

Vitamin B₁₂ Production by *Citrobacter freundii* or *Klebsiella pneumoniae* during Tempeh Fermentation and Proof of Enterotoxin Absence by PCR

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The influence of some fermentation parameters on vitamin B₁₂ formation by strains of *Citrobacter freundii* and *Klebsiella pneumoniae* isolated from Indonesian tempeh samples during tempeh fermentation was investigated. A decrease in fermentation temperature from 32 to 24°C led to a decrease in vitamin B₁₂ formation. Inoculation of soybeans with different numbers of cells of *C. freundii* at the beginning of solid-substrate fermentation showed that only the velocity of vitamin formation and not the final amount of vitamin formed depended on the number of cells. The addition of cobalt and 5,6-dimethylbenzimidazole increased the vitamin B₁₂ content of tempeh. Nevertheless, levels of incorporation of the two precursors into the vitamin B₁₂ molecule were very low. Neither *C. freundii* nor *K. pneumoniae* possessed the genes encoding the enterotoxins Shiga-like toxin SLT IIA, heat-labile enterotoxin LT I_h, and heat-stable enterotoxin ST I_h, as indicated by PCR. This result supports the suggested use of these two strains to form vitamin B₁₂ during tempeh fermentation in Indonesia.

Tempeh (tempe kedelai) is a traditional fermented soybean food in Indonesia, where it serves as a cheap basic foodstuff in the nutrition of the Indonesian population. It is produced by cooking and hulling soybeans. After a soaking process, the soybeans are cooked once more. For solid-substrate fermentation (SSF), they are inoculated with molds of *Rhizopus* spp. In the traditional process, soybeans are soaked overnight, and spontaneous bacterial acidification takes place. In modern industrial processes in the Western world, bacterial acidification is replaced by artificial acidification with lactic acid. Tempeh is highly significant because of its high content of amino acids, fatty acids, and vitamins (3, 6, 17, 18, 20, 33). The most important vitamin is vitamin B₁₂, which is normally not found in vegetarian foodstuffs (16) and which is formed by bacteria that accompany the fermentation process (20, 22, 28).

In a screening for vitamin B₁₂-producing bacteria isolated from Indonesian tempeh samples, a strain of *Citrobacter freundii* was found to produce the highest vitamin B₁₂ concentration during the tempeh SSF. A *Klebsiella pneumoniae* strain also formed large amounts of this vitamin (20). Besides vitamin B₁₂, these two strains also formed other water-soluble vitamins, such as riboflavin and vitamin B₆. *C. freundii* also produced vitamin B₁₂ during the soaking of soybeans (8).

Some strains of the family *Enterobacteriaceae* are known to be capable of producing enterotoxins (5, 14, 19). Therefore, we investigated whether the aforementioned *C. freundii* and *K. pneumoniae* strains possess three known genes for enterotoxin production: the genes for Shiga-like toxin SLT IIA (30), for heat-labile enterotoxin LT I_h (34), and for heat-stable enterotoxin ST I_h (24).

The aim of the present work was to investigate the influence of fermentation parameters and additions on vitamin B₁₂ formation. Furthermore, it was important to know whether the tested *C. freundii* and *K. pneumoniae* strains possess genes

encoding enterotoxins, because the intention is to use these strains for tempeh fermentation.

MATERIALS AND METHODS

Microorganisms. The mold *Rhizopus oligosporus* (isolate Tebo), which was isolated from Indonesian tempeh (17) and which provides high yields of water-soluble vitamins (20), was used for tempeh fermentation. *C. freundii* (isolate 259) and *K. pneumoniae* (isolate 274) were also added at the beginning of the fermentation process because they had been characterized as producing high levels of vitamin B₁₂ during SSF (20).

Microorganisms isolated from tempeh samples which had been soaked according to the traditional process in our laboratory were identified on the basis of the key to the genera in *Bergey's Manual of Determinative Bacteriology* (7) and checked against the information in *Bergey's Manual of Systematic Bacteriology* (21, 32).

