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Growth of *Bacillus cereus* in soyabean tempeh

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Bacillus cereus strains isolated from retail samples of tempeh were added in levels of approx. 10^5 cfu/g to soaking soyabeans intended for tempeh manufacture. Their growth was prevented and tempeh of good quality was obtained when the soaking resulted in acidification of the beans to pH 4.85, but *B. cereus* could grow well (approx. 10^8 cfu/g) in tempeh and cause its spoilage when the soaking did not cause acidification (bean pH 5.70–6.00). Acidification of soyabeans by addition of lactic acid prior to fungal fermentation required pH ≤ 4.4 to prevent *B. cereus* growth; at this pH, tempeh of good quality was obtained. Acidification of soyabeans with addition of acetic acid inhibited at pH ≤ 5.5 both *B. cereus* and *Rhizopus oligosporus*, the mould responsible for tempeh formation; hence, no tempeh could be obtained. Mixed inocula of *R. oligosporus* and either *Lactobacillus plantarum*, *L. casei* ssp. *alactosus* or *L. fermentum* produced tempeh of excellent quality. However, mixed inocula containing lactobacilli were not able to prevent *B. cereus* growth and subsequent spoilage when added to unacidified soyabeans of pH 6.6. This indicates that soyabean acidification, either by biological or by chemical means, is essential to inhibit *B. cereus* growth during the tempeh fermentation.

Key words: Tempeh; Soyabean; Fermented foods; *Bacillus cereus*; *Lactobacillus*

Introduction

Tempeh is an oriental fermented food obtained by fungal solid-state fermentation of soaked and cooked soyabeans, legumes, cereals or other suitable raw materials. Its traditional manufacture and properties have been excellently reviewed by Ko and Hesseltine (1979).

Soyabean tempeh may be prepared by soaking dehulled yellow variety soyabeans overnight, followed by rinsing and cooking in tapwater for 30–90 min, draining the water and allowing the adhering water to evaporate by spreading the beans in a thin layer. After cooling to ambient temperature the beans are inoculated with a spore preparation usually of *Rhizopus oligosporus*, packed into sparsely perforated containers and incubated at 30–35°C for approx. 48 h. The final product has a shiny white colour due to the profuse growth of non-sporulated mycelium and has a firm

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texture due to the mycelial penetration into the tempeh mass. Prior to consumption, tempeh is subjected to a heat treatment by boiling or frying.

A recent examination of the microbiological composition of 110 retail samples of tempeh by Samson et al. (1987) revealed the general presence of lactic acid bacteria, Enterobacteriaceae and yeasts. In addition, approx. 10% of the samples contained *Staphylococcus aureus* and *Bacillus cereus* in numbers which may cause food intoxication.

In this paper we consider the effect of some processing conditions on the growth of *B. cereus* during tempeh manufacture and storage. During the stage of soaking the beans at ambient temperatures, an uncontrolled growth of microorganisms (mainly bacteria) takes place. Ko and Hesseltine (1979) report that during the traditional Indonesian process, this spontaneous fermentation results in acidification to approx. pH 4.5. We found this to happen as well under laboratory conditions; in addition to acidification, gas production caused foaming and the soak water became viscous. However, we observed that such fermentation may unpredictably fail to materialise; in such cases, foam and viscosity were sometimes produced but the soak water was not (pH 6.6) or only slightly (pH > 5.5) acidified.

Steinkraus et al. (1965) stated that acidification during the soak was desirable in order to prevent growth of microbial contaminants during the fungal fermentation stage. They recommended that a sufficiently low pH of the cooked beans be ensured by soaking them in dilute acids, e.g. lactic or acetic acid, in order to counteract the effect of a failing acidification during soaking.

Tanaka et al. (1985) found that during the manufacture of tempeh from unacidified soyabeans, added contaminations of *Clostridium botulinum*, *Staph. aureus*, *Salmonella typhimurium* and *Yersinia enterocolitica* could grow very well and that clostridial and staphylococcal toxins could be produced.

Materials and Methods

Semi-traditional non-sterile process for tempeh manufacture

1 kg dry abrasion-dehulled yellow variety soyabeans kept at -20°C to avoid insect infestation, were rinsed twice with 11 l tapwater at 45°C , drained and soaked for spontaneous fermentation in 3 l tapwater at 45°C in an incubator at 25°C for 24 h. In most cases, such spontaneous fermentation failed to acidify resulting in soaked beans with pH of approx. 6.

