Optimization of Carbon and Nitrogen Sources for Clostridium butyricum CBM01 and Its Gastrointestinal Tolerance: A Postprint

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Date: 2017-10-23T00:00:00+00:00

Abstract

This study aimed to investigate the optimal carbon and nitrogen sources of Clostridium butyricum CBM01 and its gastrointestinal tolerance. Using a controlled variable approach and based on Clostridium enrichment medium, the carbon and nitrogen sources of C. butyricum CBM01 were optimized, while its gastrointestinal tolerance was simultaneously evaluated. The results demonstrated that: 1) the optimal carbon and nitrogen sources for C. butyricum CBM01 were glucose and peptone, respectively; 2) after 3 h of exposure to artificial gastric juice and artificial intestinal juice, the survival rates were 90.33% and 92.09%, respectively; 3) the maximum tolerable bile salt concentration for C. butyricum CBM01 was 0.40%. These findings indicate that C. butyricum CBM01 exhibits excellent tolerance to the gastrointestinal environment and represents a probiotic strain with substantial development potential and broad application prospects.

Full Text

Optimization of Carbon and Nitrogen Sources for *Clostridium butyricum* CBM01 and Its Gastrointestinal Tolerance

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Abstract

This study aimed to investigate the optimal carbon and nitrogen sources for *Clostridium butyricum* CBM01 and evaluate its tolerance to gastrointestinal con-

ditions. Using a controlled variable approach with reinforced clostridial medium (RCM) as the basal medium, we optimized the carbon and nitrogen sources for *C. butyricum* CBM01 and examined its gastrointestinal tolerance. The results demonstrated that: (1) glucose and peptone were the optimal carbon and nitrogen sources, respectively; (2) after 3 hours of exposure to artificial gastric juice and artificial intestinal juice, the survival rates were 90.33% and 92.09%, respectively; and (3) the highest tolerable bile salt concentration was 0.40%. These findings indicate that *C. butyricum* CBM01 exhibits excellent tolerance to gastrointestinal environments, making it a promising probiotic strain with substantial development potential and broad application prospects.

Keywords: *Clostridium butyricum*; carbon source; nitrogen source; bile salt tolerance; artificial gastric juice; artificial intestinal juice

Introduction

Probiotics, also known as direct-fed microbials or microbial ecological agents, are live microorganisms that confer health benefits to the host when administered in adequate amounts. As purely biological products, probiotics are characterized by being green, residue-free, and non-antimicrobial resistance-inducing. When used as feed additives, probiotics can reduce anti-nutritional factors in feed and improve feed utilization efficiency. Upon ingestion by animals, they regulate intestinal microecological balance, enhance immune function, and improve animal production performance, thereby generating substantial economic benefits for producers.

Clostridium butyricum, also known as Miyairi bacteria or butyric acid bacteria, is a Gram-positive bacterium belonging to the phylum Firmicutes, class Clostridia, order Clostridiales, family Clostridiaceae, and genus *Clostridium*. It is commonly found in the intestines of healthy humans and animals. Previous studies have demonstrated the safety of *C. butyricum* through acute oral toxicity tests and Ames tests, and its ability to form spores confers strong resistance to harsh conditions, enabling survival during feed processing and passage through the acidic gastrointestinal environment. Research has shown that *C. butyricum* exhibits excellent intestinal adhesion properties and can antagonize pathogenic bacteria such as *Vibrio anguillarum* through competitive exclusion while producing various beneficial metabolites that enhance host immune function. These characteristics align with the selection criteria for probiotics regarding safety, functional properties, and technical feasibility.

Liu et al. reported that *C. butyricum* CBM01 could improve broiler production performance, enhance antioxidant capacity, and modulate intestinal flora structure, yet its biological characteristics remain unclear. Therefore, this study optimized the carbon and nitrogen sources for *C. butyricum* CBM01 and investigated its gastrointestinal tolerance to gain deeper insights into this strain and provide a theoretical foundation for its production and application as a

probiotic.

Materials and Methods

1.1.1 Bacterial Strain *Clostridium butyricum* CBM01 was provided by the Animal Microecology Laboratory, College of Veterinary Medicine, Sichuan Agricultural University.

1.1.2 Reagents and Culture Media Artificial gastric juice and artificial intestinal juice were prepared according to the 2015 edition of the Chinese Pharmacopoeia. Porcine bile salts were obtained from Chengdu Kelong Chemical Reagent Factory. $FeSO_4 \cdot 7H_2O$ and $CuSO_4 \cdot 5H_2O$ were purchased from Chengdu Kelong Chemical Reagent Factory, while $MnSO_4$ was purchased from Chongqing Jiulong Chemical Reagent Factory.

Reinforced Clostridial Medium (RCM): Beef extract 10 g, glucose 5 g, yeast powder 10 g, sodium chloride 5 g, sodium acetate 3 g, tryptone 10 g, soluble starch 1 g, L-cysteine hydrochloride 0.15 g, agar 17 g, distilled water 1,000 mL, pH 7.0, sterilized at 1$×10^{5}$ Pa for 30 min.