For PCR analysis, the following reference strains were used: *Escherichia coli* C600 W34 (SLT IIA⁺), *E. coli* 2348/I (SLT IIA⁻), *E. coli* O:128H⁻ (LT⁻ ST⁺), *E. coli* O:6H⁻ (LT⁺ ST⁺), and *E. coli* G 1253 (LT⁺ ST⁻); they were kindly provided by H. Karch, Institut für Hygiene und Mikrobiologie, University of Würzburg, Würzburg, Federal Republic of Germany. *E. coli* C600 EWD 299 (LT⁺ ST⁻) and *E. coli* HB101 SLM 004 (LT⁻ ST⁺) have been described by Moseley et al. (23) and were kindly provided by them.

Process of tempeh fermentation. The modern tempeh fermentation process was carried out in the standardized way as developed by Hering et al. (17). The beans (300 g, wet weight) were inoculated with a spore suspension (1.8 ml) of *R. oligosporus* Tebo (10⁶ spores ml of 0.9% NaCl⁻¹ corresponding to 6 × 10³ spores g of beans⁻¹). In fermentations with *C. freundii* or *K. pneumoniae* 1.8 ml of a cell suspension (10⁷ cells ml of 0.9% NaCl⁻¹, corresponding to 6 × 10⁴ cells g of beans⁻¹) was added. After that, the beans were fermented at 32°C for 34 h.

The traditional fermentation process was carried out as

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TABLE 1. Nucleotide sequences of primer pairs and PCR conditions used for the amplification of enterotoxin genes

Primer	Nucleotide sequence	PCR conditions						Expected size of fragment (bp)
		Denaturing		Annealing		Extension		
		°C	s	°C	s	°C	s	
SLT IIA start SLT IIA stop	5'-CCCGGATCCATGAAGTGTATATTATTTAAATGG-3' 5'-CCCGAATTCCTTATTTACCCGTTGTATATAAAAA-3'	94	45	53	60	72	60	960
LT Ih start LT Ih stop	5'-GCGTTACTATCCCTCTCTATG-3' 5'-ATTGGGGGTTTATTATTTC-3'	94	40	49	60	72	45	320
ST Ih start ST Ih stop	5'-TCCCTCAGGATGCTAAAC-3' 5'-GCAACAGGTACATACGTT-3'	94	30	47	60	72	30	244

described by Baumann et al. (3) with *C. freundii* only. Soybeans (one part) were washed and cooked with demineralized water (three parts) for 30 min. After a period of cooling down, the soaking water was inoculated with *C. freundii* (3×10^7 cells ml of soaking water⁻¹), and the beans were soaked at 30°C for 15 h. After separation of the beans and soaking water, the beans were hulled, cooked again for 30 min, surface dried, and divided into two portions. One portion was autoclaved before SSF with the *Rhizopus* sp., and the other portion was not sterilized.

The portion of the artificially soaked soybeans and the two portions of the traditionally soaked soybeans were divided into three parts each. One part was inoculated with the *Rhizopus* sp. and *C. freundii*, the second part was inoculated with the *Rhizopus* sp. only, and the third part was inoculated neither with the *Rhizopus* sp. nor with *C. freundii* (control).

As five fermentations under the same conditions had shown a maximal relative standard deviation of $\leq 9.2\%$, we did not replicate each fermentation.

Variation of fermentation parameters. For analyzing the influence of incubation temperature, tempeh fermentations were carried out at 24, 28, and 32°C. The influence of different numbers of bacterial cells on vitamin B₁₂ formation was investigated by inoculating soybeans with suspensions of 10^3 to 10^8 cells ml⁻¹ (equivalent to 7×10^0 to 7×10^5 cells g of beans⁻¹).

Cobalt(II)-sulfate-heptahydrate and 5,6-dimethylbenzimidazole at a concentration range of 0 to 400 mg of bacterial suspension liter⁻¹ (equivalent to 0.135 to 2.7 $\mu\text{g g}$ of beans⁻¹) were added to the bacterial suspension, with which the beans were inoculated before SSF with the *Rhizopus* sp. The yield was determined by dividing the number of vitamin B₁₂ molecules formed by the number of precursor molecules added.

Determination of bacterial growth in tempeh. The growth of bacteria in tempeh was determined as described by Hausler (15). Bacteria were spread out on plate count agar (E. Merck, Darmstadt, Federal Republic of Germany) supplemented with cycloheximide (100 mg liter⁻¹).

