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Manufacture of *Chungkuk-jang* with Elastase Activity

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***Chungkuk-jang* (hereafter referred to simply as *jang*), a traditional fermented soybean product in Korea, is manufactured by a process similar to that of Japanese *itohiki-natto* (referred to as *natto*). The bacteria isolated from *jang* were identified to belong to *Bacillus subtilis* (Ehrenberg) Cohn. The quality of *jang* manufactured with the isolated bacteria and two kinds of *natto* bacilli, *B. subtilis* (*natto*) KFP2 and KFP419, was evaluated to be as good in sensory evaluation except for the viscosity. Therefore, the isolated bacteria were estimated as *B. subtilis* (*natto*). In *jang*, soybean proteins were degraded to increase soluble and formol nitrogens. Sticky materials were produced, and *natto*-like flavor was emitted. *Jang* prepared in the laboratory showed elastase activity, and it was twice-to-three-times higher than that of the commercial product. While *jang* is a different product from that of *natto*, both products contain the common starter and elastase.**

Keywords: *chungkuk-jang, itohiki-natto*, elastase

The zone of non-salt fermented soybean food is identified with the so-called "A triangle zone of broad-leaved-evergreen region" in Asia (Yoshida, 1985). Japan and Korea, situated at the end of East Asia, fall in the above region where *itohiki-natto* (hereafter simply described as *natto*) (Hara, 1990) and *chungkuk-jang* (referred to as *jang*) have traditionally been manufactured, respectively.

In Japan, *natto* is manufactured with advanced technology for large-scale production where temperature/moisture is programed and automatically controlled, whereas in Korea *jang* is a product prepared on a small scale by simpler techniques. Both *natto* and *jang* belong to the category of non-salt fermented products. However, while *natto* is eaten as it is with cooked rice in Japan, in Korea *jang* is mixed with 7% (w/w) NaCl, seasoned often with garlic and cayenne pepper, and utilized as one of the ingredients of the soup called *chigae* (Lee, 1995).

Among the studies on *jang* in Korea, many papers have reported the isolation and the identification of the *jang* microorganisms (Joo, 1971; Lee *et al.*, 1971; Kim *et al.*, 1982 1987; Suh *et al.*, 1982; Rhee *et al.*, 1983), the change in the constituents of *jang*, the physical and chemical properties of the sticky substances (Lee *et al.*, 1991; Lee *et al.*, 1992) and some other topics, but no studies on the physiological functions of *jang* have been reported.

Natto has been considered as a healthy food in Japan, and its physiological functions have been attracting growing attention. Sumi *et al.* (1987) found nattokinase to be effective on thrombosis, and Muramatsu *et al.* (1995) reported that elastase was produced by *natto* bacilli and was contained in the sticky substances of *natto*.

This paper reports the isolation and identification of *jang* strains and the manufacture of *jang* with the elastase activity.

Methods

Samples Commercial *jang* was purchased from a department store in Seoul, Korea, in January 1994. The product was packed in a cup with an 8.0-cm diameter of the upper end; 6.0 cm at the lower end and 5.5 cm in depth; the content of which weighed 150 g, containing garlic and cayenne pepper. On arrival in Japan, it was kept in a refrigerator and employed for the isolation of microorganisms and chemical analyses. The microorganisms were isolated 2 months after arrival.

Microorganisms *Natto* starters, *Bacillus subtilis* (*natto*) KFP2 and KFP419 were preserved in the laboratory. *B. subtilis* IFO3026 was procured from The Institute of Fermentation, Osaka. The isolation of the *jang* strains was carried out as follows: One gram of the commercial *jang* was mixed with 9.0 g of sterilized saline water and diluted stepwise. The colonies were purified on nutrient agar plates and set on nutrient agar slants. The strains were identified according to Bergey's Manual of Systematic Bacteriology (B.M.S.B.), Vol. 2 (Claus & Berkeley, 1986) in reference to Bergey's Manual of Determinative Bacteriology (B.M.D.B.), 7th and 8th editions (Smith & Gordon, 1957; Gibson & Gordon, 1974).

