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Full Length Research Paper

Determination of an economical medium for growth of *Clostridium butyricum* TK2 using orthogonal test

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Clostridium butyricum **is a widely utilized probiotics, also used as an alternative to antibiotics for humans and growth promotion in a wide variety of livestock species. The objective of this study is to develop an economical and practical fermentation medium for the growth of *C. butyricum* TK2 using orthogonal test. The optimal fermentation medium was obtained by the single factor and the orthogonal test of carbon source, nitrogen source and growth factors design. The fermentation medium was estimated to be the most economical formula (per liter): 8 g glucose, 20 g soybean meal (hydrolyzed for 3 h by neutral protease), 5 g brewer's yeast powder. After incubating for 48 h in the optimum fermentation medium, the populations of *C. butyricum* TK2 were estimated to be 8 × 10^8 CFU ml^{-1}, while the cost was 90% lower than ever. The optimized medium is not only more economical but also good for the growth of *C. butyricum* TK2. The low cost medium developed in this study can be used for large-scale commercial application where economics are quite likely to be important.**

Key words: *Clostridium butyricum*, soybean meal, brewer's yeast powder, medium optimization.

INTRODUCTION

Probiotics can improve the balance of intestinal bacterial ecosystem of the host (Audisio et al., 2001). Probiotics can prevent and treat diarrhea in weanling animals and is an effective alternative to antibiotics for growth promotion in a diverse number of livestock species. In addition, it is reported that various immune responses are influenced by probiotics and these immunomodulatory effects have been proposed for several potential applications, including management of hypersensitivity reactions, prevention of infectious diarrhea and tumour suppression (Kirjavainen et al., 1999). In recent years, probiotic microorganisms have been increasingly included in various types of food products, especially in fermented milk products (Zhou et al., 2000; Zubillaga et al., 2001). *Clostridium butyricum* is a butyric acid bacterium which is found in soil and intestines of healthy animals and humans. It can stimulate polyclonal mucosal immune activity and shows adjuvant activity for anti-chlera toxin (Murayama et al., 1995) . *C. butyricum* is effective for both the treatment and the prophylaxis of AAD (antibiotic-associated diarrhea) in children, as it normalizes the intestinal flora disturbed by antibiotics (Seki et al., 2003).

Widespread use of *C. butyricum* can bring great benefits not only for animals but also for humans. Therefore, it would be useful to develop methods to ensure that large quantities of *C. butyricum* can be produced effectively and inexpensively. The optimization of the culture medium is one of the most important steps in the development of an economical production process that produces a quality probiotic product. Many factors such as carbon and nitrogen sources, inorganic salts and growth factors are important variables affecting the growth of microbe. Currently, there are several media to cultivate *C. butyricum* (Hassiba and Marczak, 2000; He et al., 2004; Papanikolaou et al., 2000; Wang et al., 2002; Zigova et al., 1999). In spite of the differences among the medium compositions and concentrations, the cost of these mediums is high. Therefore, the objective of this study was to use inexpensive and industrial-grade materials to develop a medium of *C. butyricum* for potential industrial scale.

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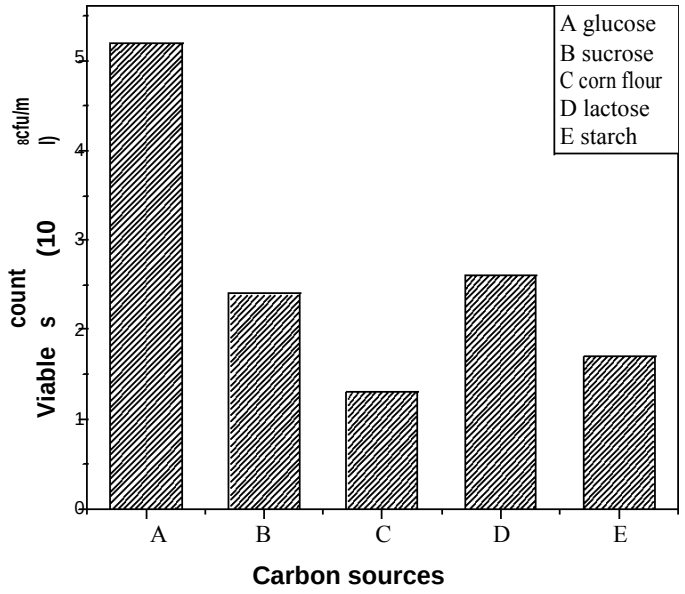

Figure 1. Effect of carbon sources on the growth of *Clostridium butyricum* TK2.

