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Fermentation Products and Carbon Balance of Spoilage *Bacillus cereus*

JINN-CHYI WANG[1]* AND HSI-HUA WANG[2]

[1.] *Department of Food Sanitation, Tajen Institute of Technology, 20 Wei-Shin Rd., Shin-Erh Village,Yen-Pu Township, Pingtung County, Taiwan*

[2.] *Department of Agricultural Chemistry, National Taiwan University, 1, Sec 4, Roosevelt Rd., Taipei County, Taiwan*

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ABSTRACT

Products of fermentation, especially volatile compounds, and the carbon balance of a spoilage microorganism usually provide useful information to understand the fermentation characteristics of the organism and as a basis for determination of contaminants. In this study, the cultivation of spoilage *Bacillus cereus* at 35˚C in a culture medium with glucose as the sole carbon source was performed in order to measure fermentation products including headspace gases, and overall carbon balance during fermentation. The organism reached their stationary phase after 20 h growth. Under anaerobic fermentation, the recovery ratio of carbon was higher than the corresponding aerobic one. The major fermentation products were 2,3-butanediol and ethanol under anaerobic fermentation, and it's 2,3-butanediol under anaerobic fermentation. The calculation of the balance of oxidation and reduction reaction yielded a theoretical value nearly equal to 1 during anaerobic fermentation. The results of this study can support to develop a system for rapid detection of headspace gases of major fermentation products specific to food spoilage by using a gas biosensor.

Key words: *Bacillus cereus*, carbon balance, volatile compounds

INTRODUCTION

Bacillus cereus is a gram-positive, spore forming, and catalase positive bacterium. That is a cause of food poisoning(1). This organism requires glucose and some amino acids for growth and consequently to form organic acids, alcohols, carbonyls compounds and carbon dioxide(2). The optimal growth temperatures for the group of *Bacillus* spp. fall in the range of 30˚C~40˚C, with only some psychrophilic species(3,4). Previous studies for *Bacillus cereus* have provided information pertaining to factors such as growth and general aspects of sporulation(5-7), effects of intoxication(8), deterioration, physiological properties, fermentation and genetics(9,10).

In general, the recovery of carbon during microbial growth is seldom complete, with perhaps only one to two percent of carbon of substrate being converted into specific constituents of the cell(11). The balance of oxidation-reduction processes during bacterial cell division is used to evaluate the level of biological energy conservation. A small-scale cultivation of the bacterium might be able to make a simple calculation of the carbon balance and avoid the loss of proliferation by-products. As a consequence of such a study, the chemical state of the oxidation-reduction of substrates and products could be determined from the overall carbon balance.

Glucose was used as the only carbon source for the fermentation of *B. cereus* in this study to explore the metabolic characteristics of the bacterium and the production of its final products including volatile metabolites. The detection, by biosensors, of the presence of such volatile metabolites as evidence of *B. cereus* bacterial activity could be applied to detect whether food had been spoiled as a result of microbial activity.

MATERIALS AND METHODS

I. *Medium and Culture Conditions*

Bacillus cereus (CCRC 14655, isolated from canned soybean milk) was grown in nutrient broth (Difco) at 35˚C for 24 h. The bacterial cells were collected by centrifugation at 4000 ×g and 4˚C. The pellet was washed with Tris-buffer (pH 7.0) three times, followed by one washing with sterile saline. The bacterial cells (10^2 cells/mL) were inoculated into 50 mL GA broth (0.55% glucose, 0.5% $NH_4H_2PO_4$, 0.02% $MgSO_4 \cdot 7\ H_2O$, pH 7.0) in a 100 mL specially designed serum flask (with a side tube of protruding fringe of 1.5 cm, 1 cm ID for sampling the fermentation products). Aerobic culture conditions were defined as follows. The mouth of the flask was stopped with a cotton plug, while a small side tube was sealed with a silicon pad along with an aluminum cap. For anaerobic culture, the cotton plug used in aerobic culture was substituted with a silicon stopper. Before culturing, the flask for carrying the medium was flushed with pure nitrogen gas (Shin An Gas Co., Taiwan) through a sterile syringe filter (0.45 μm, Gelman) at a flow rate of 30 mL/mL for 10 minutes to replace the inside air completely. The culture of microorganisms was conducted at 35˚C.