When fermentative acidification during the soak was required, this could be achieved by repeating the above procedure using 1% soaking water of the previous batch as a starter, as described elsewhere (Nout et al., in press).

After draining the soak water, the beans were rinsed twice with 11 l tapwater at 25°C , and boiled in 3 l fresh tapwater for 20 min. After draining and discarding the boiling water, the hot beans were immediately spread in a layer of approx. 1 cm thickness on a wire-mesh and turned twice to facilitate the evaporation of adhering water.

After approx. 20 min the beans were hand-dry and cooled to room temperature, 20–25°C. They were then transferred into a container and mixed with 10 ml spore suspension. This spore suspension was obtained by adding 9 ml sterile peptone saline (0.85% NaCl, 0.1% Oxoid L34 peptone, pH 7.0) to each of 2 malt extract agar (Oxoid CM 59), slant cultures of *R. oligosporus* NRRL 5905 (incubated at 30°C for 1 week) followed by scraping off the spores with an inoculation needle. The concentration of *R. oligosporus* in the inoculated beans were about 10⁶ cfu per gram.

After inoculation, 40–50 g beans were firmly packed into a 9 cm diameter petri dish with 4–5 perforations of 0.5 mm diameter in bottom and lid and incubated at 30°C for 45 h, yielding fresh tempeh. Tempeh was stored in sealed PVC bags at 4°C for 17 days.

Aseptic process for tempeh manufacture from sterile soyabeans

1 kg of soyabeans were rinsed as mentioned above, and soaked in 3 l tapwater at 4°C to avoid microbial growth. After rinsing and boiling 20 min in tapwater, they were spread and cooled on wire-mesh trays as mentioned above. Such beans had a pH of 6.6. They were transferred into screw-capped glass jars and sterilised by autoclaving at 121°C for 25 min. When required, the bean pH was adjusted by addition of predetermined amounts of concentrated lactic acid or glacial acetic acid, in order to achieve the appropriate bean pH after sterilisation.

After cooling to room temperature, the sterilised beans were inoculated with approx 10⁶ cfu/g *R. oligosporus* NRRL 5905 using a spore suspension as described above, and other microorganisms as required (see below). After mixing with a sterile spoon, the inoculated beans were packed into sterile petri dishes in a sterile laminar-flow working cabinet, and incubated as mentioned above.

Inoculation with B. cereus

B. cereus strains designated No. 8 and No. 10, isolated from retail samples of tempeh, were grown in Brain Heart Infusion (BHI) broth (Oxoid CM 225) for 24 h at 30°C. For inoculation, appropriate amounts of BHI culture were added either at the start of the soaking stage during the semi-traditional nonsterile process, or to the sterilised soyabeans during the aseptic process.

Inoculation with lactobacilli

Strains of *Lactobacillus plantarum*, *L. casei* ssp. *alactosus*, and *L. fermentum*, isolated earlier from soaked soyabeans (Nout et al., in press) were grown in de Man, Rogosa & Sharpe broth (MRS; Gibco, Scotland, No. 152-3250) for 24 h at 25°C (*L. plantarum*) or 37°C (*L. fermentum* and *L. casei* ssp. *alactosus*).

To study the influence of mixed inocula of *R. oligosporus* and lactobacilli on the growth of *B. cereus* in tempeh made from unacidified soyabeans, inoculation of sterile, unacidified soyabeans (pH 6.6) was carried out at two levels: (A) approx.

equal to the *B. cereus* inoculum, using 1 ml of the 10^{-5} diluted MRS lactobacilli cultures per 100 g soyabeans, or (B) largely in excess of the *B. cereus* inoculum, using 1 ml of the undiluted MRS lactobacilli cultures per 100 g beans. After inoculation, the exact size of the *B. cereus* and lactobacilli inocula were determined using MEPP and Rogosa agar counting plates, respectively as described below.

Tempeh acceptability

The assessment of tempeh quality for acceptability was based upon exterior colour, texture, odour and evenness of mycelial penetration into the center of the cake (Nout et al., 1985), as judged by an expert panel.