Vitamin B₁₂ analysis. Vitamin B₁₂ analysis was done with a microbiological assay as described by Okada et al. (28); this assay is considered to distinguish between different forms of corrinoids: physiologically active vitamin B₁₂ (cobalamins), which can be used by humans, and analogous forms, which cannot be so used. All vitamin B₁₂ assays were executed in triplicate at two different dilutions, each analyzed eight times. The maximal relative standard deviation was $\leq 7.5\%$.

PCR analysis. For PCR analysis, *C. freundii* (259), *K. pneumoniae* (274), and the reference strains were cultivated in 25 ml of LB medium, containing (grams liter⁻¹) tryptone (10), yeast extract (5), and NaCl (10), (pH 7.5), at 27°C on a shaker

(model G 76 Gyrotory water bath shaker; New Brunswick Scientific Co., Edison, N.J.) for 15 h at 200 rpm. In the case of SLT IIA, ampicillin (100 ng ml⁻¹; Sigma Chemical Co., St. Louis, Mo.) was added to one of two parallel cultures because the formation of SLT IIA is sometimes enhanced in the presence of this antibiotic.

The oligonucleotides used as primers for the amplification of a part of the SLT IIA gene were described by Gunzer et al. (13). The primers used for the amplification of a part of the LT Ih gene were derived from the B subunit (34). For the amplification of a part of the ST Ih gene, the primers were derived from the ST sequence (24). The nucleotide sequences of the primer pairs and the PCR conditions used for the amplification of the three genes are listed in Table 1. All primers, which were purified by high-performance liquid chromatography, were purchased from MWG-Biotech (Ebersberg, Federal Republic of Germany). The PCR analysis was done as described by Schmidt et al. (30).

RESULTS

Influence of fermentation temperature. With both bacterial strains, fermentations performed at 32°C resulted in the highest vitamin B₁₂ content (Table 2). Lowering the incubation temperature from 32 to 28°C and from 32 to 24°C resulted in a decrease in vitamin B₁₂ content in tempeh. A further effect of the lowered temperature was a prolongation of the fermentation time for the *Rhizopus* sp. from 34 h (32°C) to 50 h (28°C) and to 67 h (24°C). *C. freundii* formed higher vitamin B₁₂ concentrations than *K. pneumoniae* at all temperatures investigated.

Influence of cobalt and 5,6-dimethylbenzimidazole. The addition of cobalt resulted in an increase in vitamin B₁₂ formation (Fig. 1). Fermentations with *K. pneumoniae* which were supplemented with low concentrations of cobalt(II)-sulfate-heptahydrate (0 to 100 mg liter⁻¹) especially showed a steep increase in vitamin B₁₂ content from 73 to 170 ng g of dry weight⁻¹. The increase in vitamin B₁₂ content in fermentations

TABLE 2. Formation of vitamin B₁₂ by *C. freundii* (259) and *K. pneumoniae* (274) at different fermentation temperatures^a

Temp (°C)	Cyanocobalamin (ng g of dry wt ⁻¹)	
	<i>C. freundii</i>	<i>K. pneumoniae</i>
24	114	74
28	130	95
32	152	135

^a The maximal relative standard deviation was $\leq 7.5\%$.

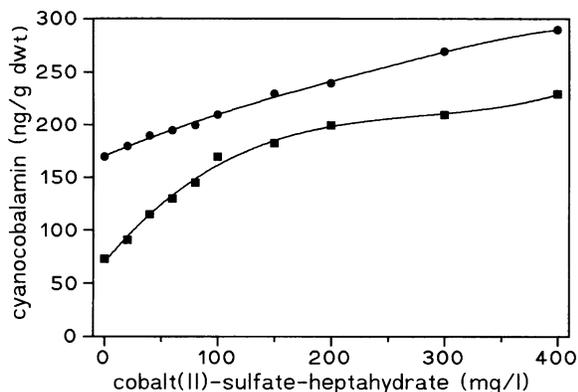


FIG. 1. Formation of vitamin B₁₂ (cyanocobalamin) during tempeh fermentations by *C. freundii* (259) (●) and *K. pneumoniae* (274) (■) after the addition of cobalt(II)-sulfate-heptahydrate. The maximal relative standard deviation was $\leq 7.5\%$. dwt, dry weight.

with *C. freundii* developed in a straight line with increasing cobalt concentrations, and vitamin B₁₂ content reached a maximal level of 290 ng g of dry weight⁻¹. Nevertheless, the incorporation of the precursor was not really effective, as the resulting yields were very low and reached a maximal value of only 0.014 (data not shown).

