Role of Microorganisms in Tempeh Manufacture

—Isolation of vitamin B_{12} producing bacteria—

By NORIYUKI OKADA

Department of Applied Microbiology, National Food Research Institute
(Tsukuba, Ibaraki, 305 Japan)

Tempeh originated hundreds of years ago in Central and East Java, and is now Indonesia's most popular fermented food. It is made of cooked soybeans bound together by a white mycelium of *Rhizopus* mold into compact cakes and is usually served as thin sliced and fried. It can be an ideal main course at meals in place of meat, chicken, or fish, and can be the protein backbone of a vegetarian diet, because tempeh contains 20% protein, a high amount of unsaturated lipids, no cholesterol and vitamin B_{12}. The last one is often lacking in vegetarian diets. Although the essential microorganism in the tempeh fermentation is a fungus or a mold, other two microbial groups are also concerned in the tempeh manufacture; a lactic acid producing group and a vitamin B_{12} producing group. This paper will present the role of these microorganisms in tempeh manufacture as well as isolation results of vitamin B_{12} producing bacteria, and prospects of the utilization of isolates are also discussed.

Manufacture of tempeh

There are many variations in Indonesian manufacturing process[9]. The process essentially requires those steps; hydration of soybeans, partial cooking, acidification, dehulling, surface drying, inoculation with a starter, packing in a container, and incubation. The process seen at Bogor was as follows.

Soybeans without dirt and stones are cooked partially with drum cans (Plate 1-a), then they are soaked in water through night (Plate 1-b). The first group of microoganisms or the lactic acid producing group works in a modest way, playing an important part at this stage. As shown in Plate 1-c, the liquid becomes viscous during soaking, indicating the occurrence of an extensive growth of microorganisms. From this liquid, lactic acid bacteria were detected about $10^8/ml$ in number and its pH value was lower than 4 as a result of propagation of lactic acid bacteria[3]. The decrease of pH value is very important for the later fungal fermentation stage, because the low pH prevents the growth of undesirable bacteria. After soaking the hulls are removed by rubbing the soaked beans between hands or by stamping them with feet in water (Plate 1-d). The loosened hulls are floated away with water, then the cotyledons are boiled shortly, cooled, surface dried, and inoculated with a starter (Plate 1-e, f) made of tapioca starch residues (Plate 1-g) containing *Rhizopus* spores. The inoculated cotyledons are packed into perforated polyethylene bags (Plate 1-h), or traditionally wrapped in banana leaves (Plate 1-i). Incubation is done on shelves at room temperature (an annual average temperature at Bogor is about 27°C) for 1 to 2 days (Plate 1-j) until the cotyledons are completely overgrown with the mold mycelium (Plate 1-k, l).

The occurrence at soybeans during fermentation is as follows. For the first few hours, there is a lag time during which germination of spores starts and rapid mold growth follows though no clear changes can be seen. When the growth of mold becomes evident,