Microbiological analysis

Sample preparation: Representative samples of 10.0 g taken under aseptic conditions, were homogenized for 2 min with 90 ml of sterile peptone saline using a Colworth type 400 Stomacher (Seward & Co., London). Decimal dilution series were prepared by mixing 1 ml of a previous dilution with 9 ml of sterile peptone saline.

Total aerobic count: Pour plate counts in Plate Count Agar (PCA, Oxoid CM 325) incubated for 72 h at 30°C. At low sample dilutions, i.e. -1 to -4 , 0.01% cycloheximide was added to PCA to prevent overgrowth by moulds.

Enterobacteriaceae: Pour plate counts in Violet Red Bile Glucose (VRBG, Oxoid CM 485) agar, with overlay, incubated for 24 h at 30°C.

Yeasts: were determined in Oxytetracyclin Glucose Yeast Extract agar pour plates (OGYE, Oxoid CM 545) to which 100 mg/l oxytetracyclin (Terramycin, Pfizer) and 0.1 g/l diphenyl (biphenyl, BDH Chemicals) were added after sterilisation; incubation 5 days at 30°C.

Lactic acid bacteria: Pour plate counts were carried out in Rogosa agar (LBS agar BBL No. 11327, Becton Dickinson & Co.) with overlay; the plates were incubated 3–5 days at 30°C.

Bacterial spores: 1:10 dilutions in sterile peptone saline were heated at 80°C for 5 min. Surviving spores were determined, by pour plate counts (PCA, 30°C, 24–72 h) for appropriate dilutions in sterile peptone saline.

B. cereus: Determinations were conducted as surface counts on Mannitol Egg-yolk Phenol red Polymyxin (MEPP, Cereus Selektiv agar, Merck no. 5267 to which 10 mg polymyxin B sulphate (Pfizer) and 40 ml 50% sterile egg yolk emulsion in 0.85% NaCl were added per liter of basal medium), incubated for 24 h at 30°C.

Reporting of counts: Microbial counts are presented as $\log_{10}$ per g of sample. The detection limit of the pour plate method is 50/g (5 colonies on plates containing 1 ml of 10^{-1} diluted sample), corresponding with $\log N = 1.7$. Similarly, the detection limit of the surface streaking method is 500/g (5 colonies on plates inoculated with 0.1 ml of 10^{-1} diluted sample), corresponding with $\log N = 2.7$.

Results and Discussion

Growth of B. cereus during semi-traditional non-sterile processes

B. cereus strains 8 and 10, isolated in our laboratory from retail samples of tempeh, were tested for their growth during a semi-traditional non-sterile process aimed at simulating conditions of industrial practice.

A non-inoculated control process (A_0) with beans which had significantly acidified during soaking, resulted in tempeh of excellent acceptability (Table I) containing mainly lactic acid bacteria with no detectable Enterobacteriaceae, *B. cereus*, bacterial spores, or yeasts.

During a non-sterile process cross-contaminations of the cooked beans take place through handling and equipment, explaining total counts of up to 2.20 log cfu/g. Such contaminations can multiply rapidly during the fungal fermentation stage, resulting in counts of 8–9 log cfu/g in fresh tempeh.

On the other hand, Table I shows that a non-inoculated control process (B_0) with poorly acidified beans yielded tempeh in which a normal mould growth had occurred but which was unacceptable due to its repellent off-odour, brown discolouration and sticky texture. This product contained lactic acid bacteria as well, but in addition 8.41 log cfu/g *B. cereus*, either originating from the raw beans or introduced as a cross-contamination.

Similarly, Table I shows that contaminations of *B. cereus* 8 (process A_8) and *B. cereus* 10 (process A_{10}) when introduced at the start of the soak, could not grow if acidification took place, giving tempeh of good acceptability and a microbiological composition similar to that of tempeh from the non-inoculated control process A_0 .

In contrast, contaminations with *B. cereus* 8 (process B_8) and *B. cereus* 10 (process B_{10}) at the start of the process where no acidification took place, resulted in tempeh with high levels of lactic acid bacteria, *B. cereus* and Enterobacteriaceae. Whereas process B_8 gave unacceptable tempeh comparable to the product of process B_0 , process B_{10} produced tempeh of acceptable sensory characteristics.