Manufacture of jang A large-type soybean, Tachinagaha variety produced in Tochigi Prefecture, Japan in 1993, was employed in manufacturing *jang* as follows (Joo *et al.*, 1987): One thousand grams of the soybean was soaked at room temperature for 18 h and then steamed at 121°C for 30 min in an autoclave. The starter strains were grown in NBP medium (Sulistyo *et al.*, 1988), and after cultivation, the cells were centrifuged at 10000×*g* for 10 min. They were washed

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twice with an equal volume of sterilized saline water. The cell suspension(10^7/ml) was inoculated on the steamed soybeans in a bowl disinfected with ethanol. After mixing, 50 g each of the soybeans was measured, packed in a polystyrene-paper (PSP) package and fermented on the programed temperature/humidity (Fig. 1) using Automatic Natto Manufacturing Equipment, Type SY-20 (Suzuyo Industrial Co., Tokyo).

Sensory evaluation Sensory evaluation was performed according to the Methods of Natto Research (Society for Study of Natto, 1990a), except for the evaluation method which was graded in 7 degrees instead of 5. Panelists were composed of 11 female students in their twenties and a man in his fifties. All of them had participated in the studies on fermented soybean foods or *natto* for more than half a year, and they had been in the training on sensory evaluation tests of those products. The difference test for sensory evaluation was carried out among the three *jang* by the analysis of variance for two-way layout. As a control, a commercial *natto* manufactured by a certain *natto* company was proposed to panelists every time sensory evaluation was performed.

Chemical analyses of constituents

Samples and methods of chemical analyses Raw samples were employed for the quantitative analyses of ammonium-nitrogen and organic acids. The samples were lyophilized and powdered for the purpose of other analyses. The analyses were carried out according to the standard method (Society for Study of Natto, 1990b). The difference test for the contents of chemical constituents was performed among the three *jang* by the analysis of variance for one-way layout.

Isolation of sticky substances and measurement of relative viscosity The products were centrifuged with centrifuge tubes with stainless steel nets at 1500 rpm for 10 min, and the precipitate fraction was obtained. One gram of the precipitate fraction was employed for the measurement of the relative viscosity by employing an Ostwald's viscometer (Society for Study of Natto, 1990c).

Free sugar analysis The analysis was performed by HPLC according to Taira *et al.* (1989) as follows: A 10-μl sample extract was injected into an LC-3A high-performance chromatograph (Shimadzu Manufacturing Co., Kyoto) equipped with an RID-2A refractive index ionization detector (Shimadzu Manufacturing Co.) and a Chromatopak CR-3A integrator (Shimadzu Manufacturing Co.). The HPLC conditions were as follows: Column; a 250 mm×4 mm i.d. packed with Lichrospher NH_2 (particle size; 5 μm, E. Merck, Darmstadt, Germany), column temp.; 40°C, solvent; water/acetonitrile (25/75), flow rate; 1.0 ml/min and sensitivity; 8×10^{-5} RIU.

Organic acid analysis Three grams of *jang* and 10 ml of DW were mixed, cooled in ice and homogenized. After the centrifugation at 3000 rpm for 10 min, the supernatant fraction was obtained. Following 3-time homogenization, the supernatant fractions were collected together and filled up to in a 50-ml volumetric flask. The solution was centrifuged at 3000 rpm for 10 min, and the supernatant fraction was filtered with a cellulose acetate membrane filter with 0.20-μm pore size. The 4-μl filtrate was injected into a GC-14A gas chromatograph (Shimadzu Manufacturing Co.) equipped with a flame ionization detector and Chromatopak CR-4A integrator (Shimadzu Manufacturing Co.) under the following conditions (Kanno *et al.*, 1984): Column; 2 m×3 mm i.d. glass column packed with Unisole F-200 (30/60 mesh, GL Sciences Co., Ltd., Tokyo), carrier gas; N_2 with a flow rate at 60 ml/min, column temp.; 140°C and injection and detector temp.; 160°C.

Measurement of enzyme activity Gamma-glutamyl transpeptidase (referred to as γ-GTP) activity was measured with a kit of γ-GTP Wako (Wako Pure Chemical Industries, Ltd., Tokyo) (Society for Study of Natto, 1990d). Elastase activity was measured with elastin-orcein (Elastin Products Co., U.S.A.) (Muramatsu *et al.*, 1995).

Reagents F-kit for urea and ammonia of Boehringer-Mannheim GmbH (Germany) was employed for the quantitative analysis of ammonium-nitrogen. The other reagents were of special grade available and purchased in the market.