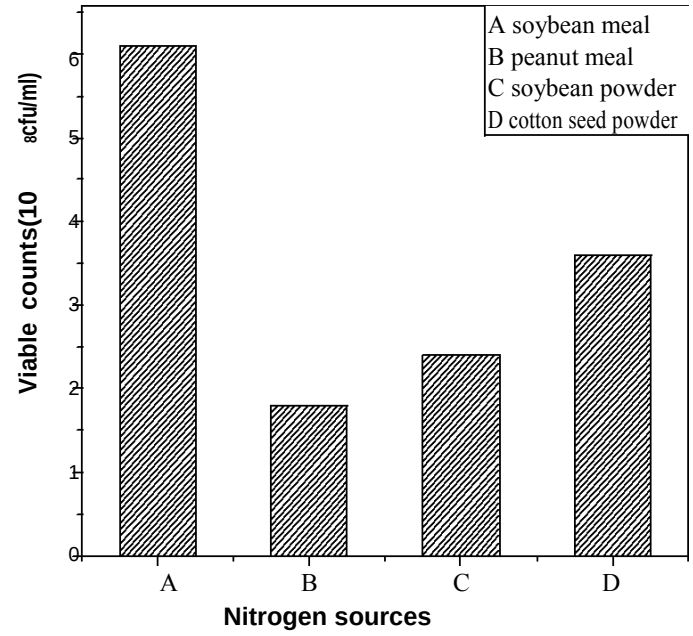

Figure 2. Effect of nitrogen sources on the growth of *C. butyricum* TK2

MATERIALS AND METHODS

Microorganism and methods

C. butyricum TK2 used in this study was originally isolated from the soil and preserved in our laboratory. The medium for inoculum preparation was CM (Wang et al., 2002) that contained the following ingredients (per liter): 24.4 g glucose, 10 g tryptone, 20.8 g yeast extract, 1 g $(NH_4)_2SO_4$, 1 g $NaHCO_3$, 0.2 g $MnSO_4 \cdot H_2O$, 0.2 g $MgSO_4 \cdot 7H_2O$, 1 g $CaCO_3$, 20 g agar (if necessary). The pH level, adjusted to 7.4. A_{650} was adopted to reflect the biomass density qualitatively using spectrophotometer at 37°C.

Fermentation studies were carried out in 100 ml anaerobic bottles containing 90 ml fermentation medium. All trials were performed in triplicate. Each bottle was inoculated with 5% of seed culture and incubated anaerobically at 37°C for 48 h.

The number of viable cells was determined using serial 10-fold dilution in sterile phosphate-buffered saline and 0.1 ml aliquots were injected in anaerobic tubes (containing CW agar, 50°C and rotated evenly in ice water immediately (the roll tube method). Anaerobic tubes (capped with black butyl rubber) were incubated anaerobically at 37°C for 48 to 72 h and the colony-forming units were estimated (He et al., 2004).

RESULTS AND DISCUSSION

Effect of carbon sources on the growth of *C. butyricum* TK2

Based on the original medium, 10 g/L glucose, sucrose, corn flour, lactose and starch were added as carbon sources to study the effects of different carbon source on the impact of *C. butyricum* TK2. The result was shown in Figure 1. Based on Figure 1, glucose had the most obvious effect on cell growth. The maximum number of viable cells was up to 5.2×10^8 CFU ml^{-1}, much higher than other carbon sources. So glucose was selected as the carbon source.

Effect of nitrogen sources on the growth of *C. butyricum* TK2

Taking into account the traditional nitrogen sources, such as beef extract, yeast extract, peptone and other kinds of material, although they had high nitrogen content, but the price were high, not conductive to large-scale production. In this study, we used four low-cost nitrogen sources: Soybean meal (residues of organic solvent extraction soybean oil), peanut meal, soybean powder (soybean oil extracted by crushing soybean residue) and cotton seed powder, which were hydrolyzed for 3 h by neutral protease. 20 g/L nitrogen sources were added to study the effects of different nitrogen source on the impact of *C. butyricum* TK2. The result is as shown in Figure 2. When the medium was supplemented with soybean meal, the number of viable cells was high, reaching 6.1×10^8 CFU ml^{-1}. Cotton seed powder was next. Peanut meal and soybean meal were less effective, which had the minimum number of viable cells. So soybean meal was chosen as the nitrogen source.