During earlier investigations (Nout et al., 1985) we found that fresh soyabean tempeh of excellent acceptability has a pH of 6.0–6.6. Considering the pH values and *B. cereus* counts of the products from processes B_0 , B_8 and B_{10} , a combination of high *B. cereus* counts (e.g. 8.80 log cfu/g) and high pH (e.g. pH 7.40) may be associated with the spoilage symptoms of brown, sticky, and malodourous tempeh.

Growth of B. cereus in tempeh from sterile soyabeans using an aseptic process

Whereas a fermentative acidification of soyabeans to pH 4.85 proved adequate to prevent the growth of naturally occurring or inoculated *B. cereus* (Table I, processes A_0 , A_8 , and A_{10}), we were interested in the maximum soyabean pH which still produces this inhibitory effect.

In order to eliminate possible interference from naturally occurring *B. cereus* on the beans, an aseptic process was used in which the pH of sterile soyabeans had been adjusted either with lactic acid or with acetic acid.

TABLE 1 – Growth of *B. cereus* during semi-traditional non-sterile tempeh processes

	Tempeh ^a quality	pH	Total aerobic	Enterobac- teriaceae	Lactic acid bacteria	<i>Bacillus</i> <i>cereus</i>	Bacterial spores	Yeasts
A. Tempeh from well-acidified soyabeans								
A0 non-inoculated control	soyabeans: soaked : cooked	4.85	9.28 2.18	2.45 <1.7	9.20 <1.7	<2.7 <2.7	<1.7 <1.7	<1.7 <1.7
	tempeh : fresh : stored	6.65	8.43 8.59	<1.7 <1.7	8.40 8.62	<2.7 <2.7	<1.7 <1.7	<1.7 <1.7
	soyabeans: soaked : cooked	4.85	9.30 <1.7	2.34 <1.7	9.15 <1.7	<2.7 <2.7	3.04 <1.7	<1.7 <1.7
	tempeh : fresh : stored	6.53	8.34 8.63	<1.7 <1.7	8.32 8.57	<2.7 <2.7	<1.7 <1.7	<1.7 <1.7
A8 soak, inoculated with <i>B. cereus</i> No. 8 at 4.81 log cfu/g beans	soyabeans: soaked : cooked	4.85	9.26 <1.7	2.36 <1.7	9.26 <1.7	<2.7 <2.7	2.98 <1.7	<1.7 <1.7
	tempeh : fresh : stored	6.30	7.86 9.18	<1.7 <1.7	8.76 9.18	<2.7 <2.7	<1.7 <1.7	<1.7 <1.7
A10 soak, inoculated with <i>B. cereus</i> No. 10 at 5.32 log cfu/g beans	soyabeans: soaked : cooked	4.85	9.26 <1.7	2.36 <1.7	9.26 <1.7	<2.7 <2.7	2.98 <1.7	<1.7 <1.7
	tempeh : fresh : stored	6.30	7.86 9.18	<1.7 <1.7	8.76 9.18	<2.7 <2.7	<1.7 <1.7	<1.7 <1.7
B. Tempeh from poorly acidified soyabeans								
B0 non-inoculated control	soyabeans: soaked : cooked	6.00	9.61 <1.7	8.61 <1.7	9.45 <1.7	3.88 <2.7	<1.7 <1.7	<1.7 <1.7
	tempeh : fresh : stored	7.40	8.63 8.86	<1.7 <1.7	7.11 5.72	8.41 8.85	7.50 8.58	<1.7 <1.7
	soyabeans: soaked : cooked	5.80	9.64 <1.7	8.23 <1.7	9.51 <1.7	7.36 <2.7	3.08 <1.7	<1.7 <1.7
	tempeh : fresh : stored	7.38	8.72 9.04	n.d. 4.56	8.08 7.90	8.52 8.90	7.50 9.04	<1.7 <1.7
B8 soak, inoculated with <i>B. cereus</i> No. 8 at 5.08 log cfu/g beans	soyabeans: soaked : cooked	5.80	9.64 <1.7	8.23 <1.7	9.51 <1.7	7.36 <2.7	3.08 <1.7	<1.7 <1.7
	tempeh : fresh : stored	7.38	8.72 9.04	n.d. 4.56	8.08 7.90	8.52 8.90	7.50 9.04	<1.7 <1.7
	soyabeans: soaked : cooked	5.70	9.65 2.20	6.40 <1.7	9.54 2.10	7.46 <2.7	2.76 <1.7	<1.7 <1.7
	tempeh : fresh : stored	6.85	8.77 9.04	8.11 8.88	8.69 8.23	7.65 7.38	6.50 7.04	<1.7 <1.7
B10 soak, inoculated with <i>B. cereus</i> No. 10 at 5.11 log cfu/g beans	soyabeans: soaked : cooked	5.70	9.65 2.20	6.40 <1.7	9.54 2.10	7.46 <2.7	2.76 <1.7	<1.7 <1.7
	tempeh : fresh : stored	6.85	8.77 9.04	8.11 8.88	8.69 8.23	7.65 7.38	6.50 7.04	<1.7 <1.7