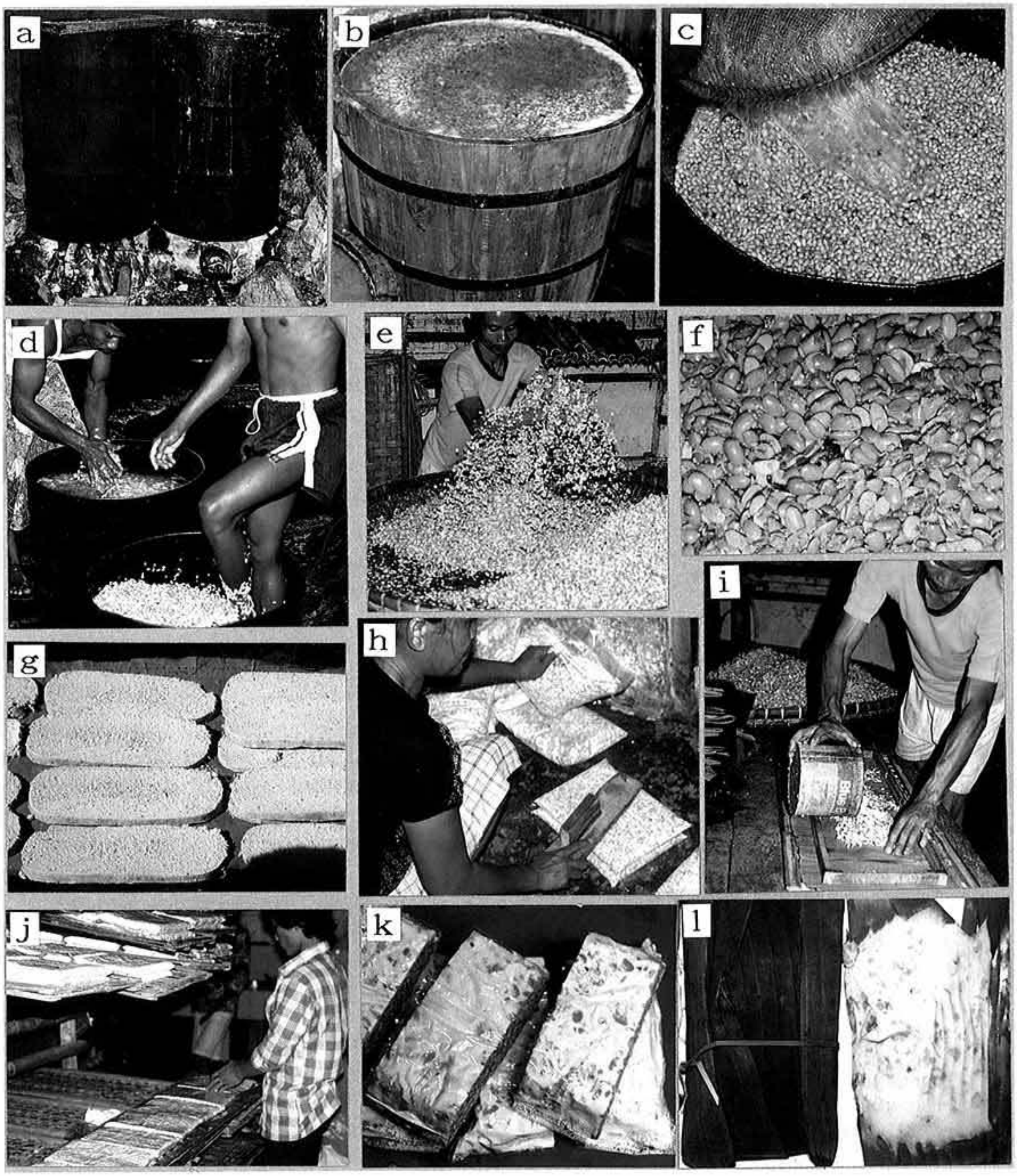

Plate 1. Manufacture of tempeh
a: Cooking of soybeans, b: Soaking, c: Soybeans after soaking, d: Dehulling, e: Mixing of starter, f: Soybeans after inoculation, g: Ragi tempeh (tempeh starter), h: Packing, i: Packing (traditional method), j: Incubation, k, l: Tempeh (final product).

the temperature of soybeans rises upper than the surrounding temperature. The extensive growth of mold makes soybeans form into a compact mass with secretion of enzymes such as proteases and lipases. Some of the fungal hyphae penetrate into the soybean cotyledons and secrete enzymes there too. Thus the soybeans become soft and easily digestible as the protein and lipids are decomposed to soluble matter. The tempeh is now ready to eat. However the mold continues to grow even after this stage, and spore formation and ammonia production due to protein breakdown appear finally. The pH value below 4 at the beginning rises to 6 to 6.5 at its optimum quality, and goes beyond 7.5 at the over ripe stage.

