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Mutagenic Analysis of Fermenting Strains and Fermented Brine for Stinky Tofu

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ABSTRACT

Stinky tofu, a popular fermented food in Taiwan and South China, is conventionally made by open-type natural fermentation in which the product quality is not easily controlled. The Food Industry Research and Development Institute (FIRDI) in Taiwan has attempted to isolate the major fermenting strains from stinky brine in order to produce stinky tofu that is sanitary and ensure its quality. This study investigated the mutagenicities of isolated fermenting strains and their fermented brine for stinky tofu by two assays: (I) Ames test and (II) mutation assay with human lymphoblast TK6 cells. In the Ames test, the number of revertants of *Salmonella typhimurium* TA98 and TA100 was at the same levels under the dose of fermenting strain lysates (10^7 cfu/ plate) and 10-fold dilution of the fermented brine compared with the respective control. In the mutation assay with TK6 cells, the same samples were tested and the mutant frequencies were comparable to the respective control. Experimental results provided no evidence of mutagenic activity attributed to the tested materials.

Key words: mutagenicity, stinky tofu, Ames test, human lymphoblast TK6 cells

INTRODUCTION

Tofu is a popular food in the Orient made from soybeans. A special fermented tofu, stinky tofu also referred to as “Stinky Chinese Cheese”, receives its name from its unusual fragrance. The conventional process of making stinky tofu initially involves mixing vegetables, shrimp and salt together in a big-mouth jar and then exposing the mixture to the air for several months by natural microbial fermentation until a stinky brine is obtained. During this period, microorganisms spontaneously grow in the mixture and secrete many enzymes, particularly protease and lipase[1]. Once the brine has developed a unique stinky smell, tofu is submerged in the brine for 4-6 hours and then washed with water and kept at 4˚C overnight for aging. After these processes, stinky tofu is formed and generally deep-fried or steamed.

However, other microorganisms easily contaminate the stinky brine made by this conventional open-type fermentation and the quality of each batch can not be ensured. The Food Industry Research and Development Institute (FIRDI) in Taiwan has attempted to isolate the major fermenting floras from stinky brine in order to produce sanitary and reliable stinky tofu[2]. Among the major fermenting strains for stinky brine having been isolated are *Bacillus sphaericus* and several other bacteria, such as *Enterococcus gallinarum*, *Acinetobacter spp*. and *Corynebacterium spp*[2]. Isolated mix-culture fermentation is characterized by a shortened period of brine-making, i.e., from 6 months to only one month, and a hygienic process that avoids other microbial contamination.

The mutagenicity of isolated fermenting strains and their fermented stinky brine must be verified to ensure their bio-safety. Two *in vitro* assays are available for mutagenicity: (I) Ames test[3-5] and (II) mutation assay with human lymphoblast TK6 cells[6]. According to our results, the mutagenic potential of the fermenting strains and their fermented brine of stinky tofu were negative: both in the Ames test and the human lymphoblast TK6 cells test.

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MATERIALS AND METHODS

I. *Fermenting Strains of Stinky Tofu*

The fermenting strains and fermented brines for stinky tofu[2] were kindly provided by Dr. F. L. Lee at the Culture Collection and Research Center, FIRDI, Taiwan. The procedure is briefly described as follows: samples of stinky brine (the fermented broth for making stinky tofu) collected from Taiwan were used to isolate the major fermenting strains of stinky tofu. The samples were first inoculated to a brine medium (see below) and incubated without shaking at 30˚C for one month. Samples with a distinct stinky odor were selected and transferred again to confirm the reproducibility. Moreover, the fermented broth with favored stinky odor was serially diluted and poured on tryptic soy agar (TSA) plates and incubated at 30˚C for 24 hr. The bacterial colonies on the TSA plates were scraped and inoculated to sterile fresh brine medium with static fermentation at 30˚C for one month. This process could reduce the population of microbial flora and maintain the ability to produce the stinky odor. A stinky brine sample A was collected from Hsinchu and its 1: 100 dilution which was referred to as A2 still had the best odor producing ability. The A2 sample was plated on TSA agar and different morphologic colonies among the A2 group were tested for their stinky odor productivity. One sub-group named

A2'group contained 8 different major colonies and had a flavor most similar to stinky tofu after sensory evaluation[2]. This sub-group was identified and grouped using an identification system of microbial fatty acid composition[2]. Bacillus *sphaericus* was dominant in this A2' group. This group also contained several other bacteria, including *Enterococcus gallinarum*, *Acinetobacter spp*. and *Corynebacterium spp*.

