KOREAN J. FOOD SCI. TECHNOL.
Vol. 20, No. 2, pp 218~224(1988)

Studies on Thermophilic Flat-sour Bacteria in Soymilk: Isolation, Indentification and Determination of Heat Resistance

Je-Bong Chung, Kyun-Hee Lee, Heon-Soo Sohn and Suk Min Kim

Central Research Institute, Dr. Chung's Food Co., Ltd.

두유내의 고온성 Flat-sour 변질균의 분리·동정 및 열저항성에 관한 연구

정제봉*·이균희·손헌수·김석민

저 식품 중앙연구소

Abstract

For the optimization of thermal processing conditions in soymilk process, 4 strains of thermoresistant flat-sour bacteria were isolated from soymilk. The isolates were aerobic spore-forming rods, and grew at 65℃. Based on the morphological and physiological properties, all of the isolated strains were identified as *Bacillus stearothermophilus*. The heat resistance of spores of 3 isolates and *Bacillus stearothermophillus* ATCC 12980 as a reference was determined in soymilk(pH 7.0) and pH 7.0 buffer solution. For each of the spores studied, linear regression equations with standard error were presented for the thermal destruction at 110, 115, 121, and 125℃. It was not obvious that the components of soymilk increased the heat resistance of spores. Between the strains studied, variability was noted in the D values at the different temperature, and no one strain was consistently the most heat resistant at all the given temperatures. The average D value for the 4 strains was 77.27, 20.20, 2.76 and 1.39 min at 110, 115, 121 and 125℃, respectively, and the average z value was 8.36℃.

Key words: soymilk, flat-sour, *Bacillus stearothermophilus*, spore, thermoresistance

Introduction

The distribution of thermophilic aerobic sporeforming bacteria in food ingredients is an important factor in processing of low-acid foods because of their potentiality as spoilage organisms. Thermophilic aerobic sporeforming bacteria cause flat-sour spoilage in low-acid foods such as sugar, milk, corn, wheat, cocoa, peas, and beans[1-4]. The presence of thermophilic bacteria in beans can be recognized as a potential source of spoilage organism in soymilk and our preliminary observations showed that flat-sour spoilage occurred in soymilk at high temperature (above 55℃), but there are few reports on the thermophilic bacterial contamination in soymilk and its production process.

The objectives of this research were to identify the thermophilic flat-sour bacteria in soymilk, and to determine the heat resistance of their spores for the optimization of thermal processing conditions in soymilk process.

Corresponding author: Je-Bong Chung, Central Research Institute, Dr. Chung's Foods Co., Ltd. 1-25, Songjung-dong, Chungju, Choongbuk-do 360-290

Materials and Methods

Test organisms

Four isolates which were designated arbitrarily as CFR-1, CFR-2, CFR-3 and CFR-4, *Bacillus stearothermophilus* ATCC 12980 and ATCC 12016 were used for identification. Three isolates (CFR-1, CFR-3 and CFR-4), and *B. stearothermophilus*

ATCC 12980 were used for determination of heat resistance.

Soybean and soymilk

Soybeans (*Glycine max L.*) imported from United States were used in this study. Soymilk was obtained from Dr. Chung's Food Co., Ltd. (KOREAN). The proximate composition of soymilk was (wt/wt): protein 3.1%, fat 3.3%,carbohydrate 4.8%, ash 0.4%, and moisture 88.4%

Isolation of the thermophile

Cultures of thermophilic flat-sour bacteria were obtained by taking the ground soybean suspension samples from the pilot process and incubating for 24hr at 65℃. The samples in which flat-sour spoilage was produced were inoculated on the dextrose-tryptone agar plate (Difco) and incubated for 24hr to 48hr at 65℃. The round surface colonies surrounded by a yellow-halo were picked arbitrarily, and restreaked on the nutrient agar (Difco) plate until single colony was obtained. The stock cultures were prepared on soil extract agar[5] slant and in nutrient broth, and stored at 4℃.

Identification of the thermophile

Morphological properties and taxonomic characteristics were examined according to the methods described in the Genus *Bacillus*[5] and in Bergey's Manual of Determinative Bacteriology[6]. All experiments were conducted at 55℃ with time intervals of 24hr to 14 days. Because the thermophilic nature of the isolates immediately suggested *B. stearothermophilus,* all the classification tests were performed simultaneously on *B. stearothermophilus* ATCC 12980(Type strain) and ATCC 12016.

