was 1 g/L, the concentration of MnSO4 and MgSO4 was 0.3 g/L, the concentration of Sodium acetate trihydrate was 3 g/L, with no inorganic salts added in control experiment.
Figure 5

Growth curve and pH curve of the two strains in optimized coculture medium.

Supplementary Files

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