II. *Brine Medium*

The medium containing Chinese green cabbage 30% (w/v), bamboo shoots 20%, Tofu 10%, shrimp 5%, salts 1% and water 34% was homogenized and autoclaved. The sterile medium was inoculated with the fermenting strains of A2 and A2' groups at 30˚C for one month to produce fermented stinky brine.

III. *Preparation of Test Samples*

A2 and A2' strains were both cultured with TSA medium (DIFCO Laboratories, Sparks, MD) at 30˚C for 24 hr. Cells were scraped and washed with phosphate buffer saline (PBS, Gibco BRL., Island, NY) and, then, lysed with dimethyl sulfoxide (DMSO, Merck KgaA, Germany) as stock samples (10^9 cells in 1 mL DMSO). Stock samples were further diluted with PBS and the final DMSO concentration was 1% in this study. The fermented stinky brines were centrifuged (12,000 xg, 10 min.) and the supernatants were collected and filtrated with 0.2 μm membrane.

IV. *Ames Test*

The strains of *Salmonella typhimurium* TA98 and TA100 were purchased from Dr. B. N. Ames of the University of California, Berkeley. In addition, both strains were routinely assessed for their sensitivity to crystal violet (*rfa* wall mutation), ampicillin resistance (pKM101 plasmid), and the frequency of autoreversion mutation (his^-) as described by Maron and Ames[7]. The preincubation procedure was used to increase the sensitivity of assays[8]. The fresh *S. typhimurium* TA98 and TA100 cultures, tested samples and S9 mix were mixed and incubated in a water bath for 20 min at 37˚C. The activation system (S9 mix) contained 4% of Aroclor 1254-induced rat liver homogenate S9 (ICN Biomedicals, Costa Mesa, CA). The his^+ revertant colonies were counted after incubation at 37˚C for 48 hr with an automated colony counter (AccuCount^{TM}1000, BioLogics, Gainesville, VA). The toxicity of the test samples was then evaluated by observing the bacterial growing lawn under an inverted microscope. Chemicals used as positive control differed according to Ames strains and with or without S9 mix. For assays containing S9 mix activation, 2-aminofluorene (10 μg/ plate) (Merck KgaA, Germany) was used as the positive control in both TA98 and TA100 strains. For assays without S9 mix activation, the positive controls were 4-nitro-*o*-phenylene-diamine (20 μg/ plate) (Sigma, St. Louis, MO) and sodium azide (1.0 μg/ plate) (Merck KgaA, Germany) for TA98 strain and TA100 strain, respectively. DMSO and fresh brine medium were used as negative controls in cell lysate and fermented stinky brine assays, respectively, for both TA98 and TA100 strains.

V. Mutation Assay with Human TK6 Cells

Human lymphoblast TK6 (thymidine kinase heterozygote, *tk*+/-) cell line was obtained from the Culture Collection and Research Center, FIRDI and grown in RPMI 1640 medium (Gibco BRL) plus 10% horse serum (HyClone, Logan, UT) at 37˚C, 5% CO_2 incubator (NuAire, Plymouth, MN). The cells were examined without mycoplasma contamination by direct culture and Hoechst 33258 fluoresent staining method on a regular basis[9]. The protocols of mutation assay were described by Liber and Thilly[6]. Plating efficiencies and mutant frequencies were performed by 96-well microtiter assay[10, 11]. The fermenting strains' lysate (10^7/mL) and their fermented brine (100-fold dilution) did not change the pH and osmolality of culture medium. The osmolality was measured by osmometer (Vapro, Logan, UT). For the positive control, cells were exposed to 160 μM ethylmethane sulfonate (EMS, Sigma, St. Louis, MO) for 16 hr[11]. DMSO and fresh brine medium were used as negative controls in cell lysate and fermented stinky brine assays, respectively.

Calculations of plating efficiency (P.E. %) and mutation frequency are summarized below[6]:

$$\text{P.E.\%} = \frac{\text{-ln P(0) x 100\%}}{\text{number of cells per well}}$$

$$\text{Where P(0)} = \frac{\text{number of empty wells}}{\text{total number of wells plated}}$$

$$\text{Mutant frequency} = \frac{\text{total mutant colonies observed}}{\text{(P.E. \%) x (total cells plated)}}$$

VI. *Statistic Analysis*

Results are expressed as the mean ± SE, with n = 3. Duncan Multiple Range Test was conducted to statistically analyze the differences between means.

RESULTS AND DISCUSSION

In this study, two mutagenic assays, i.e., the reverse mutation (his^- to his^+) by Ames test with procaryote and forward mutation (*tk*+/- to *tk*-/-), were conducted on human TK6 cells to examine whether the microbial strains of stinky tofu and their fermented stinky brine were mutagenic.