* Author for correspondence. Tel & Fax: 08-7620651; E-mail:jicy.wang@msa.hinet.net

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II. *Fermentation Data*

The number of cells was determined in triplicate at the interval of three hours during fermentation for the samples obtained from aerobic and anaerobic culture using aerobic plate counting (APC) with standard dilution method. The broth was centrifuged at 4000 ×g for five minutes, and the supernatant was removed and filtered using a 0.22 μm syringe filter (Gelman). The filtrate was collected for the analysis of soluble nitrogen using Nessler's method[12] and for the analysis of reducing sugar. The content of reducing sugar was determined using a color reaction with 2,3-dinitrosalicylic acid (DNS) and the wavelength for measuring sugar content was set at 575 nm[13].

III. *Analysis of Fermentation Products*

The carbon dioxide in the culture medium was measured by injecting 50%(v/v) sulfuric acid from the side tube of the culturing flask for acidifying the sample to a pH of 2. To ensure that all of the CO_2 was liberated from the medium and removed from the headspace gas, we used a gas-tight syringe to transfer the air in flask to a pora-pak Q column (60-80 mesh, Supelco) and analyzed by a thermal conductivity detector[14]. After the medium was cultivated for 20 h, the fermented broth was centrifuged (4000 ×g, 5 min). The collected supernatant was then filtered through a 0.22 μm syringe filter and subjected to gas chromatographic analysis. The detection of headspace gases was performed using a modified technique originally reported by Mattila & Auvien[15] and Matiella & Hsieh[16].

The fermented broth was preheated for 20 min in a water bath at 50˚C, then purged with aseptic N_2 to remove dissolved gas from the broth. The headspace gases were then trapped onto a Tenax TA tube (60~80 mesh, Supelco) via the side tube of the culturing flask, and subsequently isolated by heating the Tenax TA tube from room temperature to 185˚C in a desorption chamber, followed by purging with helium through the tube, while conducting into a GC column. The fermentation products were analyzed from 2 μL of the broth. The Shimatsu 9A gas chromatograph was equipped with a 60 m length, 0.53 mm ID. 0.5 μm film thickness, Nukol phase fused silica capillary column (Supelco, Inc.). The operating conditions were: injection port 190˚C, oven temperature programmed from 35˚C to 190˚C at 7˚C/min with helium as the carrier gas (50 mL/min) and a flame ionization detector (Shimatsu). The quantitative analysis of individual compounds and the analytes of headspace gases and fermentation broth was based on standard curve and peak area measurements. Peak identification was made by comparison of retention times with that of authentic compounds (carbon dioxide, hydrogen were purchased from Supelco Inc.; acetoin was purchased from Sigma Co.; lactic acid, acetic acid, succinic acid, formic acid, glycerol, ethanol, and 2,3-butanediol were purchased from Merck Co.). A known amount of 2-heptanol was added to the sample as an internal standard. All of the analytes were conducted in duplicates, and the Duncan's multiple range test was used to determine significant differences ($p<0.05$).

RESULTS AND DISCUSSION

I. *The Growth Curve of Bacillus cereus*

Determination of the growth curve and the changes of products during cultivation usually provide a basis for determining the relationship between the production of fermentation products and change of growth under certain conditions, and establish a growth model[17]. The growth curve of *Bacillus cereus* for cultivation in GA medium is indicated in Figure 1. At the sixth hour of the fermentation, growth of the bacterium reached the logarithmic phase; and after 20 h, the culture reached the stationary phase. The soluble nitrogen compounds increased after 20 h, but the changes of reducing sugar tended to stabilize gradually. The pH value increased during fermentation, which came along with the increase of carbon dioxide. This indicated that the growth of the bacteria had become steady after 20 h. *Bacillus cereus* manifested a different metabolic status in its growth, by comparison with the other *Bacillus* spp. summarized by Gerhard[18], Mignone & Rossa[19] and Spira & Silverman[20].

