Influence of acidity and initial substrate temperature on germination of *Rhizopus oligosporus* sporangiospores during tempe manufacture.

Journal of Applied Bacteriology

Reu, J.C.; Rombouts, F.M.; Nout, M.J.R.

<https://doi.org/10.1111/j.1365-2672.1995.tb02843.x>

This publication is made publicly available in the institutional repository of Wageningen University and Research, under the terms of article 25fa of the Dutch Copyright Act, also known as the Amendment Taverne. This has been done with explicit consent by the author.

Article 25fa states that the author of a short scientific work funded either wholly or partially by Dutch public funds is entitled to make that work publicly available for no consideration following a reasonable period of time after the work was first published, provided that clear reference is made to the source of the first publication of the work.

This publication is distributed under The Association of Universities in the Netherlands (VSNU) 'Article 25fa implementation' project. In this project research outputs of researchers employed by Dutch Universities that comply with the legal requirements of Article 25fa of the Dutch Copyright Act are distributed online and free of cost or other barriers in institutional repositories. Research outputs are distributed six months after their first online publication in the original published version and with proper attribution to the source of the original publication.

You are permitted to download and use the publication for personal purposes. All rights remain with the author(s) and / or copyright owner(s) of this work. Any use of the publication or parts of it other than authorised under article 25fa of the Dutch Copyright act is prohibited. Wageningen University & Research and the author(s) of this publication shall not be held responsible or liable for any damages resulting from your (re)use of this publication.

For questions regarding the public availability of this publication please contact openscience.library@wur.nl

Influence of acidity and initial substrate temperature on germination of *Rhizopus oligosporus* sporangiospores during tempe manufacture

J.C. de Reu, F.M. Rombouts and M.J.R. Nout

Department of Food Science, Wageningen Agricultural University, Wageningen, The Netherlands

5010/08/94: received 5 August 1994, revised 10 October 1994 and accepted 10 October 1994

J.C. DE REU, F.M. ROMBOUTS AND M.J.R. NOUT. 1995. During the soaking of soya beans according to an accelerated acidification method organic acids were formed, resulting in a pH decrease from 6.0 to 3.9. After 24 h of fermentation at 30°C, lactic acid was the major organic acid (2.1% w/v soak water), while acetic acid (0.3% w/v soak water) and citric acid (0.5% w/v soak water) were also found. During cooking with fresh water (ratio raw beans : water, 1 : 6.5) the concentrations of lactate/lactic acid and acetate/acetic acid in the beans were reduced by 45% and 51%, respectively.

The effect of organic acids on the germination of *Rhizopus oligosporus* sporangiospores was studied in liquid media and on soya beans. Germination in aqueous suspensions was delayed by acetic acid: within 6 h no germination occurred at concentrations higher than 0.05% (w/v incubation medium), at pH 4.0. When soya beans were soaked in the presence of acetic acid, the inhibitory concentration depended on the pH after soaking. Lactic acid and citric acid enhanced germination in liquid medium, but not in tempe.

Inoculation of soya beans with *R. oligosporus* at various temperatures followed by incubation at 30°C resulted in both increased and decreased periods for the lag phase of fungal growth. A maximum difference of 3 h lag phase was found between initial bean temperatures of 25 and 37°C.

When pure cultures of homofermentative lactic acid bacteria were used in the initial soaking process, less lactic acid and acetic acid was formed during soaking than when the accelerated acidification method was used. This resulted in a reduction of the lag phase before growth of *R. oligosporus* by up to 4.7 h.

INTRODUCTION

Tempe is an Indonesian food product prepared by fermenting dehulled cooked soya beans with the fungus *Rhizopus oligosporus*. The manufacturing process (Fig. 1) was reviewed by Ko and Hesseltine (1979), Winarno and Reddy (1986) and Nout and Rombouts (1990).