Effect of hydrolyzing time on the growth of *C. butyricum* TK2

We changed the hydrolysis time of soybean meal to

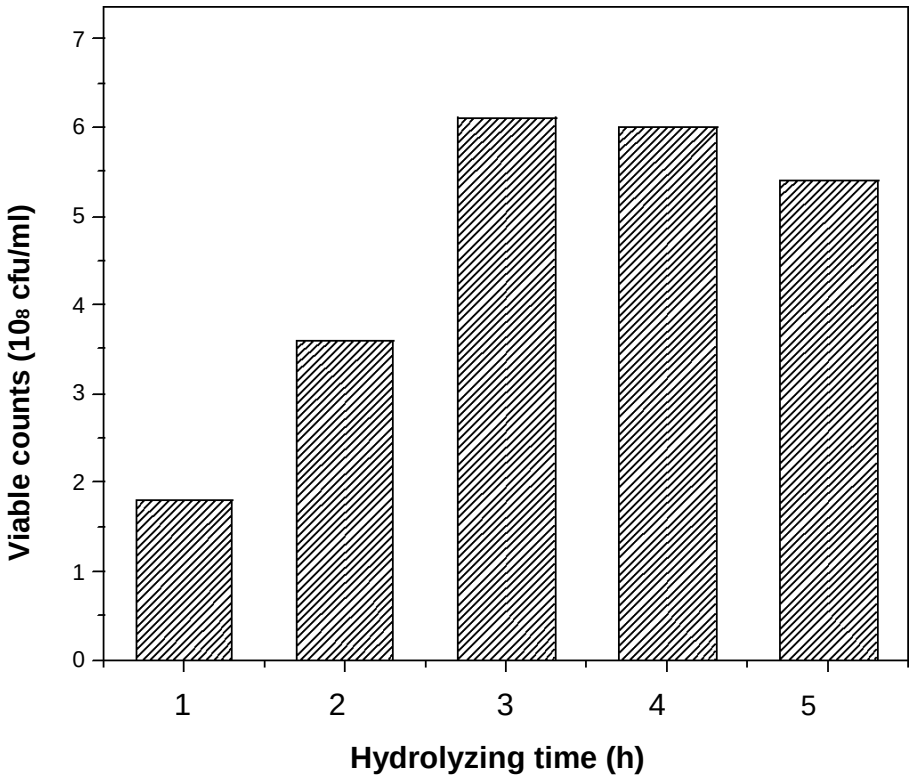

Figure 3. Effect of hydrolyzing time on the growth of *C. butyricum* TK2.

examine its impact on the number of viable cells. Figure 3 showed that the hydrolysis time had great impact on the number of viable cells. With hydrolysis time extending, the number of viable cells also increased. When hydrolysis time arrived at 3 h, the number of viable cells reached 6.1×10^8 CFU ml^{-1}. At present, *C. butyricum* was cultured with the main nitrogen sources, such as yeast extract, beef extract and peptone. In our study, soybean meal, peanut cake powder, soybean powder and cotton seed powder were chose as nitrogen source. Although the capacity of *C. butyricum* TK2 using these four substances was less than traditional nitrogen sources, soybean meal was hydrolyzed for 3 h by neutral protease and its efficiency can be greatly improved. There are rich and cheap soybean resources in China. It is useful for large-scale production of *C. butyricum.*

Effect of growth factors on the growth of *C. butyricum*

Figure 4 showed that the effect of brewer's yeast powder was better than the other growth factors. The number of viable cells reached 7.2×10^8 CFU ml^{-1}. Considering production costs, the price of brewer's yeast powder was far less than yeast extract and it was rich in vitamins, amino acids and other active factors, which was beneficial to the growth of *C. butyricum* TK2. Therefore, brewer's yeast powder was used as growth factor.

Optimization analysis of orthogonal results

The results from Tables 1 and 2 showed the hydrolysis time was the most significant factor. The fermentation medium was the most economical formula (per liter): 8 g glucose, 20 g soybean meal (hydrolyzed for 3 h by neutral protease), 5 g brewer's yeast powder. After incubating 48 h in the optimum fermentation medium, the populations of *C. butyricum* TK2 were estimated to be 8×10^8 CFU ml^{-1}.

