Kor. J. Appl. Microbiol. Bioeng.
Vol. 16, No. 5, 352-357 (1988)

The Production of Folic Acid by Microorganisms Isolated from Fermenting Corn Meal

Fu Gen Yoa,[1] Marion L. Fields,[1*] and Hee J. Chung[2]

[1]*Department of Food Science and Nutrition, University of Missouri-Columbia, 122 Eckles Hall, Columbia, MO 65211, USA*
[2]*Department of Food Science and Technology, Chonnam National University, Kwangju 500-757, Korea*

옥수수 가루 발효 과정에서 분리한 미생물에 의한 Folic Acid의 생산

Fu Gen Yoa[1]·Marion L. Fields[1]*·정희종[2]

[1]미주리주립대학교 식품가공학과 [2]전남대학교 식품공학과

Twenty-five out of 35 strains isolated from fermented corn meal produced folic acid. *Bacillus licheniformis* strain 6 and *Enterobacter cloacae* strain 18 produced the largest quantities of 1830 ± 271 ng and 1350 ± 167 ng per 100 ml of the assay broth, respectively. *B. licheniformis* produced maximum yields when initial pH values were 6, 7, or 8 and were incubated at 35 °C for 5 days. The initial pH (range 4-8) had no effect on folic acid production by *E. cloacae;* 35 °C for 5 days was optimal for this bacterium. Added carbohydrates had no effect on the production of total folic acid in the bacterial cells in pure or mixed cultures. However, in their growth media, carbohydrates enhanced the production of free and total folic acid by *E. cloacae* and in the mixed cultures. Added carbohydrates had no significant ($P < 0.05$) effect on the production of free and total folic acid by *B. licheniformis.*

The lack of folacin is one of the most common dietary deficiencies encountered in clinical practice. Megaloblastic anemia, caused by folacin deficiency, is a major world health problem, especially in pregnant women and patients with malabsorption disorders (5).

In a study of B-vitamins in natural lactic acid fermentation of corn meal, murdock and Fields (6) found that after lactic acid fermentation, the folic acid content in corn meal doubled or tripled as compared with non-fermented corn meal. Thus, natural lactic acid fermentation may be an inexpensive and simple method that can be performed at home or industrially. In this study, 35 strains originally isolated by Dyer (4) were used to study folic acid production. Primary objectives were to determine the effects of incubation time, temperature and initial pH on folic acid production in nutrient broth. We also determined the effects of carbohydrates on the production of free and total folic acid.

Materials and Methods

Survey of microorganisms

Thirty-five cultures isolated by Dyer (4) were used: *Hansenula subpelliculose* (1 strain), *Streptococcus faecalis* (9 strains), *Moraxella* sp. (1 strain), *Pseudomonas* sp. (1 strain), *Bacillus licheniformis* (3 strains), *Enterobacter cloacae* (3 strains), *Agrobacterium* sp. (4 strains), and *Lactobacillus brevis* (13 strains). The strains were maintained at 4 °C on slants of Bacto Plate Count Agar and Stabs of Bacto-Lactobacilli Agar. Fresh cultures were prepared

Key words: Folic acid production, *Bacillus licheniformis, Enterobacter cloacae*
*Corresponding author

monthly.

Preparation of inocula

Inocula for folic acid production were prepared by subculturing into Tryptic Soy Broth (TSB) or Lactobacilli Broth (LB). After 24 h incubation at 35 °C, the cells were removed by centrifugation. The cells were resuspended in 10 m*l* of sterile 0.9% NaCl solution and washed 5 additional times. Finally, the cells were resuspended in 10 m*l* of sterile 0.9% NaCl. Eighty percent transmission of the bacterial cells at 600 nm on a Sectronic-20 was used to standardize the inoculum. The inoculum was used at a level of 1% of the total sample volume. Standard curves were prepared for analyses on each run. One drop of 1 : 10 dilution of the saline suspension per tube was used.