Results and Discussion

Isolation and identification of jang bacteria Two billion and eight CFU per gram of *jang* were counted, when the colonies formed white wrinkles on the surface. All the colonies were observed to belong to the same species. Thus 6 colonies were randomly picked up and numbered from CKJ1 to CKJ6. The taxonomical characteristics are shown in Table 1. *Bacillus subtilis* IFO3026 was employed as a control culture for the tests. The main characteristics were as follows: Gram staining was positive; catalase reaction, positive; the diameter of the cell, 0.8 μm; acid produced from glucose, positive; growth in the presence of 7% (w/w) NaCl, positive; and nitrate reduction, positive. No growth was observed under the anaerobic conditions. The characteristics of 6 *jang* strains and *B. subtilis* IFO3026 were identical with the description of B.M.D.B. and B.M.S.B.

In order to investigate whether *jang* strains were the same with *B. subtilis* (*natto*) employed as a starter of *natto*, *jang* was manufactured with *natto* bacilli, *B. subtilis* KFP2 and KFP419, and CKJ1 under the same conditions. The results of the sensory evaluation of the manufactured *jang* are shown in

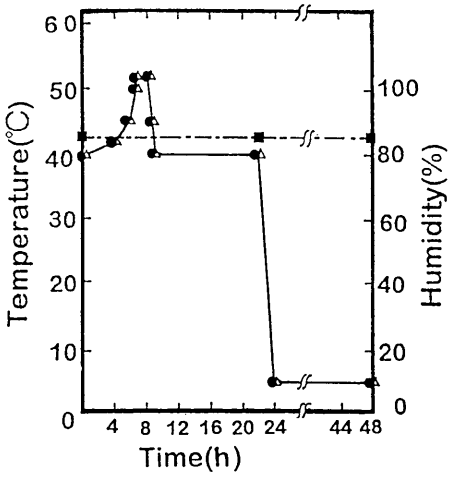

Fig. 1. Temperature and moisture conditions for *chungkuk-jang* fermentation. ●, chamber temperature; △, temperature of *chungkuk-jang*; ■, Humidity.

Table 2. *Jang*, manufactured with KFP2 and KFP419 employing the large-size soybean was evaluated to be 3.3 in taste, 3.0 in viscosity and, hence, 3.3 in the total evaluation, which places it in the mean ranks in the other aspects. Those manufactured with CKJ1 were scored 3.7 for hardness, taste, total evaluation and 4.0 in the other properties. No significant difference was shown between the quality of *jang* manufactured with CKJ1 and that of *jang* manufactured with both of the *natto* bacilli in all the evaluations, except viscosity. Significant differences were shown only in viscosity among CKJ1-*jang* and KFP2-*jang* at the 5% level and among CKJ1-*jang* and KFP419-*jang* at the 1% level on F value. CKJ1 was thus concluded to belong to the *natto* bacilli, *Bacillus subtilis* (*natto*).

Chemical analyses of jang manufactured with CKJ1

The chemical composition of *jang* is shown in Table 3. The moisture of the commercial *jang* was 55.3% (w/w), while that of the *jang* manufactured in the laboratory was 59-61% (w/w). The moisture of the former appeared slightly lower than that of the latter. The ash content of the manufactured *jang* was 2.0% (w/w), while that of the commercial *jang* was 6% (w/w) higher than the manufactured products due to the NaCl contained. Fat and protein content did not significantly differ between the manufactured *jang* and the commercial one.

The carbohydrate contents of all *jang* were less than that of the steamed soybeans. It is assumed that the uptake of low molecular compounds from sugars by the starters may be

Table 1. Characteristics and identification of CKJ1-6 and IFO3026.

Characteristics	CKJ1-6	IFO3026	Remarks[a]
Cell diameter >1.0 μm	−	−	−
Spores round	−	−	−
Sporangium swollen	−	−	−
Parasporal crystals	ND	ND	ND
Catalase	+	+	+
Anaerobic growth	−	−	−
Voges-Proskauer test	+	+	+
pH in V-P test <6	−	+	d
>7	−	−	−
Acid from D-glucose	+	+	+
L-arabinose	+	+	+
D-xylose	+	+	+
D-mannitol	+	+	+
Gas from glucose	−	−	−
Hydrolysis of casein	+	+	+
gelatin	+	+	+
starch	+	+	+
Utilization of citrate	+	+	+
propionate	−	−	−
Degradation of tyrosine	−	−	−
Deamination of phenylalanine	−	−	−
Egg-yolk lecithinase	−	−	−
Nitrate reduced to nitrite	+	+	+
Formation of indole	−	−	−
dihydroxyacetone	+	+	ND
NaCl and KCl required	ND	ND	−
Allantoin or urate required	ND	ND	−
Growth at pH			
6.8 nutrient broth	+	+	+
5.7 nutrient broth	+	+	+
Growth in NaCl 2%	+	+	+
5%	+	+	+
7%	+	+	+
10%	−	−	ND
Growth at 5°C	−	−	−
10°C	−	−	d
30°C	+	+	+
40°C	+	+	+
50°C	+	+	d
55°C	−	−	−
60°C	−	−	−
Growth with lysozyme present	+	+	d
Autotrophic with H_2+CO_2	ND	ND	−
Identification	*B. subtilis*		