II. *Fermentation Products of Bacillus cereus*

The results of the present experiment indicated that the sole carbon source for *Bacillus cereus* in GA medium was glucose. In this study, the bacterial activities were found to be broadly comparable between aerobic and anaerobic fermentation, except for the formation of acetic acid found only in aerobic condition and some variation occurred in the proportion and quantity of by-products (Table 1).

The loss of carbon dioxide from the gaseous phase of the culture medium was not measured during aerobic fermentation, while the analysis of carbon dioxide from the broth itself was conducted by acidifying the medium after 20 hours of cultivation as described above. The major products under aerobic conditions were 2,3-butanediol and carbon dioxide, and the minor products were acetic acid, lactic acid, glycerol, ethanol, acetoin, formic acid and succinic acid.

Under the anaerobic fermentation condition the major product were essentially ethanol and 2,3-butanediol, with the following minor products: lactic acid, glycerol, formic acid, succinic acid and acetoin. Under aerobic fermentation conditions, the production of acetoin was about five times the rate of that under anaerobic conditions. The product ratios of major compounds in aerobic fermentation to anaerobic fermentation were 1.42 for 2, 3-butanediol, 0.64 for lactic acid, and 0.5 for H_2. Previous studies have suggested that the glucose metabolism of *Bacillus cereus* and *Bacillus anthracis* involved butanediol-fermentation under anaerobic growth conditions. The major product of such fermentation was 2,3-butanediol, and the minor products were acetic acid, ethanol, glycerol, lactic acid, carbon dioxide, formic acid and succinic acid[21,22].