Preparation of folic acid free glassware

All glassware was precleaned with Micro-liquid laboratory cleaning solution (Taylor Chemical Co., Hanley Industrial Ct., St. Louis, MO), then rinsed with tap water 5 times and distilled water 3 times, and immersed overnight in a 32-gallon tank that contained Micro-liquid laboratory cleaning solution. The soaked glassware was rinsed free of detergent. Then, all glassware was heated to 150 °C for 2 h or longer to destroy heat labile folate.

Folic acid determination

Folic acid casei (FAC) medium was prepared according to Difco Laboratories (2). *Lactobacillus casei* ATCC 7469 was used as the test organism for assay of folic acid in FAC (2). The assay of folic acid, except the procedure for cleaning glassware, was according to the Association of Vitamin Chemists (1). Free folic acid was obtained with no conjugase being used whereas total folic acid was determined after conjugase used.

Total folic acid of 35 isolates

Folic acid content was determined after incubation with the test microorganisms at 35 °C for 72 h. In the case of 9 *S. faecalis* strains, which did not grow in folic acid assay medium, 0.4 ng/m*l* of folic acid was added to the medium to check for requirement for folic acid. For those that grew in the folic acid assay medium, cells and medium were separated by centrifuging at about 6,000 g for 10 min. The content of folic acid in the medium was determined.

Initial pH, temperature and incubation time

Nutrient broth was used in all experiments and was sterilized at 121 °C for 15 min. The broth was adjusted to pH values of 4 through 8 using 1N NaOH or 1N HCl. After inoculation, test tubes of the broth were incubated at 35 °C for 72 h and analyzed.

To determine the influence of temperature on the production of folic acid, broth cultures were adjusted to each species optimal pH and incubated at 25 °C, 30 °C, 35 °C, 40 °C, and 45 °C for 72 h.

To determine the influence of time on folic acid production, broth cultures were incubated at 35 °C (and at their optimal pH per species) for 6 days with samples taken each day.

Carbohydrates and mixed cultures

The effect of carbohydrates on the production of folic acid by pure and mixed cultures of *B. licheniformis* strain 6 and *E. cloacae* strain 18 in nutrient broth was studied. One m*l* of 30% carbohydrate stock solutions (sucrose, glucose, fructose, or sucrose plus glucose plus fructose) was injected through a 0.2 um micro-filter into 9-m*l* of sterile nutrient broth. The broths were then incubated for 5 days at 35 °C at the optimal pH (pH 7.0, 7.5, and 8.0) determined for production of folic acid by these two microorganisms and their mixed cultures. The mixed culture was inoculated at a 1:1 ratio (v/v) of *B. licheniformis* strain 6 and *E. cloacae* strain 18. After incubation, the amount of free and total folic acid in the cells and medium were determined separately.

Plate counts

Total cell counts of *B. licheniformis* strain 6 and *E. cloacae* strain 18 were determined daily during the 6-day incubation period.

pH changes during incubation

The pH of the nutrient broth was determined each day during 6 days of growth of *B. licheniformis* strain 6 and *E. cloacae* strain 18 at 35 °C at the optimal pH for each organism, pH 7.0 and 8.0, respectively.

Statistical analysis

Analysis of variance (7) was used to determine if

Table 1. Survey of microorganisms that produced folic acid in folic acid assay medium.

Microorganism	No. of Strains	Range ng/100 ml	Mean	Ranking
Bacillus licheniformis	3	1670 ± 269 to 1830 ± 271	1747 ± 256	1
Enterobacter cloacae	3	1330 ± 117 to 1350 ± 167	1337 ± 148	2
Agrobacterium spp.	4	870 ± 236 to 1052 ± 232	937 ± 232	3
Lactobacillus brevis	13	320 ± 74 to 458 ± 47	381 ± 61	4
Morexella sp.	1	73 ± 8	73 ± 8	5
Hansenula subpelliculosa	1	56 ± 19	56 ± 19	6

there were statistically significant differences among means for treatments and replications. When significant ($P < 0.05$) differences were found, Duncan's (3) new multiple range test and multiple F test was used to locate the means that differed. Standard deviations were also determined and are reported along with the means.

