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Effect of acidification on the microbiological composition and performance of tempe starter

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Using rice and soya-beans as raw materials, the effects of: (i) (fermentative) acidification of the substrate, (ii) cross-contamination with acidifying microflora of the soaked substrate and (iii) the method of spore extraction were investigated. Microbiological analyses of fresh and stored starters, and of the resulting soya-bean tempe were carried out. The acceptability of fresh tempe was also evaluated. Rice-based starters yielded tempe of higher acceptability but had a shorter shelf-life (2·5 months) than soya-bean based starters (7 months). Fermentative acidification of raw materials resulted in starters with higher counts of fungal spores and lower counts of bacteria. Starters made with cross-contaminated raw materials resulted in tempe of highest acceptability when fermentatively acidified soya-beans were used for tempe manufacture. Starter extraction by straining of fungal spores was compared with grinding the total mass of moulded substrate, and was considered inappropriate in view of its low yield compared to the grinding method. It is concluded that fermentative acidification of raw materials prior to the manufacture of starters and tempe contributes to their microbiological safety and acceptability. The latter can be increased by lactic acid bacteria which may be introduced by the starter or by cross-contamination with fermentatively acidified soaking water.

Introduction

Tempe, mostly manufactured from soya-beans, consists of a firm cake of beans and fungal mycelium, obtained by solid substrate fermentation of soaked and cooked raw material. The manufacturing process was reviewed by Ko and Hesseltine (1979).

Originating in Indonesia, tempe meets an increasing interest in other regions including the US (Wang 1984) and Europe (Berghofer 1987). In addition, tempe manufacture could be an appropriate method (Steinkraus 1978) for small- and medium-scale processing of locally available legumes and cereals into wholesome products of high nutritional value in developing countries (Djurtoft and Nielsen 1983).

In Indonesia, tempe is a widely consumed protein component of the diet. Consequently, it is daily available in most markets and has a rapid turn-over. Usually, the fresh product is prepared and consumed the same day it has been purchased. However, in societies where tempe is a minor product, distribution and marketing may require considerable time. Since fresh tempe has a limited shelf-life, such delays can cause spoilage or loss of acceptability. During a survey

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of the microbiological composition of tempe sold in the Netherlands (Samson et al. 1987), counts of *Enterobacteriaceae* were $\geq 10^6$ g^{-1} in 60% of all samples; *Staphylococcus aureus* and *Bacillus cereus* counts were $\geq 10^5$ g^{-1} in 13 and 11% of all samples, respectively. The poor bacterial quality of such samples can be related primarily to the conditions prevailing during the tempe fermentation, and secondarily to inadequate storage conditions.

Several attempts have been made to preserve tempe, e.g. by packaging (Leviton 1984), parboiling and freezing (Shurtleff and Aoyagi 1979) or dehydration (Steinkraus et al. 1965). Such added technologies increase the consumer price and decrease the competitiveness with alternative products of equivalent protein content such as meat offals. This is of particular importance for low-income consumer groups in developing countries.

Our aim is to achieve optimum product acceptability, shelf-life and microbiological composition by applying environmental conditions favouring tempe formation but inhibiting the development of undesirable microflora. In this context we reported earlier that accelerated souring of soya-beans by lactic acid fermentation inhibits the growth of *Enterobacteriaceae*, *B. cereus* (Nout et al. 1987a,b) and *S. aureaus* (Nout et al. in press) during tempe manufacture. In addition, the combined use of acidified cooked beans and fermentation conditions favouring lactobacilli contributed positively to the acceptability of the final product (Nout et al. 1987a).

Our standard tempe fermentation conditions were designed to be representative for the traditional Indonesian method as well as the commercial production in the Netherlands. The strain of *Rhizopus oligosporus* used (NRRL 5905), isolated from Indonesian tempe, has an optimum growth temperature of 33°C whereas its maximum temperature for growth is 42°C. In Indonesia, incubation temperatures vary between 20–30°C (Ko and Hesseltine 1979). Earlier experiments in our laboratory had shown that incubation at 30°C for 40–44 h gave optimum tempe acceptability and shelf-life. At this incubation temperature, the heat generated by the growing mould causes a temperature increase up to approx. 40°C in the centre of the tempe cakes.

In Indonesia, the tempe fermentation is started with 'usar' (Soetarno Hadisepoetro et al. 1979), usually consisting of *Hibiscus* ('waru') leaves covered with a mixed microflora. This includes the fungus *Rhizopus oligosporus* which is regarded as essential for tempe formation (Hesseltine 1965). Due to the simple manufacturing process, the mixed microbiological composition of 'usar' is difficult to control. For this reason, and for convenient use in medium- and larger-scale tempe manufacture, several authors (Hesseltine et al. 1976, Ko 1985, Lindajati Tanuwidjaja and Roestamsjah 1985) proposed processes for the production of optimized tempe starters consisting of pure cultures of *R. oligosporus* grown on rice or soya-beans. Little attention has been paid to the bacteriological composition of such pure or semi-pure culture starters made under laboratory conditions.