Vitamin B_{12} content of tempeh as compared with other fermented foods in the tropics

It is already known that tempeh contains vitamin B_{12}[12]. We examined V. B_{12} content of tempeh as compared with other fermented foods in the tropics[6]. Table 1 shows the V. B_{12} content of some fermented foods of the tropics. The daily requirement of V. B_{12} for man is estimated to be 3 μg. The test samples which were detected an appreciable amount of V. B_{12} were fish sauce (3-month fermentation, Thailand) 2.4 μg/100 g, fermented shrimp (Thailand) 2.5 μg/100 g, ka-pi (Thailand) 5.3 μg/100 g, tempeh (Indonesia) 4.6 μg/100 g, thua-nao (Thailand) 1.5 μg/100 g. However V. B_{12} detected in fish sauce, fermented shrimp and ka-pi may not be produced through fermentation, since they are made of animal materials. Those soy-fermented products such as tempeh and thua-nao are worthy of study, because V. B_{12} detected in them is considered to increase through fermentation, since soybeans do not contain any V. B_{12}[5].

Table 1. V. B_{12} content of fermented foods

No.	Sample name	Country	V.B_{12} (μg/100 g fresh)
1	Soy sauce mush	Tha	<0.1
2	Fish sauce, 3-month fermentation	Tha	2.4
3	Fish sauce	Tha	0.2
4	Oyster flavored sauce	Tha	0.7
5	Soy sauce	Tha	0.2
6	Fermented shrimp	Tha	2.5
7	Ka-pi, shrimp paste	Tha	5.3
8	Tempeh, fermented soybeans	Ind	4.6
9	Tauco, miso	Ind	<0.1
10	Ketchup, soy sauce	Ind	<0.1
11	Anchov fish extract, fish sauce	Tha	0.8
12	Oyster flavored sauce	Tha	<0.1
13	Shin-shin ami, fermented shrimp	Sin	0.3
14	Soy sauce mush	Sin	<0.1
15	Sufu, Chinese cheese	Sin	1.1
16	Neli com patis, fish sauce	Phi	<0.1
17	Diners patis, fish sauce	Phi	0.2
18	Mariz patis, fish sauce	Phi	<0.1
19	Dalisay patis, fish sauce	Phi	0.1
20	Thua-nao, fermented soybeans	Tha	1.5

Tha: Thailand, Ind: Indonesia, Sin: Singapore, Phi: Philippines.

Production mode of vitamin B_{12} in tempeh

Although vitamin B_{12} was detected in tempeh, not all the tempeh samples contained such an amount of V. B_{12}. The detectable amount was greatly dependent upon the freshness of samples. Only a few amount was detected in a fresh tempeh (Table 2)[6]. However, when the fresh tempeh was placed at 30°C, it was observed that its content increased significantly (Fig. 1). Tempeh prepared by using a pure *Rhizopus oligosporus* starter did not show any increase at the same condition that means microorganisms other than *Rhizopus* produce it. Vitamin B_{12} in tempeh is considered to increase as follows[9]. As stated before, the pH of soybeans is low at the beginning of fermentation that acts inhibitly on the growth of bacteria and favors for a fungal growth. This condition, on the one hand, makes a fungal fermentation successful even in an unsanitary environment and that also explains a low content of V. B_{12} in the fresh tempeh. However, as the fermentation proceeds, soybean protein is decomposed by action of mold enzymes with re-

Table 2. V. B_{12} content of tempeh from Indonesia and tempeh prepared at Japanese laboratory

Sample no.	V. B_{12} (μg/100 g fresh)	Note
1	4.6	Transported slowly from Indonesia[a] (Smell was not so good).
2	0.7	Transported quickly from Indonesia[b] (Smell was good).
3	0.05	Prepared with tempeh starter of Indonesia.
4	0.06	Prepared with *Rhizopus oligosporus* NRRL 2710
5	0.02	〃
6	0.03	〃

a) : It was exposed around 25℃ for 3 days before analysis.
b) : It was brought from Indonesia as quickly as possible. However, it was exposed around 25℃ for 8 hr before analysis.

leasing ammonia that invites an increase of pH value. Consequently, bacteria that usually favor neutral pH grow at the later stage of fermentation, and some of them contribute to yield V. B_{12}.