Results and Discussion

Survey of microorganisms

Twenty-five of the 35 cultures produced folic acid as shown in Table 1. Strain 6 of *Bacillus licheniformis* was the best producer with a yield of 1830 ± 271 ng/100 ml of the assay broth. The two highest producers were used in further research on factors influencing yields.

The 6 strains of *Streptococcus faecalis* grew in the folic acid assay medium only after the addition of 0.4 ng/ml folic acid.

Effect of pH

Bacillus licheniformis strain 6 did not grow in pH 4 nutrient broth, and yielded the most folic acid when the initial pH was above 6. NO significant differences were found at pH 6, 7, and 8 in the production of folic acid. Neither did different initial pH levels have an effect on the production of folic acid by *E. cloacae* strain 18. Based on these results, it seems that weak acidic or alkaline conditions favor production of folic acid by *B. licheniformis* strain 6. However, in the pH range 4 to 8, *E. cloacae* strain 18 produced no significant ($P < 0.05$) difference in the synthesis of folic acid.

Stokes and Larsen (8) investigated the effect of pH on the transformation of the *Streptococcus lactic* R factor to folic acid by resting cell suspensions of *Enterococci*. They reported that within the pH range 4 to 8, the optimum for *Enterococci* to convert the *S. lactic* R factor to folic acid was pH 8 with little folic acid being formed at pH 5. Their results were similar to the data obtained in this study for *B. licheniformis* strain 6 but not for *E. cloacae* strain 18.

Table 2. Effect of incubation temperature on the production of folic acid by *B. licheniformis* strain 6 and *E. cloacae* strain 18 in nutrient broth[a]

Temperature (°C)	Total folic acid (ng/100 ml) produced by *B. licheniformis*[b]	*E. cloacae*[c]
25	248 ± 60[c]	190 ± 42[c]
20	925 ± 43[b]	730 ± 84[b]
35	1415 ± 161[a]	1408 ± 100[a]
40	1509 ± 269[a]	895 ± 237[b]
45	938 ± 75[b]	0 ± 0[c]

[a]N = 4. Where letters differ within a column, means differ significantly ($P < 0.05$) from each other.
[b]Incubated at pH 7 for 72 h.
[c]Incubated at pH 8 for 72 h.

Incubation temperature

Incubation temperature influenced production of folic acid by *B. licheniformis* strain 6 and *E. cloacae* strain 18 (Table 2). The maximum yields of folic acid were produced at 35 °C and 40 °C for *B. licheniformis* strain 6 and 35 °C for *E. cloacae* strain 18.

Incubation time

The production of folic acid with both microorganisms increased progressively over 5 days and then dropped on the 6th day of incubation. The incubation period of 5 days with *B. licheniformis* strain 6 (reached yields greater than 1,200 ng/100 ml) was optimum for folic acid production. With the *E. cloacae* strain 18, there were no significant

($P > 0.05$) changes in production of folic acid on the 4th and 5th day of incubation. Yields slightly higher than 800 ng/100 ml were obtained. Therefore, five days of incubation time was also considered as optimum for *E. cloacae* strain 18.

The final pH levels after 6 days of incubation for *B. licheniformis* strain 6 and *E. cloacae* strain 18 were 8.5 and 8.6, respectively. The pH increased progressively during the 6 days of incubation for both microorganisms. This probably was due to the fact that nutrient broth contains no carbohydrates so that the microorganisms could not produce acid to reduce the pH.

Total plate count

Total plate counts for *B. licheniformis* strain 6 and *E. cloacae* strain 18 in nutrient broth are presented in Table 3. The maximum growth of *B. licheniformis* strain 6 was during the 1st to the 3rd day, and there was a progressive decrease after the 4th day of incubation. However, *E. cloacae* strain 18 achieved the highest cell count on the 3rd day and decreased after the 4th day of incubation. Thus, one may assume that the maximum yields of folic acid produced by these microorganisms was not closely related to the maximum growth of microorganisms since the maxima differed for the two bacteria. The increase in folic acid may have been due to accumulation during the 6 days of incubation.