Obviously, the microflora development during the tempe fermentation will be influenced by several factors including the incubation temperature, the a_w of the substrate, and the microbial load accompanying *R. oligosporus* in a tempe starter. Using our standardised process with 30°C incubation and with substrate from which adhering water was removed by adequate evaporation, we investigated if the principle of accelerated acidification (Nout et al. 1987a) could be

applied to reduce undesirable microbial development during starter manufacture. In addition, the effect of experimental starters on the acceptability and microbiological composition of fresh tempe was evaluated.

Materials and Methods

Culture

Rhizopus oligosporus NRRL5905 was grown and maintained on malt extract agar (MEA, Oxoid CM 59) slants. Incubation was at 30°C for 1 week, and storage at 5°C.

Usar

Traditional tempe starter ('usar') was obtained fresh from a manufacturer at Malang, Indonesia. A composite sample of 3 leaves (average weight 3·5 g per leaf) was used for analysis.

Analysis

Sample preparation, enumeration of total aerobic bacteria, *Enterobacteriaceae*, yeasts, lactic acid bacteria, bacterial spores and *Bacillus cereus*, as well as reporting of counts was described previously (Nout et al. 1987b).

Fungal propagules: pour plates in rose bengal chloramphenicol agar base (Oxoid CM 549) with addition of 0.2 g l^{-1} Rose Bengal (Fluka AG, Switzerland) and, after sterilization, 100 mg chloramphenicol/800 ml basal medium. Incubation was at 30°C for 3–5 days.

Staphylococcus aureus: surface count plates in Baird–Parker agar (BP medium base, Gibco 152–0320) with addition of 50 ml egg yolk tellurite enrichment (Oxoid 0779–73–1) per 950 ml basal medium after sterilization. Incubation was at 35°C for 24 h.

Tempe quality

The assessment of tempe acceptability was based upon exterior colour, fungal penetration, firmness, smell and pH and was carried out according to guidelines given previously (Nout et al. 1985).

Starter manufacture

Starter were prepared using two substrates, i.e. soya-beans (dehulled by dry abrasion) and brown rice. The basic process consisted of soaking the substrates for 24 h; draining and discarding the soak water; rinsing the substrates twice with tapwater; boiling in 3 times (soya-beans) or 1·5 times (rice) their weight of tapwater for 20 min; draining and discarding the boiling water; spreading the substrates in a 1 cm thick layer on wire mesh to cool during 40 min; inoculating with 0·3% v/w spore suspension of *R. oligosporus* NRRL5905 prepared as described earlier (Nout et al. 1987b); incubation in perforated containers at 30°C for 48 h (soya-beans) or 1 week (rice); drying with a fan-dryer at 50°C for 5·5 h (soya-beans) until approx. 9% moisture content. Due to the extended incubation required to obtain adequate sporulation in rice-based starters, the latter had a post-incubation moisture content of 9–12% and were not given additional drying treatment. The dried material was either ground whole, or was passed through a strainer to obtain a dry spore concentrate.

The following experimental treatments were applied. (A) and (B): soaking at 25°C with 1% v/w soak water from a stabilized acidifying soak obtained as described earlier (Nout et al. 1987a). In (B), 0·6% v/w of the soak water mentioned above was added during the inoculation with *R. oligosporus*; (C); soaking at 5°C in tapwater acidified with 1·5% w/w lactic acid and 0·3% w/w acetic acid (soya-beans) or 0·25% lactic acid and 0·05% w/w acetic acid (rice) in order to achieve acidity and pH prevailing in raw materials soaked according to procedure A (pH 4·55 in soya-beans and pH 4·70 in rice); (D): soaking at 5°C in tapwater. Storage of all finished starters was at 5°C.

Tempe manufacture

Tempe was made with soya-beans soaked in 3 times their weight of tapwater under 2 different conditions, i.e. (I) for 24 h at 5°C to avoid microbial action. These beans had pH values of 6·0–6·5 after soaking; and (II) for 24 h at 25°C with addition of 1% v/w of soaking water from a soak with stable acidification obtained as described earlier (Nout et al. 1987a), resulting in soaked beans with pH values of 4·0–4·5.

After soaking, the soya-beans were processed into tempe as described previously (Nout et al. 1987a).

Results and Discussion

Table 1 summarizes the microbiological composition of the starters obtained, and of a composite sample of traditional 'usar' leaves. Rice (3) yielded starters

Table 1. Microbiological composition of tempe starters (log cfu g^{-1}).