Basal Medium: Glucose 5 g, potassium dihydrogen phosphate 6 g, dipotassium hydrogen phosphate 4 g, ammonium sulfate 2 g, magnesium sulfate 0.2 g, sodium citrate 1 g, distilled water 1,000 mL, pH 7.2–7.4, sterilized at 1$×10^{5}$ Pa for 30 min.

Starch Medium: Peptone 10 g, sodium chloride 5 g, beef extract 5 g, soluble starch 2 g, agar 17 g, distilled water 1,000 mL, pH 7.2–7.4, sterilized at 1$×10^{5}$ Pa for 30 min.

Beef Extract Peptone Medium: Beef extract 3 g, peptone 10 g, sodium chloride 5 g, agar 17 g, distilled water 1,000 mL, pH 7.2–7.4, sterilized at 1$×10^{5}$ Pa for 30 min.

1.2 Equipment Autoclave (Hirayama, HVE-50), electronic balance (Sartorius, BS-224S), anaerobic incubator (Thermo Scientific, 1029), UV spectrophotometer (Thermo Fisher Scientific, BioMate-3S).

1.3 Experimental Procedures

1.3.1 Activation of *C. butyricum* CBM01 Two days before strain activation, mixed gas (CO_2, H_2, N_2) was introduced into the anaerobic incubator to establish strict anaerobic conditions. The lyophilized stock culture was diluted with physiological saline and inoculated onto solid RCM plates inside the anaerobic incubator. After two successive passages, the strain was inoculated into liquid RCM for enrichment. The activated strain was aliquoted, sealed with paraffin, and stored at 4 °C for subsequent experiments.

1.3.2 Growth Curve Determination Activated *C. butyricum* CBM01 was inoculated into liquid RCM at 1% inoculum and incubated anaerobically at 36 °C (5% CO_2, 10% H_2, 85% N_2) for 48 h. The optical density at 600 nm (OD_{600}) was measured every 4 hours using three replicate tubes to construct the growth curve.

1.3.3 Growth on Different Media Activated *C. butyricum* CBM01 was inoculated onto basal medium, RCM, starch medium, and beef extract peptone medium, followed by anaerobic incubation at 36 °C for 24 h. The growth performance was then evaluated.

1.3.4 Carbon Source Optimization Various carbon sources (lactose, fructose, starch, sucrose, glucose, and maltose) at 2% concentration were used to replace the carbon sources (glucose and soluble starch) in RCM. After inoculation with *C. butyricum* CBM01 and anaerobic incubation at 36 °C for 24 h, bacterial counts were determined using a hemocytometer.

1.3.5 Nitrogen Source Optimization Various nitrogen sources (beef extract, peptone, urea, ammonium sulfate, and sodium nitrate) at 2% concentration were used to replace the nitrogen sources (beef extract and tryptone) in RCM. Following inoculation with *C. butyricum* CBM01 and anaerobic incubation at 36 °C for 24 h, bacterial counts were performed using a hemocytometer.

1.3.6 Bile Salt Tolerance Test *C. butyricum* CBM01 was inoculated at 1% into liquid RCM containing 0.30%, 0.40%, and 0.50% porcine bile salts, with three replicates per concentration. After 24 h of anaerobic incubation, growth was assessed.

1.3.7 Gastrointestinal Tolerance Test *C. butyricum* CBM01 cultured for 48 h was inoculated into artificial gastric juice and artificial intestinal juice, followed by anaerobic incubation at 36 °C. Viable cell counts were determined hourly for 3 h using three tubes per time point. The average values were used to calculate survival rates.

Results

2.1 Growth Curve of *C. butyricum* CBM01 The growth curve was constructed with culture time as the x-axis and OD_{600} values as the y-axis. As shown in [Figure 1: see original paper], the lag phase lasted approximately 4 h, after which the strain entered the logarithmic growth phase with exponential bacterial proliferation and linear increase in OD_{600} values. After 12 h, the culture entered the stationary phase, reaching maximum viable cell density.

2.2 Growth Performance on Different Media Using hemocytometer counts and colony diameter measurements as indicators, we compared the growth of *C. butyricum* CBM01 on four different media (). The results revealed that *C. butyricum* CBM01 could not grow on basal medium or beef extract peptone medium but grew well on both RCM and starch medium, with superior growth on RCM. Therefore, RCM was selected for subsequent experiments.

TABLE:1 Effects of different media on growth of *Clostridium butyricum* CBM01

Medium	Growth Status	Cell Concentration/Colony Diameter
Reinforced clostridial medium	+	3.6$×10^{8}$ cells/mL
Starch medium	+	(0.18$±$0.04) cm
Basal medium	-	0
Beef extract peptone medium	-	0

(+ indicates growth; - indicates no growth. Same as TABLE:2.)