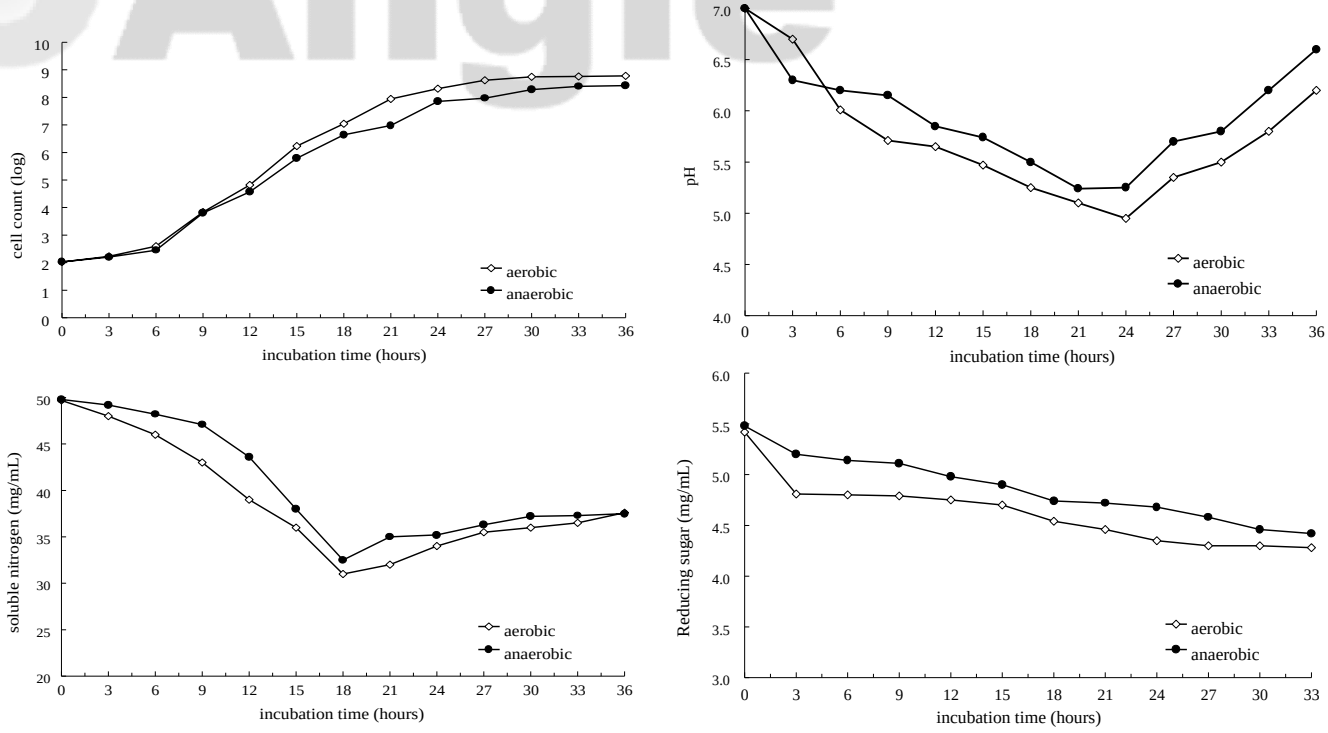

Figure 1. Growth curve and change of components for *Bacillus cereus* cultivated in GA medium. The incoulum was 10^2 cells/mL. The serum flask had a volume of 110 mL, stuffed with a cotton plug for aerobic conditions, and was displaced by a silicon stopper on the mouth of the flask for anaerobic conditions. The broth was purged with aseptic nitrogen for two minutes before inoculation.

Table 1. Fermentation products of *Bacillus cereus* and their yield, cultivated under aerobic and anaerobic conditions[a]

	Yield (mmol)	
Products	Aerobic[b]	Anaerobic
Carbon dioxide	3.82	2.87
Hydrogen	0.02	0.04
Acetoin	0.15	0.026
Acetic acid	0.86	—
2,3-Butanediol	1.63	1.15
Ethanol	0.16	1.16
Formic acid	0.12	0.45
Glycerol	0.47	0.67
Lactic acid	0.52	0.81
Succinic acid	0.05	0.36

[a] The culture method is as described in Figure 1.

[b] The loss of carbon dioxide in air was not considered during aerobic culture.

When the pH was held at 5.5 within the fermenting broth under aerobic conditions for a period of 20 h in the present experiment, *B. cereus* produced 2,3-butanediol as a major product in addition to the other above-cited compounds. The yield of 2,3-butanediol was about 2 times of acetic acid. The pH was less than 6.3, however, the acetic acid product was further transformed into acetoin and 2,3-butanediol.

Under aerobic conditions, the production of 2, 3-butanediol and carbon dioxide were about 50% higher, and acetoin about 5 times higher than that under anaerobic conditions, while the production of ethanol, lactic acid, glycerol, formic acid, and succinic acid was conspicuously lower. The production of 2,3-butanediol was corresponding to about 40% and 28% of the sugar consumed by the microorganisms in aerobic and anaerobic fermentation, respectively. The yields of the major end products (carbon dioxide and 2,3-butanediol) were corresponding to 70% of the total products obtained under aerobic conditions and 64% of anaerobic conditions.

The Enterobacteriaceae, such as *Escherichia*, *Salmonella* and *Shigella*, could decompose hexose into ethanol, succinic acid, 2,3-butanediol, lactic acid, acetic acid, formic acid and carbon dioxide[23]. Although these bacteria are gram-negative, non-spore formers, their associated fermentation pathways are similar to those of *Bacillus cereus*.

The carbon balance for the fermentation pathway of *Bacillus cereus* is referred to in Tables 2 (aerobic condition) and 3 (anaerobic condition). The recovery ratio of carbon was 98.31% for anaerobic conditions, and under aerobic conditions, the recovery ratio was 98.60%. Although the ratio of oxidation-reduction for fermentation products was not calculated for the aerobic fermentation (since the levels of carbon dioxide and oxygen could not be measured accurately), the ratio of oxidation-reduction of the anaerobic fermentation products was close to the theoretical value of unity (Table 3). Therefore the present results pertaining to the fermentation balance and oxidation-reduction ratio appear valid. Furthermore, the establishment of the complete data of fermentation balance for the main spoilage microorganisms in packaged food and the identification of the spoiled product such as 2,3-butanediol detected in the present study should

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Table 2. Fermentation balance of *Bacillus cereus* under aerobic condition[a]

Products	mmol / 100 mmol of glucose fermented	mmol of C1	O/R number[b]	Milliequivalents of oxidation / reduction	
Hydrogen	0.72	—	-1		0.72
Carbon dioxide	138.9	138.9	2	277.8	
Acetoin	5.45	21.8	-2		10.9
Acetic acid	31.27	62.54	0		
2,3-Butanediol	59.27	237.08	-3		177.81
Ethanol	5.85	11.7	-2		11.7
Formic acid	4.36	4.36	1	4.36	
Glycerol	17.08	51.24	-1		17.08
Lactic acid	18.91	56.73	0		
Succinic acid	1.82	7.28	1	1.82	
		591.63		283.98	218.21
% of C recovery = 98.60%					

[a] The culture method is as described in Figure 1.