The addition of 5,6-dimethylbenzimidazole also resulted in an increase in vitamin B₁₂ formation, especially at low concentrations (0 to 100 mg liter⁻¹) (Fig. 2). In contrast to fermentations with *K. pneumoniae*, fermentations with *C. freundii* showed a further increase with concentrations of up to 300 mg liter⁻¹. The yields for the incorporation of 5,6-dimethylbenzimidazole into vitamin B₁₂ were also very low and reached a maximal value of only 0.013 (data not shown). The numbers of cells were not influenced by the addition of the two precursors and were comparable in all fermentations.

Influence of inoculation of soybeans with different numbers of cells of *C. freundii*. The effect of inoculum size was investigated with the most effective vitamin B₁₂-producing organism, *C. freundii*. Although the initial numbers of cells differed from 10¹ to 10⁶ (g of wet weight⁻¹), after 27 h all cell numbers approached 10⁹ to 10¹⁰ (g of wet weight⁻¹) (Fig. 3A) as a result of more rapid growth rates for small inocula. After 27 h

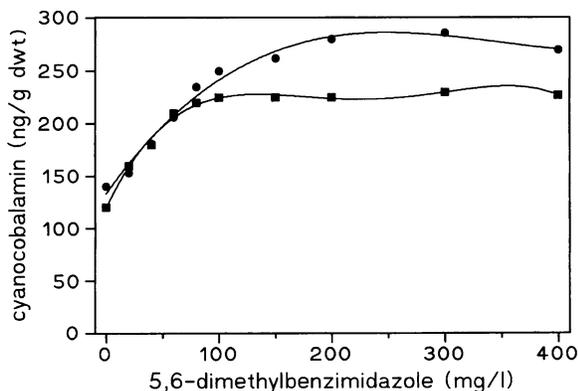


FIG. 2. Formation of vitamin B₁₂ (cyanocobalamin) during tempeh fermentations by *C. freundii* (259) (●) and *K. pneumoniae* (274) (■) after the addition of 5,6-dimethylbenzimidazole. The maximal relative standard deviation was $\leq 7.5\%$. dwt, dry weight.

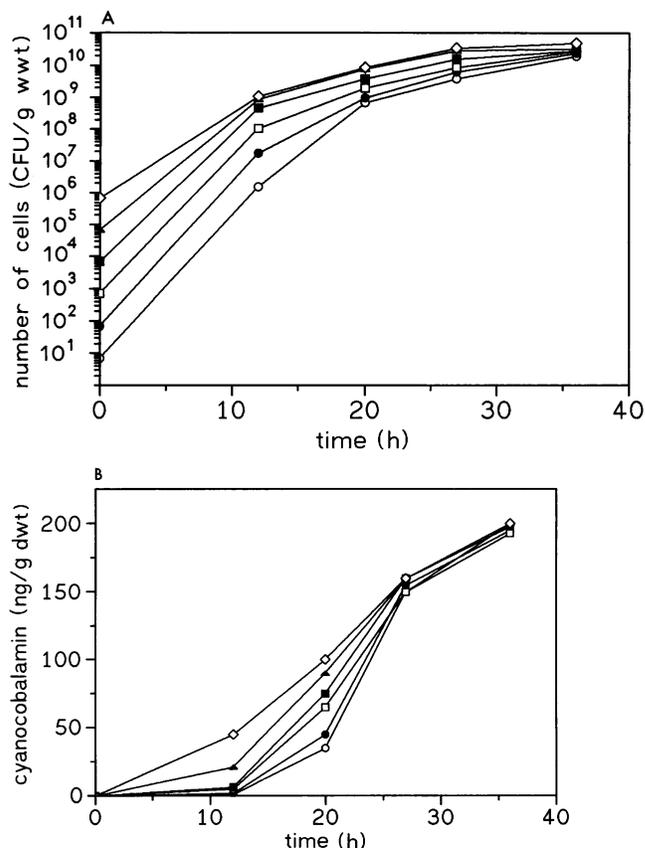


FIG. 3. (A) Numbers of cells of *C. freundii* (259) during tempeh fermentation after inoculation of soybeans with different numbers of cells. wwt, wet weight. (B) Dependence of the formation of vitamin B₁₂ (cyanocobalamin) on the inoculation of soybeans with different numbers of cells of *C. freundii* (259). The maximal relative standard deviation was $\leq 7.5\%$. dwt, dry weight. Symbols (for both panels): ◇, 10⁸; ▲, 10⁷; ■, 10⁶; □, 10⁵; ●, 10⁴; ○, 10³.

the vitamin B₁₂ contents of the different fermentations reached nearly the same range of 150 to 160 ng g of dry weight⁻¹, in accordance with the increase in the cell numbers (Fig. 3B).