Spore preparation

The spore stock solutions were prepared by the methods described by Mabsout and Stevenson[7], Pflug *et al.*[8], and Beaman *et al.*[9], with the slight modification. The spore crops were grown at 55℃ for 48hr on nutrient agar supplemented with 10mg $MnSO_4$ per liter. The surface growth was washed with sterile distilled water and centrifugation steps were repeated three times. After the final washing the spores were treated with 0.3mg/m*l* lysozyme at 37℃ for 2hr, and cleaned by centrifugation with sterile distilled water, further treated with 0.1mg/m*l* papain at 37℃ for 2hr. The spores were then washed three times in distilled water by centrifugation at 950×g for 3min. After cleaning, the spores were resuspended in 0.05M potassium phosphate buffer(pH 7.0), and stored at 4℃.

Determination of heat resistance

Heat resistance of the spores in soymilk (pH 7.0) and 0.05M potassium phosphate buffer (pH 7.0) was determined by the procedure of Beaman *et al.*[9]. The soymilk and buffer solution were sterilized at 121℃ for 15min, and were inoculated with the spore preparation to a level of about 10^6 spores/m*l*. The 1.5m*l* aliquots were distributed into sterile thin-walled glass ampoules(9mm outer diameter by 13cm length). The ampoules were heat-sealed, and were submerged in a stirred and thermoregulated oil bath. Three ampoules were removed at appropriate time intervals and cooled by immersing in an ice bath for at least 5min. The ampoules were opened and 1m*l* sample in each ampoule was transferred into 9m*l* of germination solution(0.6% alanine, 1.0% peptone, 0.4% leucine, 0.3% glucose, 0.4% adenosine), which was incubated at 55℃ for 50min. Afterwards, appropriate decimal dilutions were made in 1% peptone solution, and quintuplicate 0.1m*l* samples at several appropriate dilutions were transferred and spread on the surface of predried agar medium(Plate Count Agar, Difco). The plates with 30 to 300 colonies were selected, and the counts were averaged and plotted. D and z values were calculated as suggested by Mikolajcik[10]. Regression analysis was used to generate the best fit curves.

Results and Discussion

Identification

All of the test strains, 4 isolates and 2 ATCC strains, were aerobic spore-forming rods, and grew 65℃. The endospores were definitely swollen and located at terminal and subterminal position. These characteristics of the isolates are sufficient for their tentative identification as *B. stearothermophilus* as described in Bergey's Manual of Determinative Bacteriology[6]. Comparison of the characteristics of the test strains with the descriptions of *B. stearothermophilus* as given in Bergey's

Table 1. Morphological and physiological characteristics of the test strains.

Property \ Strains	ATCC 12980	ATCC 12016	CFR 1	CFR 2	CFR 3	CFR 4	Bergey's Mannal	Gordon *et al.*
Rods Width, μm	0.6-0.7	0.5-0.6	0.5-0.6	0.6-0.8	0.6-0.8	0.5-0.6	0.6-1.0	0.6-1.0
Length, μm	3-4	2-4	2-3	2-3	2-3	3-4	2-3.5	2-3.5
Gram reaction	v	v	v	v	v	v	d and v	v
Spore shape	E	E	E	E	E	E	E	E
Dominant position	T	T	T	T	T	T	T	T
Swelling the sporangium	+	+	+	+	+	+	+ or -	+
Motility	+	+	+	+	+	+	+	+
Catalase	-	+	+	+	+	+	d	a
Temperature of growth, °C								
Maximum	70	70	70	65	65	65	65-75	65-75
Minimum			35				30-45	30-45
Growth in								
Anaerobic agar	-	-	-	-	-	-	-	-
0.001% lysozyme	-	-	-	-	-	-	-	-
Media at pH 5.7	-	-	-	-	-	-	-	-
0.02% azide	-	-	-	-	-	-	-	-
5% NaCl	-	-	-	-	-	-	d	b
10% NaCl	-	-	-	-	-	-		-
Acid from								
glucose	+	+	+	+	+	+	+	+
arabinose	-	+	+	+	+	+	d	b
xylose	+	+	+	-	-	-	d	a
mannitol	+	+	+	+	+	+	d	b
Gas from fermented carbohydrates	-	-	-	-	-	-	-	-
V-P reaction	-	-	-	-	-	-	-	-
pH in V-P broth	5.3	5.8	5.2	6.3	5.2	5.0		4.8-5.8
Hydrolysis of starch	+	+	+	+	+	+	+	+
Use of citrate	-	-	-	-	-	-		-
Reduction of NO_3 to NO_2	+	+	+	+	+	+	d	a
Formation of								
dihydroxyacetone	-	-	-	-	-	-	-	-
indole	-	-	-	-	-	-	-	-
Decomposition of								
casein	+	-	-	+	-	-	d	a
tyrosine	-	-	-	-	-	-	-	-

d=reactions differ, positive for 11-89% of strains.
v=character inconstant in one strain
E=elliptical or cylindrical
T=terminal or subterminal
+=positive for 90-100% of strains
−=negative for 90-100% of strains
a=50 to 89% of the strains positive
b=10 to 49% of the strains positive

Manual and in the Genus *Bacillus*[5] is shown in Table 1.