[b] The number was obtained by comparing the number of oxygen and hydrogen atoms in the molecule, assuming that each oxygen is numerically equal to +1 and two hydrogens together are equal to –1[(23)].

Table 3. Fermentation balance of *Bacillus cereus* under anaerobic condition[a]

Products	mmol / 100 mmol of glucose fermented	mmol of C1	O/R number[b]	Milliequivalents of oxidation / reduction	
Hydrogen	1.45	—	-1	208.7	1.45
Carbon dioxide	104.35	104.35	2	16.32	1.9
Acetoin	0.95	3.8	-2	13.09	125.43
2,3-Butanediol	41.81	167.24	-3		84.36
Ethanol	42.18	84.36	-2		24.36
Formic acid	16.32	16.32	1		
Glycerol	24.36	73.08	-1		
Lactic acid	29.45	88.35	0		
Succinic acid	13.09	52.36	1		
		589.86		238.11	237.5
% of C recovery = 98.31%					

[a] The culture method is as described in Figure 1.

[b] The number was obtained by comparing the number of oxygen and hydrogen atoms in the molecule, assuming that each oxygen is numerically equal to +1 and two hydrogens together are equal to –1[(23)].

aid in rapidly determining the spoilage status, including the type of contaminated microorganisms and extent of spoilage.

From the presence of volatile fermentation products (Table 1), as detected by the use of a gas biosensor, it seems that the rapid detection of the spoilage of package food due to the action of *Bacillus cereus* is clearly feasible.

REFERENCES

1. Luby, S., Jones, J., Dowda, H., Kramer, J. and Horan, J. 1993. A large outbreak of gastroenteritis caused by diarrheal toxin-producing *Bacillus cereus*. J. Infect Dis. 167: 1452-1455.
2. Boone, D. R. 2001. Bergey's Manual of Systematic Bacteriology. Vol.1. 2nd. ed. p.75. Springer-Verelag, Inc. N. Y., USA.
3. Finlay, W. J., Logan, N. A. and Sutherland, A. D. 2000. *Bacillus cereus* produces most emetic toxin at lower temperatures. Letters Appl. Microbiol. 31: 385-389.
4. Jaquette, C. B. and Beuchat, L. R. 1998. Combined effected of pH, nisin and temperature on growth and survival of psychrotrophic *Bacillus cereus*. J. Food Prot. 61: 563-570.
5. Anderson, A., Granum, P. E. and Ronner, U. 1998. The adhesion of *Bacillus cereus* spores to epithelial cells might be an additional virulence mechanism. Int. J. Food Microbiol. 39: 93-99.
6. Mazas, M., Lopez, M., Gonzalez, I., Bernardo, A. and Martin, R. 1997. Effects of sporulation pH on the heat resistance and the sporulation of *Bacillus cereus*. Letters Appl. Microbiol. 25: 331-334.
7. Raso, J., Barbosa, G. C. and Swanson, B. G. 1998. Sporulation temperature affects initiation of germination and inactivation by high hydrostatic pressure of *Bacillus cereus*. J. Appli. Microbiol. 85: 17-24.
8. Granum, P. E. and Lund, T. 1997. *Bacillus cereus* and its food poisoning toxin. FEMS Microbiol. Letters 157: 223-228.
9. Asano, S. I., Nukumizu, Y., Bando, H., Iizuka, T. and Yamamoto, T. 1997. Cloning of novel exterotoxin genes from *Bacillus cereus* and *Bacillus thuringiensis*. Appl. and Environ. Microbiol. 63: 1054-1057.