During the traditional process in Indonesia the soya beans undergo a natural microbial fermentation during the hydration (soaking) process (Winarno and Reddy 1986). During this period of 12–48 h at approximately 28°C, the microflora residing in the beans and the soaking vessels acts

as a starter for mixed lactic/acetic acid fermentations that lower the pH from 6.5–7.0 (initial) to 4.5–5.5. The predominant species occurring during such soaking operations include the lactic acid bacteria *Lactobacillus casei*, *Enterococcus faecium* and *Streptococcus dysgalactiae* as well as other bacteria, e.g. *Staphylococcus epidermidis*, *Klebsiella pneumoniae*, *Kl. ozaenae*, *Enterobacter cloacae*, *Ent. agglomerans*, *Citrobacter diversus* and *Bacillus brevis*, and the yeasts *Pichia burtonii*, *Candida diddensiae* and *Rhodotorula rubra* (Mulyowidarso *et al.* 1989). In temperate climates with a different natural microflora as well as a more stringent disinfection of equipment, the natural acidification of soya beans does not always occur, or is very slow. Poor acidification and centrifugal washing/cooling results in cotyledons of near neutral pH and high moisture content providing

Fig. 1 Principle of the tempe process (adapted from Nout and Rombouts 1990)

excellent conditions for unwanted acid-sensitive bacteria, e.g. *Bacillus* spp. and Enterobacteriaceae (Nout and Rombouts 1990).

The importance of raw material pH is illustrated by the investigation of Tanaka *et al.* (1985) who carried out challenge tests with *Clostridium botulinum*, *Staphylococcus aureus*, *Salmonella typhimurium* and *Yersinia enterocolitica* in non-acidified soya beans inoculated with *R. oligosporus* NRRL 2710. Under the conditions used, these harmful organisms were able to grow and some produced their toxins.

Steinkraus *et al.* (1965) advocated the addition of lactic acid or acetic acid during soaking in order to reduce chances of microbial spoilage. Soaking of beans (1 kg dry beans: 3 l of water) with 0.125% (v/v) acetic acid was insufficient to prevent bacterial spoilage. In an attempt to control bacterial contamination of tempe, soaking in 0.25%

(v/v) acetic acid gave promising results, while soaking in a 0.5% (v/v) acetic acid solution required twice the normal time for fermentation (Steinkraus *et al.* 1965). Soaking in 1% lactic acid solution suppressed microbial growth (final total counts $< 10^2$ cfu ml⁻¹ soak water) and sliminess of the soak water (Nout *et al.* 1985). Chemical acidification (soaking in 1% lactic acid) gave a poor tempe quality with mixed culture usar-style starter (Nout *et al.* 1992) and acceptable quality with pure culture of *R. oligosporus* NRRL 5905.

Nout *et al.* (1987) found that inoculation with soak water of a previous batch provided a method of obtaining a vigorous lactic acid fermentation, under laboratory conditions. *Lactobacillus plantarum* was the major species in the soak water of soya beans treated with this method of accelerated acidification.

Mulyowidarmo *et al.* (1991) investigated the concentrations of organic acids and carbohydrates in soak water and soya beans during the traditional soaking method involving a natural fermentation. They found that lactic acid (0.65% w/v) and malic acid (0.35% w/v) were the major acids, after 24 h of soaking with natural fermentation.

The physiological requirements of *R. oligosporus* sporangiospores for germination were studied by Medwid and Grant (1984). They found that the optimum conditions for germination were 42°C and pH 4.0. Initial swelling occurred only in the presence of a suitable carbohydrate. Subsequent production of germ tubes required sources of both carbon and nitrogen.

One of the major factors determining the process capacity of tempe production is the lag time before the fungal fermentation stage (varying from 10 to 24 h, at 30°C). Removal of the skin of beans, the availability of oxygen, the relative humidity, the pH of the soaked beans and temperature were major factors influencing mould growth (Steinkraus *et al.* 1960).

The aim of this study was to determine the concentrations of organic acids present in soya beans after soaking with bacterial fermentation or acidification and after cooking, and to investigate the effect of these organic acids at representative levels, and of temperature on germination of sporangiospores of *R. oligosporus* in liquid suspensions and on soya beans. The knowledge obtained should provide the means to reduce or standardize the lag phase which would result in a better process control.

MATERIALS AND METHODS

Cultures

Rhizopus oligosporus NRRL 5905 was grown (7 d at 30°C) and maintained on Malt Extract agar (MEA, Oxoid CM59)

slants. Sporangiospore suspensions were prepared as described previously (de Reu *et al.* 1993). The viable count varied between 5×10^5 and 1×10^6 colony-forming units ml^{-1} . For each series a fresh sporangiospore suspension was prepared.