Symbols are as follows: (−) 90% or more strains are negative. (+) 90% or more strains are positive. (d) 11-89% of strains are positive. (ND) No data available.
[a] The description of B.M.S.B. (Claus & Berkeley, 1986).

Table 2. Sensory evaluation of *chungkuk-jang* manufactured with CKJ1, KFP2 and KFP419 strains.

Item	*Jang* strain		
	CKJ1	KFP2	KFP419
Appearance	4.0(0.603[a])	4.0(0.000)	4.0(0.426)
Lysis of bacilli	4.0(0.000)	4.0(0.000)	4.0(0.000)
Crack of soybean	4.0(0.426)	4.0(0.000)	4.0(0.000)
Color	4.0(0.000)	4.0(0.426)	4.0(0.603)
Flavor	4.0(0.426)	4.0(0.603)	4.0(0.603)
Hardness	3.7(0.888)	4.0(0.866)	4.0(0.426)
Taste	3.7(0.778)	3.3(0.866)	3.3(0.866)
Viscosity	4.0(1.044)	3.0(0.603)	3.0(0.426)
Total evaluation	3.7(0.778)	3.3(0.866)	3.3(0.622)

[a] The standard deviation.

Table 3. Chemical compositions of cooked soybean and *chungkuk-jang* manufactured with strains employed.

Item	Cooked soybean	*Chungkuk-jang*			Commercial *chungkuk-jang*
		CKJ1	KFP2	KFP419	
Moisture	56.7	59.1	59.7	60.4	55.3
Protein	15.0	16.5	16.8	16.5	16.6
Fat	6.8	8.0	7.8	7.5	7.6
Carbohydrate	19.6	14.4	13.7	13.7	12.1
Ash	1.9	2.0	2.0	2.0	8.4
pH	−	7.8	7.5	6.9	6.1

The data are shown in wet base content (%(w/w)).

Table 4. Nitrogen compositions of cooked soybean and *chungkuk-jang* manufactured with strains employed.

Item	Cooked soybean	*Chungkuk-jang* manufactured		
		CKJ1	KFP2	KFP419
Total nitrogen	6.1[a](0.094[b])	7.1(0.065)	6.8(0.016)	7.3(0.110)
Soluble nitrogen	0.7(0.000)	2.5(0.005)	2.7(0.008)	2.3(0.016)
Formol nitrogen	0.1(0.082)	0.4(0.082)	0.3(0.082)	0.4(0.082)
Nitrogen solubility ratio[c]	11.4(0.816)	35.5(1.633)	39.5(3.682)	31.7(3.266)
Formol nitrogen decomposition content[d]	2.0(0.016)	5.8(0.049)	5.0(0.041)	5.2(0.082)
Ammonia nitrogen	0.0(0.000)	0.3(0.082)	0.2(0.122)	0.4(0.082)

The data are shown in dry base content (%).
[a] Mean (n=3).
[b] Standard deviation.
[c] (Soluble nitrogen/Total nitrogen)×100.
[d] (Formol nitrogen/Total nitrogen)×100.