According to Gordon *et al.*[5] only two species, *B. stearothermophilus* and *B. coagulans*, can be recognized as thermophilic *Bacillus* species. *B. coagulans* can be distinguished from *B. stearothermophilus* by its failure to grow at 65℃. The most distinctive diagnostic characteristics of *B. stearothermophilus* as described in Bergey's manual are capacity to grow at 65℃, sensitivity to azide and a limited tolerance to acid. Comparision of these distinctive diagnostics of the test strains with the descriptions of *B. stearothermophilus* and *B. coagulans* as given in Bergey's Manual is shown in Table 2. Because the samples and plates were held at 65℃ before isolation of strains, the isolation method used in this experiment can select only microorganisms capable of growing aerobically at 65℃. Such a procedure would automatically exclude such organisms as *B. coagulans*, and all other *Bacillus* species. From the above results, the isolated strains can be identified as *B. stearothermophilus*.

Table 2. The most distinctive diagnostic characteristics of the isolated strains for comparison with *B. stearothermophilus* and *B. coagulans*

Characteristics / Isolates	Growth at 65°C	Growth in 0.02% azide	Growth in media at pH 5.7
B. stearothermophilus (by Bergey's manual)	+	-	-
B. coagulans (by Bergey's manual)	-	+	+
ATCC 12980	+	-	-
ATCC 12016	+	-	-
CFR-1	+	-	-
CFR-2	+	-	-
CFR-3	+	-	-
CFR-4	+	-	-

+ = positive for 90-100% of strains
- = negative for 90-100% of strains

Determination of heat resistance

Linear regression equations and standard error of estimate for the thermal destruction of the spores at 110, 115, 121 and 125℃ in soymilk and pH 7.0 buffer are presented in Table 3. The thermal death time curves for all of the test strains were essentially linear.

The D values and z values of the test strains in soymilk and pH 7.0 buffer are shown in Table 4. It has been reported that the components of heating medium such as carbohydrate, protein and lipid can increase the thermal resistance of bacterial spores[11]. CFR-3 strain was more heat resistant in soymilk which comprised carbohydrate, protein

Table 3. Linear regression equations (Y) with standard error of estimate (S.E.) for the heat resistance of *Bacillus stearothermophilus* spores in pH 7.0 buffer and soymilk(pH 7.0)

		110°C		115°C		121°C		125°C	
		Y=	S.E.	Y=	S.E.	Y=	S.E.	Y=	S.E
ATCC 12980	pH 7.0 Buffer	-0.0102X+5.757	±0.060	-0.0554X+5.498	±0.050	-0.4766X+7.582	±0.027	-0.9116X+7.636	±0.204
	Soymilk	-0.0217X+6.477	±0.186	-0.0760X+6.587	±0.173	-0.5663X+7.240	±0.028	-1.0562X+7.748	±0.191
CFR-1	pH 7.0 Buffer	-0.0142X+6.206	±0.065	-0.0381X+6.631	±0.078	-0.3018X+6.943	±0.095	-0.5043X+7.505	±0.206
	Soymilk	-0.0150X+6.287	±0.081	-0.0418X+6.209	±0.134	-0.3714X+7.087	±0.046	-0.5495X=7.842	±0.137
CFR-3	pH 7.0 Buffer	-0.0147X+6.106	±0.257	-0.0550X+6.711	±0.071	-0.2781X+6.784	±0.108	-0.8628X+7.995	±0.143
	Soymilk	-0.0100X+5.862	±0.077	-0.0301X+5.729	±0.027	-0.2579X+6.958	±0.189	-0.7425X+8.138	±0.131
CFR-4	pH 7.0 Buffer	-0.0085X+5.191	±0.021	-0.0628X+5.595	±0.156	-0.4797X+7.684	±0.078	-0.7416X+7.594	±0.067
	Soymilk	-0.0191X+5.920	±0.056	-0.0774X+5.129	±0.122	-0.3821X+6.949	±0.142	-0.7168X+7.534	±0.205

and lipid, than buffer solution. ATCC 12980 and CFR-1 strains, however, were more heat resistant in buffer solution than soymilk, and CFR-4 strain was not consistently more heat resistant in one medium than the other medium at a given temperature. From these results, it was not obvious that the components of soymilk increased the thermal resistance. Variability was noted in the D values between the test strains, and no one strain was consistently the most heat resistant at all the given temperatures. The average D value for the 4 spores studied regardless of the heating medium was 77.27, 20.20, 2.76, and 1.39 min at 110, 115, 121 and 125℃, respectively. The average z value was 8.36℃.