Table 3. Counts of *B. licheniformis* strain 6 and *E. cloacae* strain 18 in nutrient broth[a]

Incubation period (day)	Bacteria counts per ml	
	B. licheniformis[b]	*E. cloacae*[c]
0	142.3×10^{3c}	162.3×10^{4e}
1	235.8×10^{7a}	153.0×10^{7b}
2	190.0×10^{7a}	210.3×10^{7a}
3	226.0×10^{7a}	148.0×10^{7b}
4	96.8×10^{7b}	73.5×10^{7c}
5	55.5×10^{7b}	44.8×10^{7d}
6	53.5×10^{7b}	38.8×10^{7d}

[a]N = 4. Where letters differ within a column, means differ significantly ($P < 0.05$) from each other.
[b]Incubated at pH 7 and 35 °C.
[c]Incubated at pH 8 and 35 °C.

Effect of carbohydrates free and total folic acid

The effect of carbohydrates (sucrose, glucose, fructose, and a combination of sugars sucrose plus glucose plus fructose) on the production of free and total folic acid by pure and mixed cultures of *B. licheniformis* strain 6 and *E. cloacae* strain 18 are shown in Tables 4 and 5.

Except for fructose, carbohydrates were found to exert no significant ($P > 0.05$) effect on the production of free folic acid by *B. licheniformis* strain 6 as compared to the control (without carbohydrate, Table 4). The lowest yields of free folic acid were obtained when fructose was the added carbohydrate. Sucrose had no significant ($P > 0.05$) effect on the production of free folic acid by *E. cloacae* strain 18 and the mixed culture. Production of free folic acid by *E. cloacae* strain 6 and the mixed

Table 4. Effect of 3% sucrose, 3% glucose, 3% fructose, and a combination of 2% sucrose plus 0.5% glucose plus 0.5% fructose on the production of cellular free and total folic acid by *B. licheniformis* strain 6 and *E. cloacae* strain 18[a]

Carbohydrate	Free folic acid[b] in the cells (ng/100 ml)			Total folic acid[c] in the cells (ng/100 ml)		
	B[d]	E[e]	M[f]	B	E	M
Control	24.0 ± 6.7^{a}	$18.0 \pm 5.7^{a,b}$	$18.0 \pm 5.7^{a,b}$	133.3 ± 6.1^{a}	52.8 ± 14.1^{b}	81.8 ± 25.8^{a}
Sucrose	$19.3 \pm 7.0^{a,b}$	23.3 ± 9.0^{a}	25.0 ± 3.0^{a}	110.7 ± 1.9^{a}	$97.0 \pm 35.0^{a,b}$	110.0 ± 24.6^{a}
Glucose	$21.0 \pm 7.0^{a,b}$	$9.8 \pm 2.1^{b,c}$	7.7 ± 2.1^{c}	102.0 ± 41.6^{a}	$78.8 \pm 24.1^{a,b}$	102.0 ± 47.5^{a}
Fructose	10.5 ± 2.5^{b}	7.0 ± 3.5^{c}	$8.3 \pm 2.4^{b,c}$	67.3 ± 19.7^{a}	64.5 ± 10.4^{b}	102.5 ± 31.3^{a}
Combination	24.8 ± 8.2^{a}	7.0 ± 3.5^{c}	$16.3 \pm 6.3^{b,c}$	88.5 ± 23.5^{a}	116.5 ± 39.4^{a}	88.5 ± 23.6^{a}

[a]N = 4. Where letters differ within a column, means differ significantly ($P < 0.05$) from each other.
[b]Without conjugase treatment.
[c]With conjugase treatment.
[d]B = *B. licheniformis* strain 6.
[e]E = *E. cloacae* strain 18.
[f]M = mixed cultures of *B. licheniformis* strain 6 and *E. cloacae* strain 18.