^a Based on scores for appearance, consistency, odour, as follows: + +, excellent (creamy-white colour, firm consistency, fresh, nutty, soya odour); +, good (yellowish colour, moderately firm consistency, slight off-odour only); –, unacceptable (brown-yellow colour, soft and sticky consistency, repulsive odour).

TABLE II

Effect of pH adjustment with lactic or acetic acid on growth of *B. cereus* and *R. oligosporus* in tempeh made from sterile soyabeans (data given in log cfu/g tempeh)

pH	Lactic acid			Acetic acid		
	<i>B. cereus</i> No. 8	<i>B. cereus</i> No. 10	<i>R. oligo- sporus</i>	<i>B. cereus</i> No. 8	<i>B. cereus</i> No. 10	<i>R. oligo- sporus</i>
6.00				6.50	6.25	++
5.95	8.75	8.90	++ ^a			
5.90	8.20	7.60	++	6.65	7.20	++
5.80	8.25	8.70	++			
5.50	7.70	8.55	++	< 2.7	< 2.7	0
5.20	7.20	7.10	++			
5.15	3.85	3.00	++			
5.10	5.70	7.60	++			
5.00	7.20	7.15	++	< 2.7	< 2.7	0
4.90	6.65	7.15	++	< 2.7	< 2.7	0
4.80	5.40	6.20	++			
4.70	6.20	6.30	++			
4.55	< 2.7	2.75	++			
4.45	4.30	4.40	++			
4.40	< 2.7	< 2.7	++			
4.35	< 2.7	< 2.7	++			
3.95	< 2.7	< 2.7	+			

^a ++, dense mycelial growth required for tempeh; +, inadequate (floccose) mycelial growth with undesirable sporulation; 0, fermentation failure by complete inhibition of mycelial growth.

Table II shows that when adjusted with lactic acid, a bean pH of 4.40 was adequate to prevent the growth of *B. cereus* strains 8 and 10 on sterile soyabeans inoculated with *R. oligosporus*. Whereas undesirable fungal sporulation occurred on beans adjusted to pH 3.95 with lactic acid, soyabeans of pH 4.35 produced tempeh of excellent acceptability, i.e. the growth of *R. oligosporus* had not been affected, and undesirable colour defects due to sporulation were absent.

On the other hand, if acetic acid was used to adjust the pH of sterile soyabeans, pH ≤ 5.5 was already sufficient to prevent the growth of both strains of *B. cereus*. Unfortunately, the growth of *R. oligosporus* was prevented as well by this treatment, so the product tempeh could not be obtained. At pH 5.9 *R. oligosporus* was not inhibited by acetic acid, but neither were the added strains of *B. cereus*.

These results indicate that if soyabean acidification were to be obtained by the addition of acid, acetic acid must not be used.

During the non-sterile processes A₀, A₈, and A₁₀ (Table I), pH 4.85 of beans acidified by fermentation was adequate to prevent *B. cereus* growth during the following stage of fungal fermentation. Although we did not analyse the nature of the organic acids present in naturally acidified soaked beans, it is highly probable that they contain both lactic and acetic acids. Considering the effect of acetic acid observed in Table II, this offers an explanation why naturally acidified beans with

pH 4.85 achieve a significantly better inhibitory effect on *B. cereus* than beans acidified to the same pH using pure lactic acid.