2.3 Determination of Optimal Carbon Source As illustrated in [Figure 2: see original paper], *C. butyricum* CBM01 could utilize all six carbon sources tested: starch, sucrose, lactose, fructose, glucose, and maltose. Notably, glucose yielded the highest bacterial cell count.

2.4 Determination of Optimal Nitrogen Source [Figure 3: see original paper] demonstrates that among organic nitrogen sources, peptone supported the best growth, followed by beef extract. Among inorganic nitrogen sources, ammonium sulfate was superior to sodium nitrate. When urea was used as the nitrogen source, the total bacterial count was 1.12$×10^{8}$ cells/mL.

2.5 Bile Salt Tolerance of *C. butyricum* CBM01 As shown in TABLE:2, *C. butyricum* CBM01 could grow in medium containing 0.30% bile salts and also in medium with 0.40% bile salts, but failed to grow at 0.50% bile salt concentration. These results indicate that *C. butyricum* CBM01 possesses good bile salt tolerance, highlighting its excellent application potential.

TABLE:2 Assessment of bile tolerance of *Clostridium butyricum* CBM01 to different bile concentrations

Item	Bile Salt Concentration	Growth Status
	0.30%	+
	0.40%	+
	0.50%	-

2.6 Tolerance to Artificial Gastric and Intestinal Juice Using artificial gastric and intestinal juices prepared according to Chinese Pharmacopoeia standards to simulate human gastrointestinal conditions, we evaluated the tolerance of *C. butyricum* CBM01. As shown in [Figure 4: see original paper], survival rates gradually decreased over time. After 3 h of exposure, the survival rates in artificial gastric juice and artificial intestinal juice were 90.33% and 92.09%, respectively.

C. butyricum has been widely used as a livestock feed additive in Japan and South Korea. As a strict anaerobe, *C. butyricum* is relatively difficult to cultivate, and research in China started relatively late due to technology and equipment limitations. However, as a spore-forming probiotic bacterium, it offers excellent development prospects and application value, attracting widespread attention and research. Numerous studies have confirmed its beneficial probiotic effects, leading to its approval as a new feed additive in China in 2009, with specified application scopes defined in the 2013 Feed Additive Catalogue.

Discussion

3.1 Growth Curve Construction The growth curve of *C. butyricum* CBM01 was constructed by measuring OD_{600} values ([Figure 1: see original paper]). The strain exhibited a 4 h lag phase, indicating rapid environmental adaptation, followed by a logarithmic growth phase from 4–12 h. After 12 h, the culture entered the stationary phase with peak bacterial counts. The growth curve pattern was similar to that reported for *C. butyricum* JMY1. The inability of *C. butyricum* CBM01 to grow on basal medium and beef extract peptone medium may be attributed to the lack of essential carbon/nitrogen sources or growth factors required for proliferation.

3.2 Screening of Optimal Carbon and Nitrogen Sources Successful microbial research requires the isolation and continuous cultivation of pure bacterial cultures. This study employed a single-factor multivariate approach to identify the optimal carbon and nitrogen sources for *C. butyricum* CBM01. Glucose as the carbon source and peptone as the nitrogen source yielded the highest bacterial counts. Considering cost-effectiveness, glucose is the most economical carbon source, while peptone, though more expensive, provided the best growth performance. Therefore, glucose and peptone were determined as the optimal carbon and nitrogen sources, respectively. The optimal carbon source aligns with findings by Qi et al. but differs from those of Song et al., while the optimal nitrogen source is consistent with Zhao' s report but differs from Jiang et al.' s optimization results for *C. butyricum* C.L24. Such discrepancies may be attributed to strain-specific characteristics.

3.3 Tolerance to Bile Salts and Artificial Gastrointestinal Fluids With the increasing drawbacks of antibiotic use as feed additives, countries worldwide

have enacted legislation to restrict or ban their application in animal feed. Consequently, probiotics have emerged as ideal antibiotic alternatives. Effective probiotics must possess strong acid and bile salt tolerance to maintain viable cell numbers in the digestive tract. *C. butyricum* CBM01 demonstrated superior bile salt tolerance compared to *C. butyricum* ZJU-F1, capable of growing in medium containing 0.40% bile salts. Since intestinal bile salt concentrations typically do not exceed 0.30%, this suggests that *C. butyricum* CBM01 can rapidly colonize and exert beneficial effects in the intestine. Additionally, the strain showed good tolerance to artificial gastric and intestinal juices, likely attributable to spore formation during the later growth stages. These results demonstrate that *C. butyricum* CBM01 possesses excellent biological characteristics suitable for further development as a microecological preparation or biological feed additive.

Conclusion

The optimal carbon and nitrogen sources for *C. butyricum* CBM01 are glucose and peptone, respectively. The strain exhibits good tolerance to bile salts and artificial gastric/intestinal fluids, enabling prolonged survival in gastrointestinal environments. In summary, *C. butyricum* CBM01 demonstrates excellent biological characteristics and represents an ideal potential probiotic strain.

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Note: Figure translations are in progress. See original paper for figures.