Table 5. Effect of 3% sucrose, 3% glucose, 3% fructose, a combination of 2% sucrose plus 0.5% glucose plus 0.5% fructose on the production of free and total folic acid by *B. licheniformis* strain 6 and *E. cloacae* strain 18 in the medium[a]

Carbohydrate	Free folic acid[b] in the medium (ng/100 ml)			Total folic acid[c] in the medium (ng/100 ml)		
	B[d]	E[e]	M[f]	B	E	M
Control	1805 ± 396[a]	770 ± 469[b]	1205 ± 305[b]	2153 ± 931[a]	1040 ± 274[b]	1365 ± 272[b]
Sucrose	1475 ± 899[a]	2010 ± 477[a]	2059 ± 302[a,b]	2353 ± 701[a]	2495 ± 739[a]	2290 ± 821[a]
Glucose	1755 ± 259[a]	1980 ± 499[a]	2263 ± 558[a]	2240 ± 401[a]	2453 ± 671[a]	2483 ± 331[a]
Fructose	1720 ± 430[a]	1850 ± 191[a]	2288 ± 391[a]	1978 ± 864[a]	2353 ± 485[a]	2315 ± 307[a]
Combination	1887 ± 282[a]	2100 ± 190[a]	2150 ± 451[a]	2490 ± 507[a]	2565 ± 597[a]	1865 ± 627[a]

[a]N = 4. Where letters differ within a column, means differ significantly ($P<0.05$) from each other.
[b]Without conjugase treatment.
[c]With conjugase treatment.
[d]B = *B. licheniformis* strain 6.
[e]E = *E. cloacae* strain 18.
[f]M = mixed cultures of *B. licheniformis* strain 6 and *E. cloacae* strain 18.

culture was decreased by the addition of glucose, fructose and a combination of sugars to nutrient broth. Production of total folic acid by *E. cloacae* strain 18 increased when the combination of sugars was added. No other significant differences caused by carbohydrates were found.

Based on the results that some carbohydrates decreased the production of free folic acid in the cells, one may assume that the form of folic acid produced may be attributed to the carbohydrates in the cells of microorganisms. Tramura *et al.* (10) reported that *L. casei* responded equally to mono-, di-, and triglutamates and more slowly to longer-chain glutamate folic acid derivates. If microorganisms produce long-chain glutamate folic acid, it will not be detected equally as mono-, di-, or triglutamate by the test organism. However, the enzyme conjugase was applied to cleave the long-chain glutamate folic acid to mono-, di-, or triglutamate. This might be the reason that the production of free folic acid but not total folic acid was detected.

Similar results were obtained for the production of free and total folic acid by pure and mixed cultures of *B. licheniformis* strain 6 and E. cloacae strain 18 in the medium (Table 5). Carbohydrate had no effect on the production of free or total folic acid by *B. licheniformis* strain 6. However, carbohydrate significantly ($P<0.05$) increased the production of free and total folic acid by *E. cloacae* strain 18 and mixed cultures as compared to the control. This was in agreement with the findings of Stokes and larsen (8).

In the present study, no significant difference was found in the production of free and total folic acid by pure and mixed cultures of *B. licheniformis* strain 6 and *E. cloacae* strain 18.

Thompson (9) found that more than 50 to 88% of folic acid was excreted by microorganisms into the medium. Most of the folic acid produced by the pure and mixed cultures of *B. licheniformis* strain 6 and *E. cloacae* strain 18 used in this study was excreted into the medium. Only small amounts of folic acid were utilized by the microorganisms for their metabolic needs (Tables 4 and 5).

요 약

옥수수 가루의 발효 과정에서 folic acid를 생성하는 25균주를 분리하였는데 그 중에서 *Bacillus licheniformis*와 *Enterobacter cloacae*가 가장 많이 생산하였다.

최적배양조건은 두 균주가 모두 35℃에서 5일간 배양했을 때 최대의 생산량을 보였으나 초기 pH는 *B. licheniformis*의 경우는 pH 6.0-8.0에서 거의 변화가 없었고 *E. cloacae*는 pH 4.0-8.0에서 folic acid 생성량이 거의 일정하였다. 탄소원을 첨가하면 균체내의 folic acid 생성량은 단독 또는 혼합배양에서도 변화가 없었으며 생육배지내의 folic acid 함량은 *E. cloacae*는 상당히 증가하였으나 *B. licheniformis*의 경우는 별다른 영향을 받지 않음을 알 수 있었다.

Acknowledgement

Contribution from the Missouri Agricultural Experiment Station. Journal Series No. 10582.

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(Received August 8, 1988)