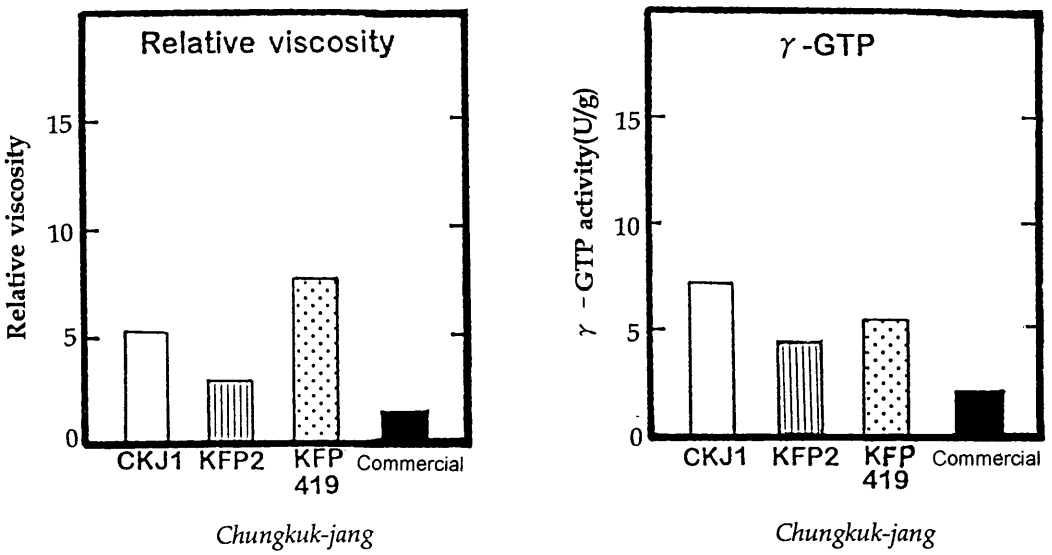

Fig. 2. Relative viscosity and γ-GTP activity of *chungkuk-jang* manufactured with strains employed.

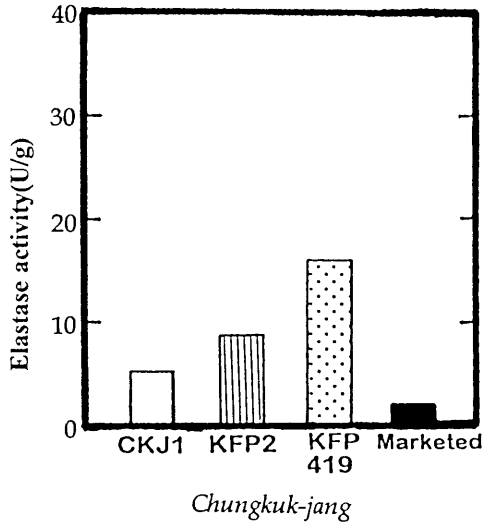

Fig. 3. Elastase activity of *chungkuk-jang* manufactured with strains employed.

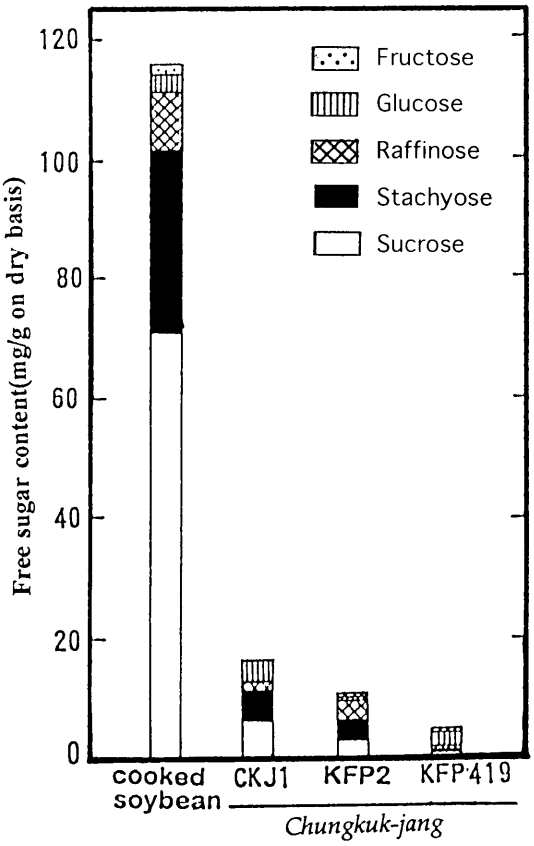

Fig. 4. Contents of free sugars in cooked soybean and *chungkuk-jang* manufactured with strains employed.

responsible for the decrease in the carbohydrate contents.

Table 4 shows the nitrogen content of *jang*. Nitrogen solubility ratios were higher in three *jang* than in soybeans, while no significant difference was observed between the nitrogen solubility ratio in *jang* manufactured with CKJ1 and that of the *jang* manufactured with the two *natto* bacilli. More ammonia-nitrogen and formol-nitrogen were contained in *jang* than in soybeans. It showed hydrolysis of protein by the starters. There was no significant difference between the nitrogen contents in *jang* manufactured with CKJ1 and those in the *jang* manufactured with the two *natto* bacilli.