Growth of B. cereus during aseptic processes using sterile unacidified soyabeans and mixed inocula of R. oligosporus and Lactobacillus spp.

Having established the inhibitory effect of natural or artificial acidification of soyabeans on the growth of *B. cereus*, we investigated whether a mixed inoculum of *R. oligosporus* and acidifying organisms could be used to inhibit the growth of *B. cereus* in unacidified beans (pH 6.6) resulting from a soak during which the acidification had failed.

Table III shows that mixed inocula of *R. oligosporus* with either *L. plantarum*, *L. casei* spp. *alactosus*, or *L. fermentum* yielded tempeh of excellent acceptability when added to sterile soyabeans of pH 6.6. However, even high levels of added lactobacilli were not able to prevent the outgrowth of relatively small inocula of the tested *B. cereus* strains when these were added simultaneously. Apparently, the acid production by the lactobacilli is either not sufficient or not rapid enough to avoid the multiplication of *B. cereus* in unacidified soyabeans.

TABLE III

Influence of mixed inocula of *R. oligosporus* and *Lactobacillus* spp. on the growth of *B. cereus* in tempeh made from sterile unacidified soyabeans (pH 6.6) using an aseptic process

<i>Lactobacillus</i> spp.	Inoculation level (log cfu/g beans)	<i>B. cereus</i> (log cfu/g tempeh)	Lactic acid bacteria	Tempeh acceptability ^a
Control (not inoculated with <i>B. cereus</i>)				
<i>L. plantarum</i>	2.20	< 2.7	9.08	++
<i>L. casei</i> ssp. <i>alactosus</i>	1.36	< 2.7	8.48	++
<i>L. fermentum</i>	1.89	< 2.7	6.26	++
Inoculated with <i>B. cereus</i> No. 8 at 1.32 log cfu/g beans				
<i>L. plantarum</i>	2.20	8.83	8.85	—
<i>L. plantarum</i>	7.70	7.81	9.64	+
<i>L. casei</i> ssp. <i>alactosus</i>	1.36	8.65	8.08	—
<i>L. casei</i> ssp. <i>alactosus</i>	6.56	9.00	9.49	+—
<i>L. fermentum</i>	1.89	9.00	6.76	—
<i>L. fermentum</i>	7.15	7.66	9.04	+—
Inoculated with <i>B. cereus</i> No. 10 at 1.54 log cfu/g beans				
<i>L. plantarum</i>	2.20	9.00	8.71	—
<i>L. plantarum</i>	7.70	7.78	9.62	+
<i>L. casei</i> ssp. <i>alactosus</i>	1.36	9.00	7.77	—
<i>L. casei</i> ssp. <i>alactosus</i>	6.56	8.62	9.36	+—
<i>L. fermentum</i>	1.89	9.04	6.20	—
<i>L. fermentum</i>	7.15	8.45	8.95	+—

^a See Table I for explanation. ++, excellent; +, good; +—, fair; —, unacceptable.

Inoculation of sterile unacidified soyabeans with *R. oligosporus* and approx. equal quantities of *B. cereus* and *Lactobacillus* spp. resulted in a malodourous, brown and sticky product.

Although the addition of an excess of lactobacilli as compared to *B. cereus* yielded products of fair to good appearance, consistency and odour, it can be seen from Table III that these products contained dangerously high numbers of *B. cereus*. This does not only illustrate that heavy inoculation of unacidified beans with lactobacilli cannot prevent the multiplication of a contamination with *B. cereus*. It also indicates that the processing of unacidified beans with a mixed inoculum of *R. oligosporus* and lactobacilli creates a public health risk, as the spoilage which would have otherwise occurred due to the large number of *B. cereus* is apparently masked by the lactobacilli.

Conclusion

It is concluded that acidification of soyabeans, either by fermentation or by lactic acid addition, during soaking for tempeh manufacture is an essential requirement for the prevention of *B. cereus* growth and associated risks of poisoning and spoilage. Furthermore, the presence of a majority of lactobacilli may contribute to the acceptability of tempeh and may offer a secondary inhibition to undesirable microorganisms by providing a competitive environment.

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