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Effect of pH, Temperature, and added Sucrose on the Production of Vitamin B_{12} and Riboflavin by *Bacillus megaterium* and *Enterobacter aerogenes*

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온도, pH 및 첨가된 Sucrose가 *Bacillus megaterium*과 *Enterobacter aerogenes*에 의한 비타민 B_{12}와 Riboflavin 생산에 미치는 영향

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Optimal pH, temperature and sucrose content for the production of vitamin B_{12} and riboflavin by *Bacillus megaterium* and *Enterobacter aerogenes* was studied by microbiological analysis. Optimal pH for the production of B_{12} was 6.0 by *B. megaterium* while the pH for *E. aerogenes* was 5.0. However, upon the addition of sucrose the optimal pH for *B. megaterium* shifted to 7.5 but *E. aerogenes* remained at pH 5.0. In the absence of sucrose, pH did not influence the yields of riboflavin produced by either bacterium. Addition of sucrose stimulated synthesis of riboflavin by both bacteria. Temperature had little effect on the production of vitamins by either bacterium.

According to Cromwell *et al.* (1), corn does not provide adequate amounts of vitamin B_{12} or riboflavin for humans. Vitamin B_{12} is widely disseminated in nature but higher animals cannot synthesize it. Therefore, it must be obtained directly from bacterial sources (2). Previous studies in our laboratory showed that vitamin B_{12} and riboflavin could be increased in cornmeal by a natural indigenous microflora fermentation (3-4).

Vaughn (5) listed *Enterobacter aerogenes* and *Bacillus megaterium* as essential to the primary stages of fermentating cabbage, olives, and cucumbers. Both bacteria are found in the soil, and both are known to produce vitamin B_{12} and riboflavin (6-10).

Because *B. megaterium* and *E. aerogenes* are part of the soil microflora present on plant material when it undergoes natural fermentation by indigenous microflora, and factors might be adjusted prior to fermentation to increase the yield of vitamins, this study was instituted. Nutrient broth was selected because it is a simple proteinaceous medium that can simulate a food waste material that might be used to produce vitamins. Also, it can be used to determine the optimal pH, temperature, and sucrose content to be used in fermentations of cereal grains such as corn. The effects of the two bacteria on the production of both vitamin was studied together as well as singly.

Materials and Methods

Microorganisms

A strain of *Enterobacter aerogenes,* isolated by Dyer (10), was used in this study since it had been shown to produce both vitamin B_{12} and riboflavin. Also, a strain of *Bacillus megaterium* ATCC 13639, known to produce vitamin B_{12}, was obtained

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from The American Type Culture Collection.

Both stock cultures were maintained on nutrient agar (BBL, Cockeysville, MD) and held at 4 °C until needed. The inoculum for each vitamin was prepared by subculturing into 10 ml of nutrient broth, from the stock culture of each bacterium. After 24 hr at 30 °C, the cells were centrifuged and the broth was decanted. Cells were resuspended in 10ml of sterile 0.85% NaCl solution. The inocula of *B. megaterium* ATCC 13639 and *E. aerogenes* ranged, respectively, from 6.3×10^5 to 1.5×10^6 and from 1.6×10^8 to 3.1×10^8 per ml. Standard curves for each bacterium were prepared by plotting the absorbance at 600 nm vs number of cells. The number of cells of each bacterium was determined by plating with nutrient agar.

Vitamin determination

Vitamin B_{12} was determined with *Lactobacillus leichmanii* ATCC 7830 and assay medium of Difco. The assay of vitamin B_{12} was essential that of the Association of Vitamin Chemists (11) with the exception that the extraction was according to Bell (12).

Riboflavin was analyzed by the procedure of the Association of Vitamin Chemists (11). *Lactobacillus casei* ATCC 7469 and Difco assay medium for riboflavin were used.

Effect of pH

The optimal beginning pH for the production of vitamin B_{12} and riboflavin was determined in nutrient broth. Four g of nutrient broth dried medium was dissolved in 500 ml of distilled water. Fifty ml of the broth was transferred to a 125-ml Erlenmeyer flask where the pH was adjusted to 5.0, 6.0, 6.5, 7.0, 7.5, and 8.0 using 1 N HCl or 1 N NaOH. Media were autoclaved for 15 min at 121 °C. The flasks were cooled and inoculated. Flasks were shaken at 100 rpm on a gyratory water bath shaker (Model G76, New Brunswick Scientific Co., Inc., New Brunswick, NJ) for 72 hr at 30 °C.