Thus tempeh is produced through cooperative works of microorganisms: lactic acid bacteria are to prepare a condition for the fungal growth by lowering pH, a mold is to nit and to solubilize soybeans by myceria and enzymes, and V. B_{12} producing bacteria are to make tempeh grade up to a substitutive food for meat by adding V. B_{12}.

Isolation and identification of vitamin B_{12} producing bacteria

In order to obtain V. B_{12} producing microorganisms, bacteria were isolated from those samples that were collected in Bogor and its

Table 3. V. B_{12} productivity of bacteria isolated from tempeh

Isolate no.	V. B_{12}[a]	Isolate no.	V. B_{12}[a]	Isolate no.	V. B_{12}[a]	Isolate no.	V. B_{12}[a]	Isolate no.	V. B_{12}[a]
3	> 66	120	0.4	169	72	232	1.0	309	181
4	>165	126	0.5	170	0.9	235	0.9	310	105
5	88	132	>165	171	0.3	238	1.3	315	170
7	143	133	39	172	103	240	10	327	33
10	>110	137	4	177	0.4	251	0.2	328	>110
12	>110	141	0.4	178	1.0	252	30	330	>110
13	>165	142	0.3	180	0.8	254	1.1	333	> 66
16	58	143	0.3	186	31	256	0.8	336	> 66
22	1350	144	5	187	0.5	263	0.9	337	380
25	81	145	0.4	189	0.7	264	1.0	348	0.5
31	>110	146	0.3	190	0.9	266	44	355	>110
32	>110	150	>110	191	54	267	0.6	362	83
36	253	151	0.4	192	1.0	270	510	363	>110
39	299	152	0.9	197	>110	271	0.6	366	51
70	92	153	0.6	199	0.4	273	0.9	368	120
72	56	154	0.6	200	188	274	0.7	372	178
81	99	155	247	201	0.6	275	125	373	1.0
84	>110	156	>110	205	145	276	97	375	0.1
90	78	157	0.9	210	178	289	125	380	0.5
91	>110	158	0.4	217	0.7	290	68	387	0.4
95	122	159	0.3	222	0.4	291	112	389	0.2
110	0.1	160	0.6	224	0.8	307	168		
114	0.7	162	0.7	228	168	308	171		

a) : V. B_{12} productivity (ng/cells in 5 m*l* of culture).

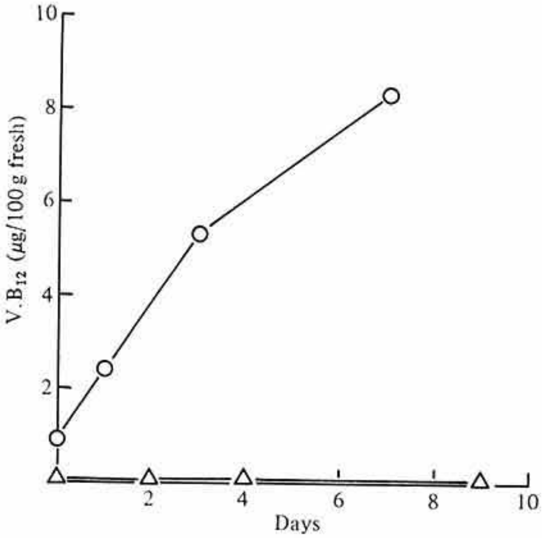

Fig. 1. Changes of V. B_{12} content
Samples were put in the plastic bag and placed at 30℃.
○ : Indonesian tempeh, △ : Tempeh prepared with *Rhizopus oligosprus* NRRL 2710.