Starters	Total aerobic	Aerobic bacterial spores	*Entero-bacteriaceae*	Lactic acid bacteria	Yeasts	Moulds
1 A[a]	2·60	2·00	<1·70	2·85	<1·70	8·64
1B	6·38	4·85	3·36	6·54	<1·70	8·15
1C	5·04	2·48	<1·70	4·94	<1·70	8·36
1D	8·40	6·70	<1·70	6·20	<1·70	7·38
2A	5·28	2·00	<1·70	4·34	<1·70	8·28
2B	7·70	5·28	<1·70	7·78	<1·70	8·20
2C	ND[b]	6·48	<1·70	6·65	<1·70	7·95
2D	8·53	6·48	<1·70	7·36	<1·70	7·15
3A	2·30	2·30	<1·70	<1·70	<1·70	8·20
3B	6·04	1·78	3·30	4·63	<1·70	7·99
3C	4·65	4·49	<1·70	4·48	<1·70	7·61
3D	4·00	3·30	<1·70	3·11	<1·70	7·64
'Usar'	7·28	3·15	7·00	6·60	6·46	8·20

[a] 1 = soya-bean grown, strained; 2 = soya-bean grown, ground; 3 = rice grown, ground; A = substrate acidified by fermentation; B = same as A, with addition of acidified soak water; C = substrate acidified with lactic and acetic acids; D = neutral substrate.
[b] ND = not determined.

with lower numbers of contaminating bacteria than soya-beans (2). The numbers of viable fungal spores in rice-based starters 3A and 3B were similar to those obtained by Lindajati Tanuwidjaja and Roestamsjah (1985). Lower bacterial counts in rice-based starters could result from reduced nutrient availability, but also from the gradual dehydration taking place during the longer incubation of rice. The resulting a_w might have been less favourable for bacterial growth. Interestingly, Wang et al. (1975) reported that the moisture content of raw material should be 34–48% for maximum fungal activity of *R. oligosporus* starter, depending on substrate used. However, they did not study the bacterial development in those starters.

Using soya-beans, the straining method (1) gave slightly higher mould counts with somewhat less contaminating bacteria. However, the weight yield of starter produced by straining was only 5–10% of the yield of ground starter. The straining method has been recommended (Shurtleff and Aoyagi 1979) to obtain starter of high quality and long shelf-life. Although the bacterial counts are indeed lower, they are still considerable; this and the low yield make straining less appropriate.

In all cases, procedure A yielded the highest concentration of mould propagules, with the lowest number of bacteria. As could be expected there was less bacterial growth with A due to the low initial pH of the beans. However, from these results it cannot be concluded whether the higher yield of fungal spores is due to decreased bacterial competition or to other factors (e.g. pH) which may stimulate sporulation.

Procedure C was carried out to simulate the combined effect of lactic and acetic acids, and pH prevailing in beans prepared according to A. The resulting starter contained higher bacterial and lower fungal counts. This implies that organic acids and pH are not the only environmental factors determining the ecological niche in C occupied by bacteria.

Procedure B was carried out to enforce

a cross-contamination from the soak water to the cooked beans via the starter. It is thus not surprising to find relatively high numbers of acid tolerant lactic acid bacteria, but also some *Enterobacteriaceae*.

Procedure D yielded the lowest fungal activity. It had been carried out as a comparison to C, in order to assess the effect of the combined acidity and pH. Only in strained soya-beans (1) did acidity have the expected reducing effect on total aerobic bacteria and bacterial spore counts. This underlines the earlier suggestion that additional environmental factors are responsible for the reduced bacterial counts in A.

Compared with the experimental starters, the traditional 'usar' leaves had very high counts of *Enterobacteriaceae* and yeasts. Other counts, including fungal spores, were of the order of magnitude of the experimental starters. The occurrence of *Enterobacteriaceae* and yeasts on 'usar' may be explained by their common presence on plant material. However, further research is required to assess the influence of these organisms on the quality of tempe.

Table 2 summarizes the effect of storage on the fungal activity of the experimental starters. The viability of soya-bean-based starters 1A, 2A and 2C was hardly affected by 7 months storage, even at room temperature. Starter 2B also had a stable count of fungal spores after an initial decrease. Rice-based starters 3A and 3D retained most of their activity after 2·5 months but had lost 80% of their viability after 4·5 months of storage at room temperature.

Table 3 summarizes the microbiological composition of soya-bean tempe using neutral (I) and fermentatively acidified (II) beans. Since the performance of the mixed microflora of 'usar' leaves cannot be compared with that of the pure culture starters, we did not include 'usar' in the experimental tempe manufacture.

From a microbiological point of view, starters prepared using procedure A and those based on ground soya-beans (2) gave the best results. In addition, tempe made with acidified beans (II) had lower counts of accompanying bacteria. These data confirm the beneficial effect of fermentative acidification of raw material on the bacteriological composi-

Table 2. Influence of storage on fungal spore count of experimental tempe starters (log cfu g^{-1}).