Pure cultures of *Lactobacillus plantarum*, *Pediococcus pentosaceus* or *Lactobacillus acidophilus* were isolated from soya bean soak water and identified as described by Hounhouigan *et al.* (1993). The pure cultures were harvested from MRS-broth (Oxoid, CM 359) which was incubated for 2 d at 30°C.

Preparation of tempe

Soaking conditions. Tempe was prepared from dehulled yellow soya beans (*Glycine max*). Raw beans, 300 g, were soaked with 900 ml of water for 24 h under the following sets of conditions: (I) at 5°C, to minimize microbial action; (II) at 5°C with the addition of acetic acid (0.125, 0.25 or 0.5% w/v) to the soak water; (III) at 5°C with the addition of lactic acid (1.5%) and acetic acid (0, 0.125, 0.25 or 0.5% w/v) to the soak water; (IV) at 30°C with the addition of lactic acid (2.0% w/v) to the soak water; (V) at 30°C with the addition of acetic acid (0.5% w/v) to the soak water; (VI) at 30°C with the addition of soaking water from an accelerated acidification (Nout *et al.* 1987) (10% v/v) to the soak water; (VII) at 30°C with the addition of 90 ml of pure cultures of *Lact. plantarum*, *Ped. pentosaceus* or *Lact. acidophilus*, to 810 ml of soak water.

Cooking. After soaking the beans were rinsed gently with approximately 0.5 l of tap water, drained, and cooked in 2 l of tap water in an uncovered pan (5 l; diam. 25 cm) for 20 min boiling time. The water was then drained away and the beans were spread on a wire mesh at room temperature to cool.

Fermentation of cooked beans. The cooked beans were inoculated with *R. oligosporus* by mixing a sporangiospore suspension (1% v/w) with 450 g of cooked beans which were then packed into a perforated box (1 × h × w: 20 × 3 × 10 cm) and incubated at 30°C in an incubator with forced ventilation (IKS, Leerdam, The Netherlands).

In order to determine the effect of the initial bean temperature of the cooked beans on germination of sporangiospores of *R. oligosporus*, beans that had been soaked by method (VI), cooked and dried were transferred to polythene bags and incubated at 25°, 30°, 37° or 42°C for 1 h were inoculated, packed into a perforated box, re-incubated at the same temperature for 1 h then transferred to 30°C for further incubation.

Determination of pH and organic acids

The pH of duplicate samples of soak water and of soaked soya beans was measured with a pH meter (WTW, type 522, Weilheim, Germany) and a pH electrode (Schott-Geräte, type N61, Hofheim a.Ts., Germany).

To determine organic acids, soak water samples were deproteinated by the addition of 5% trichloroacetic acid to give a final concentration of 0.1% (v/v). After centrifugation (10 min, 1350 g) the supernatant fluids were stored at -20°C until analysis. Just prior to HPLC analysis, the thawed supernatant fluid was filtered through 8 µm filters (Millipore, SCWP04700, Ireland) and 0.45 µm filters (Schleicher & Schuell, FP030/2, Dassel, Germany) filters. Organic acids were determined by HPLC using an Aminex HPX-87H stainless steel column (BioRad) and a refractive index detector (Shodex, SE-61) at 35°C. The acids were eluted with H_2SO_4 , 0.005 mol l^{-1} as the mobile phase and a flow rate of 0.6 ml min^{-1} .

Determination of titratable acidity

To determine titratable acidity, 10 g of soya beans were homogenized with 90 ml of distilled water in a Waring blender (30 s at high speed). This mixture was titrated to pH 8.5 using 0.1 mol l^{-1} NaOH and maintained at that level for 5 min. Subsequently the total volume of NaOH used was measured, and the titratable acidity was expressed as ml of 0.1 mol l^{-1} NaOH per g of sample. Duplicate determinations were made.