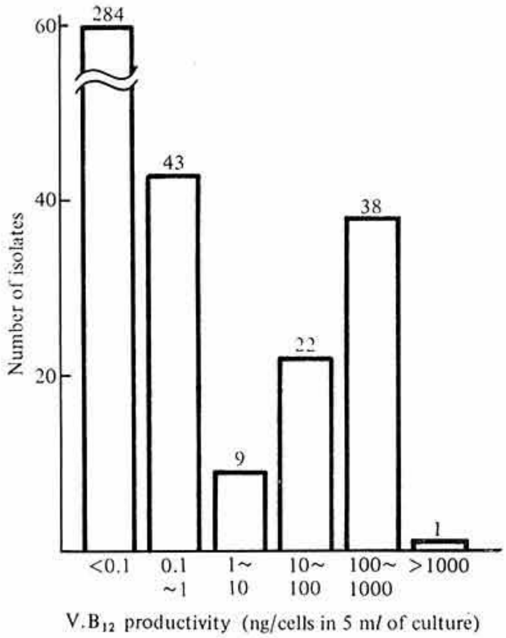

Fig. 2. Distribution of V. B_{12} productivity

Table 4. Idenfitication of isolate no. 22

Characteristics	*Klebsiella pneumoniae*	Isolate no. 22
Gram test	—	—
Motility	—	—
Shape	Short rod	Short rod
Oxidase	—	—
Indole	—	—
Methyl red test	—	—
Voges-Proskauer test	+	+
Nitrate reductase	+	+
H_2S production	—	—
Urease	+	+
Phenylalanine deamination	—	—
Lysine decarboxylase	+	+
Arginine dihydrolase	—	—
Ornitihine decarboxylase	—	—
β-Galactosidase	+	+
Utilization of:		
Citrate	+	+
Malonate	+	+
Fermentation of:		
D-Glucose	+	+
Adonitol	d[a]	+
L-Arabinose	+	+
Inositol	+	+
Raffinose	+	+
D-Sorbitol	+	+
Lactose	+	+
L-Rhamnose	+	+
Sucrose	+	+

a) : differs among strains.

surrounding towns[7]. Table 3 shows V. B_{12} productivity of the respective isolates and Fig. 2 shows the distribution of productivity of them. Sixty-nine out of 397 isolates showed some degrees of response to *Lactobacillus leichmannii* ATCC 7830. This microorganism requires a very careful handling[4], though it is generally used for a V. B_{12} assay. One isolate showed V. B_{12} productivity at the level of more than 1,000 ng/(cells in 5 *ml* of culture). Thirty-eight isolates showed 100 ng to 1,000 ng (the same as the above unit), 22 isolates showed 10 ng to 100 ng, and 9 isolates showed 1 ng to 10 ng. The known V. B_{12} producer, *Bacillus megaterium* IAM 1166 used as a control microorganism showed a value of 402 ng (the same as the above unit). Vitamin B_{12} productivity by various microorganisms seen in the textbook is 2.3 to

Table 5. Representative vitamin B_{12} producing bacteria in tempeh

No.	Sampling place	V. B_{12} producer numbers/Total numbers	Representative V. B_{12} producing bacteria	Isolate no.	V. B_{12} productivity[a)]
1	Pasar, Bogor	14/42	*Klebsiella pneumoniae*	22	1350
			Resembles either *K. terrigena* or *K. planticola*	5	88
			K. pneumoniae sub. *ozaenae*	31	>110
2	Tegallega, Bogor	6/31	Resembles *K. terrigena*	81	99
			K. pneumoniae	91	>110
3	Semplak, Bogor	1/34	G(+), rod (*Bacillus* sp.)	95	122
4	Cipanas, Pacet	3/12	*K. pneumoniae*	132	>165
			Escherichia coli	133	39
5	Cianjur	3/15	Resembles either *K. terrigena* or *K. planticola*	150	>110
			K. pneumoniae	155	247
6	Caringin, Cicurug	2/14	*K. pneumoniae*	169	72
7	Kongsi, Cicurug	1/ 8	*K. pneumoniae*	172	103
8	Caringin, Cicurug	5/22	G(+), rod (*Bacillus* sp.)	186	31
			Resembles *K. planticola*	191	54
9	Pasar, Bogor	3/15	Resembles *K. planticola*	200	188
			Resembles *K. terrigena*	210	178
10	Rawasigdin, Cicurug	2/10	G(+), rod (*Bacillus* sp.)	228	168
11	Bababan, Ciparay, Bandung	2/13	*Enterobacter cloacae*	252	30
12	Sukasari, Bogor	5/17	Resembles *K. terrigena*	266	44
			K. pneumoniae	270	510
13	Sukasari, Bogor	3/16	*K. pneumoniae*	275	125
14	Abesin, Bogor	5/13	*K. pneumoniae*	307	168
			Resembles *K. terrigena*	308	171
15	Menteng, Bogor	5/11	*K. pneumoniae*	328	>110
			Resembles *K. planticola*	330	>110
16	Kedungwaringin, Kedunghalang	2/25	*K. pneumoniae*	337	380
17	Ciomas, Ciomas	4/ 7	G(+), rod (*Bacillus* sp.)	362	83
			G(−), oxidase (+), rod	366	51
			K. pneumoniae	368	120
18	Ciluar, Kedunghalang	2/ 9	*K. pneumoniae*	372	178