The Ames mutagenic tests were performed with stinky tofu fermenting strains (10^7, 10^6, 10^5 cfu/ plate) and their fermented stinky brine (10-, 100-, 1000-fold dilution) from A2 group and its reduced-microbial flora A2' group. Each sample was tested for the mutagenic potential by *S. typhimurium* TA98 and TA100 in the absence and presence of 4% S9 mix

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activation.

None of the test doses caused cytotoxicity for the Ames strains TA98 and TA100 as evaluated by observing the bacterial growing lawn under an inverted microscope (data not shown). According to Table 1, revertants of the test samples (cell lysates and stinky brine) in TA98 were 14 to 51 and 5 to 47 with and without S9 mix activation, respectively. Furthermore, revertants of the test samples in TA100 were 125 to 176 and 123 to 165 with and without S9 mix activation, respectively. Azizan and Blevins (1995) suggested that a mutagenic ratio (revertants of sample/ revertants of negative control) must be greater than two to infer a positive mutagenic response[12]. Although statistically greater than spontaneous revertants, some results were still lower than the ratio of two. This finding suggested that there should be no mutagenic potential activity of the A2 and A2' group of stinky tofu fermenting strains and their fermented stinky brine as evaluated by the Ames TA98 and TA100 assays.

In addition to the Ames mutagenic test, mammalian cell mutation assay is conventionally used not only to evaluate the intrinsic response of the mammalian genome to mutagens, but also to provide quantitative safety data in food ingredi-

Table 1. Mutagenicity of fermenting strains and fermented brine of stinky tofu in the Ames test

		No. of histidine revertants/ plate[a]			
		TA98		TA100	
Compound and amount (/ plate)		+S9	-S9	+S9	-S9
Spontaneous revertants		32 ± 4	49 ± 1	127 ± 8	158 ± 9
Control					
DMSO (10 μL) [b]		30 ± 1	41 ± 3	147 ± 4	136 ± 4
Fresh brine (0.1 mL) [c]		40 ± 3	49 ± 3	134 ± 5	148 ± 4
2-Aminofluorene (10 μg) [d]		>1000*	–	>1000*	–
4-Nitro-*o*-phenylene-diamine (20 μg) [e]		–	>1000*	–	–
Sodium azide (1.0 μg) [f]		–	–	–	>500*
Cell lysates	(cfu)				
A2 group [g]	10^5	34 ± 4	46 ± 4	137 ± 5	127 ± 4
	10^6	35 ± 1	40 ± 1	154 ± 6*	149 ± 7
	10^7	31 ± 2	40 ± 4	157 ± 5*	145 ± 6
A2'group [h]	10^5	32 ± 2	45 ± 1	125 ± 5	165 ± 9
	10^6	26 ± 1	38 ± 1	130 ± 5	146 ± 10
	10^7	33 ± 3	47 ± 4	137 ± 13	153 ± 8
Fermented brine	dilution (fold)				
A2 group	1000	35 ± 3*	11 ± 4#	176 ± 5#	151 ± 3
	100	14 ± 4	25 ± 4#	145 ± 6	141 ± 4#
	10	32 ± 6	27 ± 6#	129 ± 2	127 ± 5#
A2' group	1000	25 ± 2	5 ± 2#	141 ± 6	147 ± 8
	100	31 ± 5	38 ± 5	149 ± 8	137 ± 2#
	10	51 ± 0*	38 ± 6	128 ± 8	123 ± 3#

[a] Results are expressed as mean ± SD of triplicates.
[b] DMSO was used as negative controls for cell lysates.
[c] Fresh brine was used as negative control for stinky brine.
[d] Positive control in TA98 and TA100 with S9 mix.
[e] Positive control in TA98 without S9 mix.
[f] Positive control in TA100 without S9 mix.
[g] The primitive formulation of fermenting strains.
[h] The partial-purified strains formula of A2 group (see Materials and Methods).
* Significantly greater than spontaneous revertants at $p \leq 0.05$.
# Significantly lower than spontaneous revertants at $p \leq 0.05$.