10. Helgason, E., Caugant, D. A., Lecadet, M. M., Chen, Y., Mahillon, J., Lovgren, A., Hegna, I., Kvaloy, K. and Kolsto, A. B. 1998. Genetic diversity of *Bacillus cereus* / *Bacillus thuringiensis* isolates from natural sources. Current Microbiol. 37: 80-87.
11. Brock, T. D. and Madigan, M. T. 1988. Biology of Microorganisms. 9th ed. pp. 602-603. Prentice Hall Int. Inc. New Jersey, USA.
12. Williams, S. 1984. Official Methods of Analysis. 14th ed. Method, 6.025. Association of Official Analysis Chemists. Washington D. C., USA.
13. Fevre, M. 1984. ATP and GTP stimulate membrane bound but not dignition-solubilization β-(1→3)glucan synthetases from *Saprolegia monoica*. J. Gen. Microbiol. 130: 3279-3284.
14. Thomas, B. and Douglas, O. M. 1981. Cellulose fermentation by a rumen anaerobic fungus in both the absence and the presence of rumen methanogenes. Appli. and Environ. Microbiol. 42: 1103-1110.
15. Mattila, T. and Auvinen, M. 1990. Headspace indicators monitoring the growth of *Bacillus cereus* and *Clostridium perfringens* in aseptically packed meat soup (part I). Lebensen. Wiss. U. Technol. 23: 7-13.
16. Matiella, J. E. and Hsieh, T. G. Y. 1990. Analysis of crab-meat volatile compounds. J. Food Sci. 55: 962-966.
17. Chorin, E., Thuault, D., Cleret, T. T. and Bourgeosis, C.M. 1997. Modelling *Bacillus cereus* growth. Int. J. Microbiol. 38: 229-233.
18. Gerhard, G. 1986. Bacterial Metabolism. 2nd ed. pp.167-169. Springer-Verelag, Inc. N. Y., USA.
19. Mignone, C. F. and Rossa, C. A. 1996. Analysis of glucose carbon fluxes in continuous cultures of *Bacillus thuringiensis*. Appl. Microbiol. Biotechnol. 46: 78-84.
20. Spira, W. M. and Silverman, G. J. 1979. Effect of glucose, pH, and dissolved-oxygen tension on *Bacillus cereus* growth and permeability factor production in batch culture. Appl. and Environ. Microbiol. 37 : 109-116.
21. Prescott, L. M., Harley, J. P. and Klein, D. A. 1990. Microbiology. pp. 154~157. Wm. C. Brown. Publ. Inc. USA.
22. Volk, W. A. and Brown, J. C. 1997. Basic Microbiology. 8th ed. pp. 108-113. Addison-Wesley Educational Publ. Inc.USA.
23. Caldwell, D. R. 1995. Microbial Physiology and Metabolism. pp. 133-140. Wm. C. Brown Publ. Inc. USA.

腐敗性仙人掌桿菌之發酵產物及碳平衡

王進琦[1]*　王西華[2]

[1] 大仁技術學院　食品衛生系　屏東縣鹽埔鄉新圍村維新二路20號
[2] 台灣大學　農業化學系　台北市羅斯福路四段一號

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摘　要

探討腐敗細菌於特定基質內所產生的發酵產物（尤其是揮發性化合物）及其碳平衡，有助於了解該細菌的發酵特性及做為鑑定污染源之基礎。本實驗以腐敗性仙人掌桿菌（*Bacillus cereus*）恆溫培養在含葡萄糖做為唯一碳源之培養基中，檢測發酵產物，並計算發酵平衡。由菌體之生長曲線發現，仙人掌桿菌在生長20小時後即進入穩定期；而在好氣狀態下之生長速率高於厭氣態。在厭氣態發酵時，碳的回收率較好氣態者高，其主要產物為2,3-butanediol及ethanol；而好氣態者為2,3-butanediol。計算氧化還原反應之平衡關係顯示，厭氣態發酵之氧化還原比值較接近理論值的1，這些結果將可提供發展使用氣體生物感測器檢測腐敗食品時，針對產生於上部空間之揮發性發酵產物，進行快速檢測之依據。

關鍵詞：仙人掌桿菌，碳平衡，揮發性發酵產物