a) : ng/cells in 5 m*l* of culture.

59 mg/*l*[1]. The highest value obtained by this study was 1,350 ng/(cells in 5 m*l* of culture) which corresponds to 0.27 mg/*l*.

Identification tests were performed on those isolates that produced V. B_{12} more than 10 ng/(cells in 5 m*l* of culture)[8]. Table 4 shows the identification test on isolate no. 22. Table 5 shows the summary of results. Most of V. B_{12} producing bacteria were identified as *Klebsiella pneumoniae*, already published by Shurtleff and Aoyagi[12], namely 13 out of 33 isolates were *Klebsiella pneumoniae*. However, other bacteria than *Klebsiella pneumoniae* were also found. Twelve isolates were closely resembled either *K. terrigena* or *K. planticola*, one was identified as *K. pneumoniae* sub. *ozaenae*. sp., two were identified as *Enterobacter cloacae* and *Escherichia coli*, four were unidentified Gram-positive rods (*Bacillus* sp.), and one was unidentified Gram-negative rod which differs from *Klebsiella* sp. in the oxidase test.

Prospects

Indonesia has not only a climate of high temperature and high humidity where molds propagate well and where tempeh is easily produced without failures, but also is an

Islamic society where most people are vegetarians who are tend to be short of an animal factor or vitamin B_{12}. It is possible to mention that the health of people, in particular that of poor people, has been sustained by tempeh which supply not only cheap protein but also an animal factor or $V.B_{12}$ that is usually taken from other animals through meat diet.

The $V.B_{12}$ poducing bacteria isolated from tempeh are expected to be utilized for other fermented foods. For instance, Japanese natto which is also made of soybeans may be possible to be improved the $V.B_{12}$ content. To examine this possibility, investigations are now underway from two points of view. Whether Japanese natto bacteria have $V.B_{12}$ producing ability or not, and are there any useful methods of transferring $V.B_{12}$ producing ability to a natto bacterium?

On the first question, it was found that any Japanese natto bacteria have not $V.B_{12}$ producing ability[5]. In old reference, it was reported that natto contained $V.B_{12}$ and natto bacterium had $V.B_{12}$ producing ability. However, we have found that the $V.B_{12}$ producing ability is missing in natto bacteria.

On the second question, we are now under investigation by the cell fusion method which is one of the promising methods of gene transfer in improvement of microorganisms for practical use. Intraspecific cell fusion between mutants of *Bacillus subtilis* IAM 1207 was so far successful[10,11]. Most of $V.B_{12}$ producing bacteria found in tempeh were *Klebsiella* sp. whose genus is different from *Bacillus natto* and that means almost impossible to perform cell fusion between them. However, other bacteria than *Klebsiella* sp. were also found in tempeh as $V.B_{12}$ producers. Among them there were *Bacillus* sp. that are worthy to be focused on for cell fusion counterparts. Recently a success of interspecific cell fusion between *Bacillus natto* and *Bacillus megaterium* was reported[2], that is very encouraging us to continue our works.

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