Determination of the effect of pH, organic acids and temperature on germination of sporangiospores of *Rhizopus oligosporus*

Sporangiospore suspensions (1 ml) were centrifuged for 2 min at 10 000 g in an Eppendorf centrifuge (Microcen 13, Herolab, Wiesloch, Germany). The pellets were resuspended in the required media.

The basal medium for spore germination consisted of a solution of 0.37% w/v glucose, 0.09% w/v $(\text{NH}_4)_2\text{SO}_4$ and 0.1% w/v $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ sterilized by autoclaving for 20 min at 121°C, to which 0.1% v/v of filter-sterilized stock solution of minerals (0.2 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.2 mg $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ per ml) was added. To the basal medium, lactic acid (90% (DL), Cat no. 366, Merck, Germany) or acetic acid (100%, Cat no. 63, Merck, Germany) or citric acid (Cat no. 244, Merck, Germany) were added in concentrations ranging from 0 to 1.5% w/v incubation medium. Subsequently, the pH of the medium was adjusted to $\text{pH } 4.0 \pm 0.05$ using 0.5 mol l^{-1} HCl or 0.5 mol l^{-1} NaOH, the resulting media were filter-sterilized (0.45 µm) and 1 ml was added to the pellet of a 1 ml sporangiospore suspension. The spores were

resuspended and the suspensions were incubated in a water bath at 25°, 30°, 37° or 42°C. At hourly intervals each sample was mixed, 20 µl was removed to measure germination, and the incubation was continued. Germination was defined as the extension of a germ tube to a length equal to one-half the diameter of the spore (Medwid and Grant 1984). The percentage of germinated spores was determined microscopically (Carl Zeiss, Standard 20, 400×, Germany). For each sample eight microscopic fields with at least 10 spores were counted. The average percentage of germination and the standard deviation were calculated. The Student's *t*-test was used to evaluate statistically significant differences between the results.

Measurement of germination of, and growth from, sporangiospores of *Rhizopus oligosporus* during production of tempe

Because direct measurement of the germination of the fungal spores on soya beans is impractical, increase in temperature of tempe was used as an indicator of germination of spores and growth of the fungus. The temperatures were monitored with thermocouples (Tempcontrol, Voorburg, The Netherlands) in the centre (h 1.5 cm, 5 cm from the longest side, 8 cm from the shortest side) of the box. Thermocouples were connected with a data logger (Squirrel 1205 Grant, UK). The data were processed using Lotus 1-2-3 (Lotus Development Corporation, Cambridge, MA, USA). The lag phase was defined as the time required to reach the stage of exponential temperature increase.

RESULTS

Bacterial fermentation, pH and organic acids in soak water

Figure 2 shows changes of pH and the concentrations of organic acids in soak water when soaking method (VI) was used. During the first 15 h of soaking, the pH decreased rapidly towards pH 3.9. After 15 h of soaking a further increase in organic acid concentrations did not affect the pH value very much.

Lactic acid was the major organic acid in soak water: after 24 h of soaking 2.1% (w/v soak water) was found. Acetic acid increased from 0 to 0.3% (w/v soak water) after 24 h of soaking. During soaking the amount of citric acid varied between 0.35 and 0.5% (w/v soak water). Other organic acids were not detected.

Table 1 shows the acidification of soak water using pure cultures of homofermentative lactic acid bacteria compared to the accelerated acidification method. The data represent averages of duplicate results. Compared with the acceler-

Fig. 2 Change in pH and organic acids during accelerated acidification of soya beans at 30°C. ▲, Acetic acid; ○, lactic acid; □, citric acid; ▽, pH

ated acidification method, soaking of soya beans with pure cultures of lactic acid bacteria resulted in less total acidity with relatively less acetic acid.

Reduction in concentration of organic acids during cooking

After soaking in 2.0% (w/v soak water) lactic acid at 5°C or in 0.5% (w/v soak water) acetic acid, the beans were rinsed with tap water and cooked in fresh tap water. The decrease of titratable acidity in beans is shown in Table 2. The results were based on duplicate trials. A reduction of 45% of titratable acidity in soya beans soaked in 2% (w/v soak water) lactic acid was observed after 20 min of boiling. For acetic acid, a reduction of 51% was found. After the major reduction between 0 and 20 min cooking a slight apparent increase of titratable acidity was found. This might be

Table 1 The concentration of acetic and lactic acids in the liquid after soaking soya beans with added lactic acid bacteria, or with accelerated acidification for 24 h at 30°C in static conditions*

Strain	pH after soaking	Acetic acid (% w/v)	Lactic acid (% w/v)
<i>Lactobacillus plantarum</i>	4.15	0.14	1.78
<i>Lactobacillus acidophilus</i>	4.20	0.14	1.61
<i>Pediococcus pentosaceus</i>	4.24	0.16	1.56
Accelerated acidification	4.12	0.29	2.14

* The figures are the averages of duplicate results.