Effect of added sucrose

The effect of added sucrose (5%, w/v) also was used to measure the effect on fermentation of single and mixed-cultures of *B. megaterium* ATCC 13639 and *E. aerogenes.*

Results and Discussion

The optimal beginning pH for the production of vitamin B_{12} by *B. megaterium* ATCC 13639 was 6.0 in nutrient broth whereas it was 5.0 for *E. aerogenes* (Table 1). However, when sucrose was added to the nutrient broth, the optimal beginning pH for the production of vitamin B_{12} by *B. megaterium* ATCC 13639 was 7.5 (a difference of 1.5 pH units). With *E. aerogenes,* the optimal beginning pH for vitamin B_{12} was 5, just as it was without added sucrose (Table 2).

After inoculation with either species in nutrient broth, the pH rose to 8.3 or higher indicating the production of basic compounds, probably ammonia and/or ammonia salts (Table 3). On the

Table 1. Effect of the beginning pH on the production of vitamin B_{12} and riboflavin by *B. megaterium* ATCC 13639 and *E. aerogenes* in nutrient broth[1,2]

pH	*B. megaterium* Vitamin B_{12} ng/ml	*B. megaterium* Riboflavin μg/ml	*E. aerogenes* Vitamin B_{12} ng/ml	*E. aerogenes* Riboflavin μg/ml
5.0	3.21 ± 0.7b	0.31 ± 0.0a	1.95 ± 0.1a	0.59 ± 0.1b
6.0	3.93 ± 1.3a	0.29 ± 0.0b	0.96 ± 0.1b	0.63 ± 0.1a
6.5	3.08 ± 0.6b	0.28 ± 0.0b	0.94 ± 0.1bc	0.57 ± 0.1bc
7.0	2.71 ± 0.5b	0.28 ± 0.0b	0.86 ± 0.1cd	0.57 ± 0.2bc
7.5	2.10 ± 0.6c	0.28 ± 0.0b	0.82 ± 0.0d	0.55 ± 0.1c
8.0	1.76 ± 0.5c	0.27 ± 0.0b	0.60 ± 0.1e	0.56 ± 0.2c

[1]N = 3. Where letters differ within a column, means differ $P < 0.05$ from each other.
[2]Incubated for 3 days at 30°C.

Fig. 2. Effect of sucrose (5%) on the optimal pH for the production of vitamin B_{12} and riboflavin by *B. megaterium* and *E. aerogenes* in nutrient broth[1,2].

pH	*B. megaterium* Vitamin B_{12} ng/ml	*B. megaterium* Riboflavin μg/ml	*E. aerogenes* Vitamin B_{12} ng/ml	*E. aerogenes* Riboflavin μg/ml
5.0	0.81 ± 0.3e	0.37 ± 0.1e	4.88 ± 0.4a	2.54 ± 0.2a
6.0	4.38 ± 0.4c	0.57 ± 0.1b	4.03 ± 0.8b	2.32 ± 0.3b
6.5	5.11 ± 0.9b	0.63 ± 0.1a	3.40 ± 0.9c	2.25 ± 0.2bc
7.0	5.45 ± 0.7ab	0.59 ± 0.1b	3.10 ± 0.6c	2.15 ± 0.2c
7.5	5.97 ± 0.9a	0.53 ± 0.1c	2.94 ± 0.5cd	2.21 ± 0.1bc
8.0	2.83 ± 0.4d	0.43 ± 0.1d	2.18 ± 0.2d	2.16 ± 0.1c

[1]N = 3. Where letters differ within a column, means differ P< 0.05 from each other.
[2]Incubated for 3 days at 30°C.

Fig. 3. Effect of incubation[1] of *B. megaterium* ATCC 13639 and *E. aerogenes* on pH of nutrient broth with and without added sucrose (5%)[2].