Table 2. Plating efficiencies and mutant frequencies of *tk* mutants in cultures of human TK6 cells exposed to fermenting strains and fermented brine of stinky tofu strains

Compound	P. E.[e] (%)	Mutant frequency (x 10^{-6})	
		10 days (NG[f])	20 days (SG[g])
1% DMSO[a]	73.7 ± 2.4	1.2 ± 0.2	6.9 ± 2.3
A2 group[c] (10^7cfu/mL)	76.0 ± 2.5	0.3 ± 0.3	5.7 ± 0.9
A2' group[d] (10^7cfu/mL)	118.4 ± 6.3	0.0 ± 0.0	3.4 ± 0.8
EMS[b]	46.3 ± 1.4	6.4 ± 0.8*	46.1 ± 1.1*
Fresh brine[a]	51.9 ± 5.6	7.9 ± 0.5	14.6 ± 0.5
A2 group[c] (1/100)	41.3 ± 2.3	1.3 ± 0.0	17.7 ± 1.3
A2' group[d] (1/100)	51.9 ± 2.7	1.3 ± 0.3	15.3 ± 0.3
EMS[b]	47.0 ± 3.3	33.2 ± 4.4*	42.9 ± 2.5*

The mutagenicity experiments for fermenting strains and fermented brine of stinky tofu strains were executed separately.
[a] Negative control.
[b] Positive control: ethylmethanol sulfoxide.
[c] The primitive formulation of fermenting strains.
[d] The partial-purified strains formula of A2 group.
[e] Plating efficiencies.
[f] Normal growth mutants.
[g] Slow growth mutants.
* Significantly different from negative controls at $p \leq 0.05$.

ents, chemicals and bioproducts[13-16]. In the mutagenicity test of human lymphoblast TK6 cell, the phenotype of mutants at the *tk* locus can be divided into two subsets: normal growth mutants and slow growth mutants[17]. Normal growth *tk*-/- mutants may originate from base-pair substitution, frameshifts, insertions and deletions in the gene sequence and require 10 days of cultivation. Slow growth *tk*-/- mutants may arise from large alterations in the *tk* region, which remove the entire copy of the active *tk* gene and require a longer (20 days) cultivation. Both mutants should be counted to evaluate the mutagenic potential[17].

The cell lysates of A2 and A2' strains were tested for their mutagenic potentials by human lymphoblast TK6 cell assays. Those results indicated no cytotoxicity effect when the concentration of cell lysates (10^7 cfu/ mL) was used as a pre-test experiment (data not shown). According to Table 2, the plating efficiencies (P.E.%) of TK6 cells treated with cell lysates of the fermenting strains of A2 and A2' groups were 76.0 and 118.4%, respectively. In addition, the mutant frequencies of both normal growth mutants (NG) and slow growth mutants (SG) of A2 and A2' strains were no significantly greater than the negative controls (1% DMSO) at $p \leq 0.05$.

The fermented stinky brine of A2 and A2' strains were also tested for potential mutagenicity at TK6 cells. TK6 cells were exposed to the fermented stinky brine of A2 and A2' groups (1:20, 1:50 and 1:100 dilution with the culture medium) for two days. Both 1:20 and 1:50 dilution of stinky brines exhibited a toxic effect towards cells, with more than 30% cell dead. A dilution of 1:100 had no cytotoxicity (data not shown), thus only the 1:100 dilution of stinky brine was tested. The plating efficiencies of the fermented stinky brine of A2 and A2' groups (1: 100 dilution) were 41.3 and 51.9%, respectively, as shown in Table 2. The mutant frequencies of normal growth (NG) mutants and slow growth (SG) mutants did not significantly differ with respect to the respective negative controls ($p \leq 0.05$). These results suggest that there should be no mutagenic potential of the stinky tofu fermenting strains and their fermented stinky brine for human lymphoblast TK6 cells.

This study evaluated the mutagenicity of the microbial strains of stinky tofu and stinky brine by both the Ames test and the mutation assay with TK6 cells. Experimental results provided no evidence of mutagenic activity attributed to the tested materials.

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臭豆腐發酵菌株及其發酵液之致突變性分析

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摘　要

臭豆腐是我國頗具鄉土風味的發酵食品，很受一般消費大眾的喜愛。市面上的臭豆腐發酵過程採用傳統開放式製造，菌相複雜，品質不易控制。食品工業發展研究所由台灣各地收集臭豆腐浸漬液樣品，經純化分離後篩選出發酵臭豆腐的優良菌株，成功的開發出利用純菌發酵之臭豆腐技術。為進行其安全性評估，本研究進一步測試浸漬臭豆腐之臭滷水和發酵菌株之致突變性，所採用的測試方法有二：分別為安氏試驗法及人類淋巴母細胞TK 6致突變試驗。結果顯示，在安氏試驗方面不論是否添加大鼠肝臟萃取液，菌體在10^7以下的濃度及臭滷水10倍以上稀釋的劑量，皆對安氏測試菌株*Salmonella typhimurium* TA98與TA100不具有致突變性。同樣在此劑量下，測試樣品對於人類的TK6細胞也沒有發現有致突變的結果。

關鍵詞：致突變性，臭豆腐，安氏試驗，人類淋巴母細胞TK 6