Pretreatment: Cooking time (min)	Control I (ml g ⁻¹)	Titratable acidity			
		Soaked in 2.0% (w/v) lactic acid (IV)		Soaked in 0.5% (w/v) acetic acid (V)	
		ml g ⁻¹	%	ml g ⁻¹	%
0	0.42	1.70	100	1.00	100
20	0.16	0.93	55	0.49	49
40	0.17	0.98	58	0.53	53
60	0.17	0.99	58	0.53	53

Table 2 Reduction of titratable acidity (ml of 0.1 mol l⁻¹ NaOH (g sample)⁻¹) by cooking and cooling of non-acidified soya beans and soya beans acidified by soaking in lactic acid and acetic acid*

* The figures are the averages of duplicate results.

caused by a different buffering capacity resulting from permeability alterations due to prolonged cooking.

The effect of organic acids and temperature on germination of sporangiospores of *Rhizopus oligosporus* in liquid media

Based on the observed concentrations of organic acids in soaked and cooked beans, the effects of lactic acid, acetic acid and citric acid on spore germination were investigated in liquid media.

In Table 3 the effects of lactic, acetic and citric acids, the spore density of the suspension, and the incubation temperature on the germination of *R. oligosporus* sporangiospores are shown. After 2 h of incubation the various stages of fungal growth became visible, viz. spore swelling, germ tube formation, hyphal extension and branching. The percentage of germinated spores increased with time of incubation up to 6 h, after which it became very difficult to distinguish between germinated and non-germinated spores, due to the dense network formed by the growing hyphae. After 6 h of incubation the pH decreased from 4.0 to 3.6. The addition of lactic acid (1.0% w/v, Table 3, experiment 1) to a liquid medium resulted in a significant ($P < 0.05$) increase from 45.1 ± 14.1 to $63.5 \pm 11.7\%$ germination. Citric acid at concentrations of 0.25% and 0.50% (w/v incubation medium) (experiments 2 and 4) increased ($P < 0.05$) the germination percentage of *R. oligosporus* sporangiospores, to a similar extent as lactic acid.

In contrast, acetic acid (Table 3, experiments 3 and 4) delayed the germination. No germination at all was found within 6 h at concentrations of 0.10 and 0.15% (w/v incubation medium), while only a low percentage of spores germinated at 0.05% (w/v incubation medium) acetic acid.

In experiment 4 (Table 3) the effects of the three organic acids on germination of a single spore suspension were tested. After 6 h of incubation a slight increase in the germination percentage was found for lactic (1.5% w/v,

$P < 0.01$) and citric acid (0.5% w/v, $P < 0.05$), while with acetic acid germination remained significantly ($P < 0.01$) lower.

The influence of the spore density on germination was also investigated. To that purpose, 10-fold diluted and four-fold concentrated spore suspensions were compared with the usual spore suspension (Table 3, experiment 5). The percentage of spores that germinated increased with increasing dilution. To investigate whether this phenomenon was due to substrate limitation, germination in Malt Extract Broth was measured; this medium is richer in organic carbon and nitrogen. Germination percentages increased significantly ($P < 0.05$) from 43.9 ± 22.2 in our experimental medium to 74.0 ± 22.0 in the Malt Extract Broth (pH 4.0).

Germination percentages at 25°C were low, but increased with temperature to a maximum at 37°C (Table 3).