Bacterium	pH Before incubation	pH After incubation Without sucrose	pH After incubation With sucrose
B. megaterium	5.00	8.30	4.30
ATCC 13639	6.00	8.50	5.30
	6.50	8.55	5.35
	7.00	8.60	5.45
	7.50	8.70	5.60
	8.00	8.75	5.80
E. aerogenes	5.00	8.45	4.80
	6.00	8.60	4.85
	6.50	8.65	4.85
	7.00	8.70	5.00
	7.50	8.75	5.05
	8.00	8.80	5.15

[1]Incubated at 30°C for 3 days.
[2]N = 3.

other hand, with sucrose added to the nutrient broth, both species produced acid which lowered the pH. With *B. megaterium* ATCC 13639, the pH dropped to 4.3 to 5.8 depending on the initial pH. During the three day fermentation, *E. aerogenes* also caused the pH to fall within a range of 4.8 to 5.15 (Table 3). Both pH and sucrose made a significant ($P < 0.05$) difference in the amount of both vitamins (Tables 1 and 2).

Temperature influenced only the production of riboflavin by *B. megaterium* and vitamin B_{12} by *E. aerogenes*. Riboflavin production was less ($P < 0.05$) at 35°C than at 25°C or 30° with *B. megaterium* ATCC 13639, and amount of vitamin B_{12} was less ($P < 0.05$) when *E. aerogenes* was incubated at 35°C than when incubated at 25°C and 30°C (Table 4).

When mixed cultures of *E. aerogenes* and *B. megaterium* ATCC 13639 were studied, the optimal beginning pH was used unless the manufacturer's

Table 4. Effect of incubation temperature on the production of vitamin B_{12} and riboflavin by *B. megaterium* ATCC 13639 and by *E. aerogenes* in nutrient broth[1].

Temperature °C	*B. megaterium*[2] Vitamin B_{12} ng/ml	*B. megaterium*[2] Riboflavin μg/ml	*E. aerogenes*[3] Vitamin B_{12} ng/ml	*E. aerogenes*[3] Riboflavin μg/ml
25	3.86 ± 0.1a	0.31 ± 0.0a	1.77 ± 0.1a	0.50 ± 0.0a
30	3.93 ± 1.3a	0.31 ± 0.0a	2.11 ± 0.4a	0.63 ± 0.1a
35	2.81 ± 0.1a	0.19 ± 0.0b	0.96 ± 0.1b	0.41 ± 0.0a

[1]N = 3. Where letters differ within a column, means differ P< 0.05 from each other.
[2]Incubated at pH 6.0 for 3 days.
[3]Incubated at pH 5.0 for 3 days.

Table 5. Effect of sucrose (5%) on the production of vitamin B_{12} and riboflavin by pure and mixed culture of *B. megaterium* ATCC 13639 and *E. aerogenes* in nutrient broth[1].

Vitamin	Microorganism *B. megaterium*[2]	*E. aerogenes*[2]	Mixed culture[3]
Vitamin B_{12} (ng/ml)			
Without sucrose	8.20 ± 0.4a	1.79 ± 0.1b	3.02 ± 0.1b
With sucrose	8.37 ± 1.0a	9.01 ± 0.9a	9.40 ± 0.7a
Starting pH	7.5	5.0	6.8
Riboflavin (ug/ml)			
Without sucrose	0.42 ± 0.0b	0.20 ± 0.0b	0.50 ± 0.0b
With sucrose	0.84 ± 0.1a	3.01 ± 0.1a	3.19 ± 0.1a
Starting pH	6.5	5.0	6.8

[1]N = 3. Where letters differ within a column and vitamin, means differ $P<0.05$ from each other.
[2]Incubated at 30°C at the optimal pH for 3 days.
[3]Inoculated at 1:1 ratio of bacteria.

original pH was 6.8. A trend of increased ($P<0.05$) vitamin production with added sucrose occurred except for vitamin B_{12} produced by *B. megaterium* ATCC 13639 (Table 5). An explanation of this anomaly is not known.

요 약

B. megaterium ATCC 13639와 *E. aerogenges* 의 비타민 B_{12} 생산을 위한 최적 pH는 각각 6.0과 5.0으로 pH의 영향을 크게 받았으나 두 세균에 의한 riboflavin 생산은 pH에 따른 변화가 거의 없었다.

Sucrose를 첨가하면 두 비타민 생산량이 크게 증가되었고 최적 pH도 변함을 알 수 있었다. 두 세균에 의한 두 비타민 생산에 미치는 온도의 영향은 pH에 비하여 아주 적었다.

이상의 결론은 장차 여러가지 식품 폐기물을 이용한 비타민 B_{12}와 riboflavin의 생산이 가능함을 보여 주었다.

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