Sticky substance production Figure 2 shows the viscosity and γ-GTP production of *jang* manufactured with the three strains. The relative viscosity of CKJ1-*jang* was significantly higher than that of the KFP2-*jang*, but significantly lower than that of the KFP419-*jang*. γ-GTP was proposed by Fujii (1963) as an enzyme associated with the synthesis of polyglutamate which was the main component of the sticky substances. The γ-GTP activities of CKJ1 were significantly higher than those of the KFP2-*jang* and KFP419-*jang*.

Elastase activity Mammalian pancreas elastase, such as rabbit and rat pancreas, was reported to be related to cholesterol or lipoprotein metabolism (Nakamura & Ishi-

kawa, 1971; Kametani, 1978; Koyama *et al.*, 1984), and that it can improve the abnormal condition of hyperlipemia by the physiological adjustment of the transfer of the lipid components of the human body (Kishimoto *et al.*, 1973; Nakamura *et al.*, 1985). Elastase of pig pancreas has been employed in a medical treatment of hyperlipemia. Muramatsu *et al.* (1995) identified KFP419 to be an elastase-producing *B. subtilis* (*natto*) and manufactured *natto* with it, which showed high elastase activity.

Figure 3 shows the elastase activity of the commercial *jang* and the laboratory-prepared one. The commercial *jang* exhibited 2.4 U/g of elastase, but the other *jang* manufactured with CKJ1 showed 5.8 U/g, which activity was 2.3 times as high as that of the commercial *jang*. The elastase activity was the highest in the *jang* manufactured with KFP419, the second with KFP2, and the third with CKJ1. The activity of CKJ1-*jang* was one third that of the KFP419-*jang*, or 64.0% (w/w) of the KFP2-*jang*.

The physiological functions of *jang* have not been reported earlier in Korea. The functionality of *jang* should be an interesting subject for future study.

Free sugars Free sugar constituents of steamed soybeans and 3 kinds of *jang* were compared (Fig. 4). The free sugar content of the cooked soybeans was 115.9 mg/g in total and consisted of sucrose, stachyose, raffinose, glucose and fructose in descending order. However, the total free sugar contents of *jang* were 16.4 mg/g in CKJ1 and 10.5 mg/g in KFP2, respectively. During fermentation, 92 to 98% (w/w) of the saccharose of soybeans and 85 to 97% (w/w) of the stachyose of soybeans decreased. There were significant differences among the cooked soybeans and 3 *jang* in the contents of 6 sugars and total sugar content, at least at the 5% level on *F* value, except there was no significant difference between the soybean and KFP419-*jang* in glucose.

Organic acids Organic acids in cooked soybeans were identified to be acetic, propionic, isobutyric and isovaleric acids. All of the acids generally increased in the three kinds of *jang*. Furthermore, isovaleric, *n*-valeric, 4-methyl valeric and hexanoic acids were also detected, especially in the *jang* of KFP419 (Fig. 5). Other organic acids, except *n*-butyric acid, were detected in *jang* manufactured with CKJ1. The main organic acids of the three kinds of *jang* were acetic, isobutyric and isovaleric acids. The content and the composition of organic acids of the CKJ1-*jang* were different from those of the KFP2-*jang* and the KFP419-*jang*.

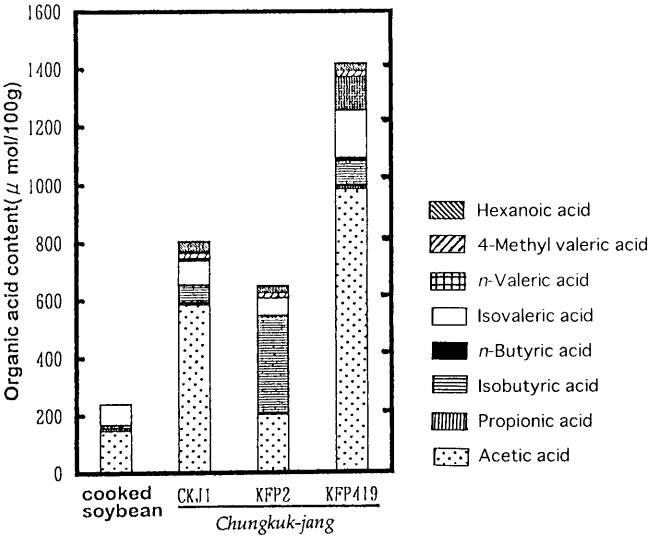

Fig. 5. Contents of organic acids in cooked soybean and *chungkuk-jang* manufactured with strains employed.

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