Changes in temperature during fermentation of cooked beans

After soaking and cooking, evaporative cooling reduced the temperature of the soya beans to approximately room temperature. Variations of initial bean temperatures influenced the growth rate and the length of the lag phase of fungal growth as measured by increase in temperature (Fig. 3). A maximum difference of 3 h was found between 37° and 25°C for the periods of most active heat production. However, if the incubation temperature was maintained at 25°C or 37°C a longer and a shorter lag phase, respectively, were observed (data not shown).

The effects of various soaking conditions on the length of the period before excessive heat production (lag phase) during tempe fermentation are shown in Table 4. The addition of acetic acid to the soak water failed to cause a significant increase in the lag phase (Table 4, experiment A). A combination of 0.5% (w/v) acetic acid and 1.5% (w/v) lactic acid in the soak water resulted in an increase in the lag phase from 14.0 to 19.5 h (Table 4, experiment B).

Table 3 The effect of lactic acid, citric acid and acetic acid on germination (%) of spores of *Rhizopus oligosporus* incubated at 30°C for 6 h in a liquid medium* adjusted to pH 4.0, and the effect of temperature on germination

Experiment	Treatment	Acid concentration % (w/v medium)	Spores/field	Germination percentage (%)
1	Control	0.00	13.0 ± 3.3†	45.1 ± 14.1†
	Lactic acid	0.50	12.5 ± 3.9	59.2 ± 7.6‡
	Lactic acid	1.00	12.8 ± 2.3	63.4 ± 11.7‡
	Lactic acid	1.50	12.4 ± 2.8	56.5 ± 15.1
2	Control	0.00	29.5 ± 6.5	19.1 ± 3.3
	Citric acid	0.25	24.1 ± 6.8	28.3 ± 7.9‡
	Citric acid	0.50	19.4 ± 3.4	37.7 ± 7.4‡
	Citric acid	1.00	20.8 ± 3.9	17.5 ± 7.6
3	Control	0.00	18.1 ± 3.2	42.7 ± 5.1
	Acetic acid	0.05	15.5 ± 6.3	10.6 ± 5.6‡
	Acetic acid	0.10	23.6 ± 10.2	0.5 ± 1.4‡
	Acetic acid	0.15	22.4 ± 10.8	0.0 ± 0.0‡
4	Control	0.00	17.9 ± 4.8	43.4 ± 10.3
	Lactic acid	1.00	13.3 ± 3.3	50.2 ± 5.8
	Lactic acid	1.50	11.5 ± 2.3	64.0 ± 12.4‡
	Citric acid	0.25	16.4 ± 4.4	46.0 ± 8.7
	Citric acid	0.50	20.4 ± 9.8	59.4 ± 13.7‡
	Acetic acid	0.05	11.5 ± 1.6	22.6 ± 11.2‡
	Acetic acid	0.10	12.7 ± 2.3	0.0 ± 0.0‡
5	10* diluted		5.8 ± 2.8	70.1 ± 27.8‡
	Control		59.3 ± 12.1	11.1 ± 4.6
	4* concentrated		196.3 ± 20.0	2.4 ± 1.1‡
6	25°C		20.6 ± 12.2	4.3 ± 3.0
	30°C		39.5 ± 6.30	12.7 ± 6.5
	37°C		31.2 ± 10.5	20.6 ± 6.6
	42°C		44.7 ± 12.6	11.4 ± 5.3

* Medium composition: 0.37% w/v glucose, 0.09% w/v $(\text{NH}_4)_2\text{SO}_4$, 0.1% w/v $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and $(0.2 \mu\text{g ml}^{-1})$ of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ and $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$.

† Mean ($n = 8$) ± S.D.

‡ Significant difference ($P < 0.05$) compared to the control.

Fig. 3 Change in the temperature of soya beans undergoing fungal fermentation with *Rhizopus oligosporus*, as a function of the initial bean temperature: —, 25°C; ---, 30°C; ·····, 37°C; - · - ·, 42°C.