Influence of the preparatory treatment of soybeans for SSF on vitamin B₁₂ formation. Different techniques were compared with regard to vitamin B₁₂ formation during SSF. The fermentation carried out with lactate soaking of soybeans and in which the soybeans were inoculated with the *Rhizopus* sp. and *C. freundii* for SSF resulted in the highest vitamin B₁₂ level (Table 3). Tempeh prepared with traditionally soaked (soaking water inoculated with *C. freundii*) soybeans which were also inoculated with the *Rhizopus* sp. and *C. freundii* before SSF showed low vitamin B₁₂ levels.

There was also a difference between tempeh prepared with autoclaved and nonautoclaved soybeans. In a sample prepared with autoclaved soybeans, only *C. freundii* could be detected after SSF with *C. freundii*. In a sample prepared with nonautoclaved soybeans inoculated with *C. freundii*, *Bacillus cereus* was also isolated. Tempeh prepared with traditionally soaked (with *C. freundii*), nonautoclaved soybeans inoculated with the *Rhizopus* sp. and *C. freundii* for SSF contained higher vitamin B₁₂ concentrations than tempeh prepared with traditionally soaked (with *C. freundii*), nonautoclaved soybeans inoculated with only the *Rhizopus* sp.

TABLE 3. Formation of vitamin B₁₂ (cyanocobalamin) and numbers of cells during tempeh fermentations carried out under various conditions^a

Method of fermentation ^b	Cyanocobalamin (ng g of dry wt ⁻¹) produced	Total plate count of <i>C. freundii</i> on plate count agar (10 ⁹)
<i>R. oligosporus</i> + <i>C. freundii</i>		
Lactate soaking	53.9	50.0
Autoclaving	48.8	35.0
No autoclaving	33.0	50.0
<i>R. oligosporus</i>		
Lactate soaking	ND ^c	ND
Autoclaving	ND	ND
No autoclaving	23.2	5.0
Control		
Lactate soaking	ND	ND
Autoclaving	ND	ND
No autoclaving	3.5	0.1

^a The maximal relative standard deviation was $\leq 7.5\%$.

^b For no autoclaving, beans were soaked overnight with *C. freundii* and cooked but not autoclaved after soaking. For autoclaving, beans were soaked overnight with *C. freundii*, cooked, and autoclaved. For lactate soaking, beans were soaked with lactate and autoclaved after soaking and cooking.

^c ND, not detectable.

Control soybeans (without any inoculation before SSF) soaked in the traditional way (with *C. freundii*) and not autoclaved also contained vitamin B₁₂. In these beans, *C. freundii*, *B. cereus*, and *Micrococcus luteus* could be detected. Fermentations carried out with traditionally soaked and with lactic acid-soaked beans, both of which were autoclaved after soaking, resulted in no vitamin B₁₂ when inoculation for SSF was done with the *Rhizopus* sp. alone.

Detection of the enterotoxin-encoding genes by PCR. Tests for the three enterotoxin (SLT IIA, LT I_h, and ST I_h) genes were negative for *C. freundii* and for *K. pneumoniae*. Figure 4 shows the test for the enterotoxin ST I_h gene.

DISCUSSION

The fact that vitamin B₁₂ production by *C. freundii* or *K. pneumoniae* is increased by raising the fermentation temperature is in contrast to the amounts of free amino acids (2, 6) and the content of γ -linolenic acid (17) in tempeh, which are improved by lowering the fermentation temperature from 32 to 24°C. As vitamin B₁₂ production is coupled to the growth of the bacteria (11), it is obvious why more vitamin B₁₂ is formed by the bacteria at 32 than at 24°C.