Storage time (months):	0	2·5		4·5		7	
Storage temperature (°C):		5	21	5	21	5	21
Starters							
1A[a]	8·64	8·45	8·40	8·75	8·72	ND[b]	8·70
2A	8·28	7·92	7·98	8·08	8·48	7·92	8·20
2B	8·20	7·66	7·58	7·56	7·81	7·69	7·78
2C	7·95	7·86	7·76	8·04	8·04	7·89	7·99
3A	8·20	7·83	7·83	7·80	7·51	ND	ND
3D	7·64	7·54	7·62	7·46	6·94	ND	ND

[a] See Table 1 for explanation of sample codes.
[b] ND = not determined.

Table 3. Microbiological composition (log cfu g^{-1}) and acceptability score of tempe.

Starters	Soya-bean pre-treatment	Lactic acid bacteria	*Micro-coccaceae*	*S. aureus*	*B. cereus*	*Entero-bacteriaceae*	Acceptability (mean of scores)
1A[a]	I[b]	7·81	7·36	<2·70	<2·70	<1·70	7·4
	II	6·26	2·60	<2·70	<2·70	<1·70	6·2
1B	I	8·98	8·30	<2·70	4·81	7·54	6·0
	II	8·75	6·70	<2·70	<2·70	6·68	5·2
1C	I	8·79	7·18	<2·70	<2·70	6·40	7·4
	II	8·72	7·34	<2·70	<2·70	<1·70	6·2
1D	I	8·62	7·75	<2·70	<2·70	6·56	4·8
	II	7·88	5·11	<2·70	5·49	<1·70	6·6
2A	I	7·32	4·18	<2·70	<2·70	<1·70	6·8
	II	5·79	<1·70	<2·70	<2·70	<1·70	7·0
2B	I	8·79	6·41	<2·70	<2·70	7·08	6·6
	II	8·30	<1·70	<2·70	<2·70	<1·70	7·4
2C	I	8·78	2·72	<2·70	<2·70	<1·70	6·8
	II	7·40	<1·70	<2·70	<2·70	<1·70	6·6
2D	I	8·57	7·29	4·00	<2·70	<1·70	5·8
	II	7·32	5·20	<2·70	<2·70	3·18	5·6
3A	I	8·75	7·11	<2·70	<2·70	6·94	6·6
	II	8·53	<1·70	<2·70	<2·70	<1·70	8·0
3B	I	8·68	7·45	<2·70	<2·70	6·98	6·6
	II	8·68	<1·70	<2·70	<2·70	5·70	8·4
3C	I	8·62	6·51	<2·70	<2·70	6·64	6·6
	II	8·49	<1·70	<2·70	<2·70	2·30	7·4
3D	I	8·75	6·83	<2·70	<2·70	6·85	6·6
	II	8·86	<1·70	<2·70	<2·70	<1·70	8·2

[a] See Table 1 for explanation of sample codes.
[b] I = tempe made with neutral soya-beans; II = tempe made with soya-beans acidified by fermentation.

tion of tempe reported earlier (Nout et al. 1987a).

From the point of view of acceptability, the best tempe quality was obtained using starter derived from rice (3) and acidified beans (II). Other variations did not result in significant effects on tempe acceptability except for starters made using procedure (D) in combination with neutral beans (I), which resulted in poor acceptability.

The acceptability of tempe can be associated with a combination of various factors, including:

(1) Fermentative acidification of the substrate, which results in significantly better acceptability and lower counts of non-lactic acid bacteria. This cannot be fully explained by the direct effect that lower non-lactic acid bacteria counts have on increased acceptability; curve-fitting resulted in a coefficient of determination $r^2 = 0{\cdot}45$.

(2) Counts of potentially spoilage-causing microorganisms in the starter. No significant relationship was found between total aerobic count or the total of all counts in the starters and the acceptability scores of the resulting tempe.

(3) Nature of bacteria present in the starter. Note that in cases 2 (soya-bean) and 3 (rice) the best acceptability was achieved with cross-contaminated starters. From Table 1 it appears likely that lactic acid bacteria, dominating the bac-

terial flora of the B starters, contribute to the acceptability of the finished tempe. Although similar results had been reported by us (Nout et al. 1987a) with pure cultures of lactobacilli, it is interesting to note that cross-contamination has the potential to give similar effects.

In conclusion, it was found that the microbiological composition of the starters is influenced by the nature of the raw material, prevailing acidity and pH combination, previous fermentation, and additional cross-contamination during starter manufacture.

The bacteriological composition and acceptability of fresh tempe was primarily influenced by initial fermentative acidification of the substrate, and also by the addition of certain lactic acid bacteria from fermenting soak water. On the other hand, the microflora of fresh tempe was not influenced significantly by the substrate (i.e. rice or soya-beans) used for starter manufacture.

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