Based on experiment B (Table 4) and experiment 3 (Table 3) it is clear that acetic acid might inhibit the temper fungal growth stage. To avoid such inhibition, two alternative approaches to soaking were tested. The first one was to use pure cultures of homofermentative lactic acid bacteria as a means of reducing the accumulation of acetic acid. Under anaerobic conditions homofermentative strains produce only lactic acid from hexoses, but as shown in Table 1 acetic acid was also found, implying oxidase activity and/or that certain substrates (e.g. pentoses) were available in the soak water enabling acetic acid formation. Compared with the method of accelerated acidification a reduction from 19.5 to 14.8 h in lag phase was seen when the beans were soaked with an inoculum of *Ped. pentosaceus* (Table 4, experiment C). Compared with acidification by *Ped. pentosaceus* an increase of the fungal lag phase was found for beans soaked with *Lact. plantarum* and *Lact. aci-*

Table 4 The effect of the soaking treatments on the pH of the soak water and of soya beans, the lag phase of *Rhizopus oligosporus* and the temperature increase during the tempe process (incubation temperatures: experiments A and B at 5°C, experiments C and D at 30°C)

Experi- ment	Soak condition	Treatment	pH* (after soaking)	pH† (after cooking)	Lag phase (h)	Temperature increase (°C h ⁻¹)
A	Non-acidified	I	6.57	6.85	11.4	1.7
	0.125% (w/v) acetic acid	II	6.00	6.55	10.8	1.6
	0.25% (w/v) acetic acid	II	5.55	6.15	10.5	1.6
	0.50% (w/v) acetic acid	II	5.10	5.69	11.4	1.6
B	1.5% (w/v) lactic acid	III	4.23	4.81	13.3	1.3
	1.5% (w/v) lactic acid 0.125% (w/v) acetic acid	III	4.24	4.77	14.0	1.2
	1.5% (w/v) lactic acid 0.25% (w/v) acetic acid	III	4.24	4.68	14.1	1.2
	1.5% (w/v) lactic acid 0.50% (w/v) acetic acid	III	4.24	4.60	19.5	1.2
C	<i>Lactobacillus plantarum</i>	VII	4.15	4.35	16.3	1.0
	<i>Lactobacillus acidophilus</i>	VII	4.20	4.45	16.5	1.0
	<i>Pediococcus pentosaceus</i>	VII	4.24	4.52	14.8	1.0
	Accelerated acidification (24 h)	VI	4.12	4.36	19.5	1.1
	Lactic acid (2.0% w/v soak water)	IV	4.01	4.40	12	1.0
	Acetic acid (0.5% w/v soak water)	V	4.73	4.57‡	> 48	0
D	Accelerated acidification (4 h)	VI	4.86	6.54	11.3	2.1
	Accelerated acidification (12 h)	VI	4.37	5.34	11.8	1.8
	Accelerated acidification (16 h)	VI	4.21	4.75	11.9	1.5
	Accelerated acidification (24 h)	VI	4.11	4.37	13.5	1.3

* Soak water.

† Soya beans.

‡ Cooked in 0.5% (w/w water) acetic acid.

dophilus from 14.8 to 16.3 and 16.5 h, respectively. A further reduction of the fungal lag phase was obtained with soaking in 2.0% (w/v soak water) lactic acid. Soaking in 0.5% (w/v soak water) acetic acid at 30°C, and subsequent cooking in 0.5% (w/w) acetic acid resulted in a lag phase beyond 48 h. The second alternative to prevent inhibitory concentrations of acetic acid is to reduce the soaking period during accelerated acidification, for example from 24 to 12 h. By doing this, spoilage of tempe by acid-sensitive bacteria might cause problems if the soya beans are not adequately acidified. A small reduction in the lag phase was found from 15.3 to 11.3 if the soaking period was reduced from 24 to 4 h.

In Table 4 it is shown that the temperature increased faster when the soya beans were less acidified. A temperature increase varying between 1.0 to 1.5°C h⁻¹ was observed for beans with a pH between 4.35 and 4.8, while a maximum increase of 2.1°C h⁻¹ was found for beans (pH of beans 6.54) soaked during 4 h according to the method of accelerated acidification.