The increase in vitamin B₁₂ concentrations with the addition

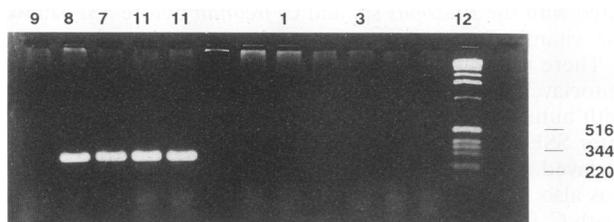


FIG. 4. PCR products of the gene for heat-stable enterotoxin ST I_h. 1, *C. freundii* 259; 3, *K. pneumoniae* 274; 7, *E. coli* O:128H⁻ (LT⁻ ST⁺); 8, *E. coli* O:6H⁻ (LT⁺ ST⁺); 9, *E. coli* G 1253 (LT⁺ ST⁻); 11, *E. coli* HB101 SLM 004 (LT⁻ ST⁺); 12, ladder (in base pairs).

of cobalt shows that the cobalt content in soybeans is suboptimal for vitamin B₁₂ production by *C. freundii* or *K. pneumoniae*. Although the addition of cobalt and 5,6-dimethylbenzimidazole leads to an increase in vitamin B₁₂ production by both strains, their incorporation is poor, as shown by the low yields. Moreover, supplementation with these precursors is not necessary, as the vitamin B₁₂ concentration produced in tempeh by either of these two strains would be high enough to meet the daily requirement of an adult person.

Inoculation of soybeans with different numbers of cells of *C. freundii* showed that vitamin B₁₂ production is correlated with the growth of the bacterium. This was also confirmed for *Propionibacterium freudenreichii* (11). The rate of vitamin B₁₂ production by *C. freundii* depended on the number of cells in the inoculum but not the final amount.

The low level of vitamin B₁₂ in the fermentation with traditionally soaked, nonautoclaved soybeans additionally inoculated with *C. freundii* before SSF can be explained by the existence of a mixed culture in this tempeh sample. In mixed cultures with other bacteria *C. freundii* does not produce as much vitamin B₁₂ as it does in pure cultures, as proved in another fermentation (data not shown).

The appearance of *C. freundii* and *B. cereus* in tempeh prepared with traditionally soaked, nonautoclaved soybeans can be explained by the nonsterile conditions of the tempeh fermentation process, which imitates the process performed in Indonesia. The results show that bacteria which are present during the soaking of soybeans can be transferred to SSF. In Indonesia, this fact is important, as the soybeans are not inoculated with vitamin B₁₂-forming bacteria. In conclusion, vitamin B₁₂ formation only takes place if vitamin B₁₂-forming bacteria that occur by chance are transferred from the soaking stage by handling of the soybeans under nonsterile conditions after the second cooking.

Rhizopus spp. have a positive effect on bacterial growth. The reason is the hydrolyzing capacity of the mold, which supplies growth substrates, such as amino acids, for the nutrition of the bacteria (2, 20). Furthermore, protease inhibitors, such as the Bowman-Birk inhibitor, are found in soybeans and can inhibit bacterial serine proteases (4). In contrast, the fungus forms proteases of the aspartate type; these are not sensitive to the inhibitor (1) and therefore can release amino acids in the presence of serine protease inhibitors.

In conclusion, the comparison of the different preparatory treatments of soybeans for SSF shows that the best way of reaching a high vitamin B₁₂ level is lactate soaking of soybeans and inoculation with a vitamin B₁₂-producing bacterium, such as *C. freundii* 259, before SSF.

The family *Enterobacteriaceae* includes many pathogenic organisms, such as *Salmonella typhi*, *Shigella dysenteriae*, *K. pneumoniae*, and *Yersinia pestis*. In addition, strains of *E. coli* and *C. freundii*, which belong to the intestinal flora of humans, can cause diarrhea via the production of enterotoxins (9, 10, 12, 19, 23, 30, 31). Although tempeh contains many members of the family *Enterobacteriaceae* (25–27, 29), no reports exist about diarrhea occurring after the consumption of tempeh. Nevertheless, it had to be determined whether the two strains of *C. freundii* and *K. pneumoniae* used here possess the ability to produce enterotoxins before they could be used for tempeh fermentation. As both strains were negative for three known enterotoxins and as they were originally isolated from Indonesian tempeh, it can now be stated that their use in tempeh fermentation should have no negative effect on tempeh consumers.

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