Conclusions

In the present study, the viable count of *C. butyricum* TK2 could reach 8×10^8 CFU ml^{-1} in the optimized fermentation medium. The optimized fermentation medium was composed of (per liter) 8 g glucose, 20 g soybean meal (hydrolyzed for 3 h by neutral protease), 5 g brewer's yeast powder. The nitrogen sources and growth factors are industrial-grade materials, which are inexpensive and easily obtained in China. It will decrease the cost of fermentation medium significantly. In summary, the optimized medium is not only more economical but also good for the growth of *C. butyricum* TK2. Therefore, it

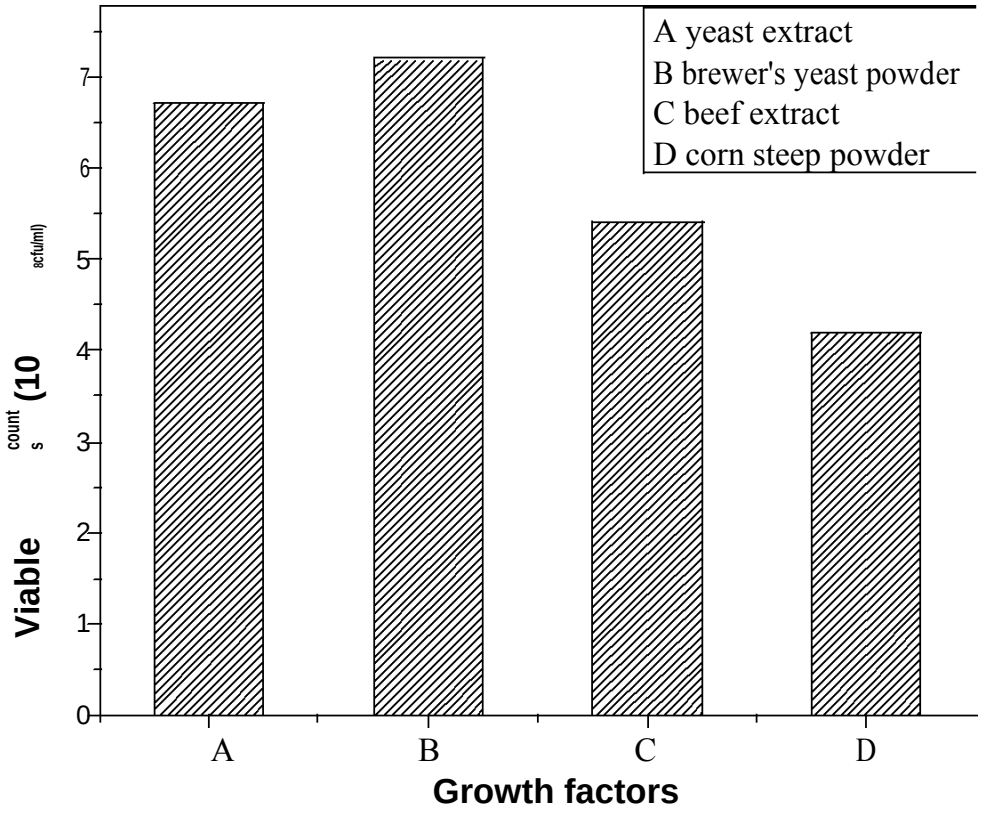

Figure 4. Effect of growth factors on the growth of *C. butyricum* TK2.

Table 1. Optimization analysis of orthogonal results.

Factor	Soybean meal (g/L)	Hydrolyzing time (h)	Brewer's yeast powder (g/L)	Viable count (10^8 ml^{-1})
1	10	1	1	4.2
2	10	3	3	6.5
3	10	5	5	5.2
4	20	1	3	5.4
5	20	3	5	8.0
6	20	5	1	5.6
7	30	1	5	4.4
8	30	3	1	6.2
9	30	5	3	6.7
Mean 1	5.300	4.667	5.333	
Mean 2	6.100	6.667	6.200	
Mean 3	5.767	5.833	5.633	
Range	0.800	2.000	0.867	

Table 2. Variance analysis of orthogonal results.

Factor	Squared deviation	Degree of freedom	Variance	F
Soybean meal	0.969	2	0.834	19.000
Hydrolyzing time	6.056	2	5.212	19.000
Brewer's yeast powder	1.162	2	1.000	19.000
Error	1.16	2		

it could be useful in large-scale application.

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