Variable results have been reported in the literature regarding the thermal characteristics of *B. stearothermophilus* strains. Some of these results are shown on Table 5. The heat resistance of *B. stearothermophilus* spores varies between the strains. Within the same strians, considerable variability does exist according to the workers. The D values for ATCC 12980 strain were found to be higher at 110, 115℃, but to be lower at 121 and 125℃ than those reported by Navani *et al.*[12]. Mallidis *et al.*[13] reported slight higher D values

Table 4. Heat resistance of *Bacillus stearothermophilus* spores in pH 7.0 buffer and soymilk(pH 7.0)

	ATCC 12980		CFR-1		CFR-3		CFR-4	
	pH 7.0 buffer	Soymilk	pH 7.0 buffer	Soymilk	pH 7.0 buffer	Soymilk	pH 7.0 buffer	Soymilk
D-Value(min)								
D_{110}	97.67	46.17	70.42	66.49	68.20	99.64	117.28	52.31
D_{115}	18.04	13.15	26.22	23.90	18.19	33.24	15.92	12.91
D_{120}	2.10	1.76	3.31	2.69	3.60	3.88	2.08	2.62
D_{125}	1.09	0.94	1.98	1.82	1.16	1.35	1.35	1.40
z-Value(°C)	7.45	8.52	9.09	8.93	8.49	7.48	7.53	9.37

Table 5. Heat resistance of *Bacillus stearothermophilus* strains quoted from the literature

Strain	Heating medium	D value (min)					z Value (°C)	Reference
		110°C	115°C	120°C	121°C	125°C		
NCIB 8919	water				3.1		7.0	Briggs, 1966(14)
ATCC 7953 (NCIB 8157)	phosphate buffer pH 7.0				2.1		8.5	Jonsson *et al.*, 1977(15)
NCDO 1096	water			16.6			7.56	Davies *et al.*, 1977(16)
NCIB 8710	Sorensen's buffer pH 7.0	31.30	10.86		3.50	1.80	12.10	Navani *et al.*, 1970(12)
NCIB 8919	Sorensen's buffer pH 7.0	17.99	6.07		2.48	1.36	14.25	Navani *et al.*, 1970
ATCC 12980 (NCIB 8923)	Sorensen's buffer pH 7.0	25.54	10.86		2.84	1.77	12.31	Navani *et al.*, 1970
NCIB 8924	Sorensen's buffer pH 7.0	35.52	15.81		3.77	1.95	11.68	Navani *et al.*, 1970
ATCC 12980 (NCIB 8923)	water		45.0	8.00		1.60	7.00	Mallidis *et al.*, 1985(13)
ATCC 7953 (NCIB 8157)	water		35.5	5.70		1.05	6.90	Mallidis *et al.*, 1985
NCIB 8920	water		15.6	3.00		0.63	7.04	Mallidis *et al.*, 1985
NCIB 8919	water		19.2	3.70		0.68	7.80	Mallidis *et al.*, 1985
NCIB 8924	water		6.0	1.35		0.36	7.80	Mallidis *et al.*, 1985

for ATCC 12980 strain than those obtained in the present study. The z value for ATCC 12980 strain was in good agreement with that reported by Mallidis *et al.*[13]. However Navani *et al.*[12] had reported higher z value.

From the results of this study, the order of D value at 121℃ for *B. stearothermophilus* can be considered as about 3 min, therefore, the Fo value of thermal process for soymilk based on spore inactivation of *B. stearothermophilus* can be calculated:

Fo=3×5=15(min) where 5 is m value that is used for commercial processing of low-acid foods[11].

요 약

두유제조공정 중 열처리 공정을 최적화 하기 위하여, 두유에서 4균주의 열저항성이 큰 flat-sour 변질균을 분리하였다. 분리균주들은 호기성 포자형성간균으로서 65℃에서도 성장하였으며, 형태학적 및 생리학적인 특징들로부터 모두 *Bacillus stearothermophilus*로 동정할 수 있었다. 3분리균주 및 *Bacillus stearothermophilus* ATCC 12980의 두유(pH 7.0) 및 pH 7.0 완충용액 내에서의 열저항성을 측정하기 위하여, 각각의 균주에 대한 110, 115, 121 및 125℃에서의 열치사에 관한 직선회귀식을 구하였다. 두유의 구성성분이 포자의 열저항성을 증가시킨다는 점은 뚜렷하게 확인되지 않았으며, 균주간에 있어서도 D값의 대소관계가 온도에 따라 변화하여, 주어진 모든 온도에서 일관성있게 가장 큰 열 저항성을 보인 균주는 없었다. 4균주의 평균 D값은 110, 115, 121 및 125℃에서 각각 77.27, 20.20, 2.76 및 1.39분이었으며, 평균 z값은 8.36℃이었다.

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(1987년 11월 10일 접수)