DISCUSSION

The formation of both lactic acid (2.1% w/v soak water) and acetic acid (0.3% w/v soak water) during soaking,

according to the method of accelerated acidification for 24 h at 30°C, was expected since the major species in the soak water, *Lact. plantarum*, is a facultative heterofermentative lactic acid bacterium. Compared with the method of accelerated acidification, lower concentrations of acetic acid (0.14% vs 0.29% w/v soak water) were obtained with pure cultures of *Lact. plantarum*. This might be due to a higher initial count, or to adaptation of the microflora to the substrate method, or to accompanying heterofermentative lactic acid bacteria in the method of accelerated acidification. The increase of citric acid observed during the first 6 h of soaking is possibly due to endogenous metabolism that would accompany the initial stages of bean germination (Mulyowidarso *et al.* 1991). Compared with the results of Mulyowidarso *et al.* (1991) less variety in organic acids but higher concentrations of lactic acid (2.0 vs 0.65% w/v soak water) and acetic acid (0.3 vs 0.08% w/v soak water) were obtained using the accelerated method. This is due to the acidification method used. In the traditional soaking method with natural acidification a variety of microorganisms were active resulting in the production of 10 different organic acids. In a method with accelerated acidification, the higher initial count of lactic acid bacteria and the lower initial pH favour the production of lactic acid and acetic acid.

- Hounhouigan, D.J., Nout, M.J.R., Nago, C.M., Houben, J.H. and Rombouts, F.M. (1993) Characterization and frequency distribution of species of lactic acid bacteria involved in the processing of mawè, a fermented maize dough from Bénin. *International Journal of Food Microbiology* 18, 279–287.
- Ko, S.D. and Hesseltine, C.W. (1979) Tempe and related foods. In *Economic Microbiology, Vol. 4, Microbial Biomass* ed. Rose, A.H. pp. 115–140. London: Academic Press.
- Medwid, R.D. and Grant, D.W. (1984) Germination of *Rhizopus oligosporus* sporangiospores. *Applied and Environmental Microbiology* 48, 1067–1071.
- Mulyowidarso, R.K., Fleet, G.H. and Buckle, K.A. (1989) The microbial ecology of soybeans soaking for tempe production. *International Journal of Food Microbiology* 8, 35–46.
- Mulyowidarso, R.K., Fleet, G.H. and Buckle, K.A. (1991) Changes in the concentration of organic acids during the soaking of soybeans for tempe production. *International Journal of Food Science and Technology* 26, 607–614.
- Nout, M.J.R. and Rombouts, F.M. (1990) Recent developments in tempe research. *Journal of Applied Bacteriology* 69, 609–633.
- Nout, M.J.R., Bonants-Van Laarhoven, T.M.G., De Dreu, R. and Gerats, I.A.G.M. (1985) The influence of some process variables and storage conditions on the quality and shelf-life of soybean tempe. *Antonie van Leeuwenhoek* 51, 532–534.
- Nout, M.J.R., De Dreu, M.A., Zuurbier, A.M. and Bonants-Van Laarhoven, T.M.G. (1987) Ecology of controlled soyabean acidification for tempe manufacture. *Food Microbiology* 4, 165–172.
- Nout, M.J.R., Martoyuwono, T.D., Bonné, P.C.J. and Odamtten, G.T. (1992) Hibiscus leaves for the manufacture of Usar, a traditional inoculum for tempe. *Journal of the Science of Food and Agriculture* 58, 339–346.
- Steinkraus, K.H., Yap Bwee Hwa, Van Buren, J.P., Providenti, M.I. and Hand, D.B. (1960) Studies on tempeh—an Indonesian fermented soybean food. *Journal of Food Science* 25, 777–788.
- Steinkraus, K.H., Van Buren, J.P., Hackler, L.R. and Hand, D.B. (1965) A pilot-plant process for the production of dehydrated tempeh. *Food Technology* 19, 63–68.
- Tanaka, N., Kovats, S.K., Guggisberg, J.A., Meske, L.M. and Doyle, M.P. (1985) Evaluation of the microbiological safety of food tempeh made from unacidified soybeans. *Journal of Food Protection* 48, 438–441.
- Tünçel, G., Nout, M.J.R. and Rombouts, F.M. (1989) Effect of acidification on the microbiological composition and performance of tempe starter. *Food Microbiology* 6, 37–43.
- Winarno, F.G. and Reddy N.R. (1986) Tempe. In *Legume Based Fermented Foods* ed. Reddy, N.R., Pierson, M.D. and Salunkhe, D.K. pp. 95–117. Boca Raton: CRC Press.