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Changes in soya bean lipids during tempe fermentation

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Soya beans were fermented with pure cultures of *Rhizopus oligosporus* and *Rhizopus oryzae* in perforated petri dishes at 25°, 30° and 37°C for 69 h. During fermentation, samples were taken at different time intervals. Lyophilised samples were analysed for total crude lipid (CL), fatty acids present in glycerides (GFA) and free fatty acids (FFA). With *R. oligosporus*, the level of GFA decreased from 22·3 to 11·5% (w/w, dry matter) after 69 h fermentation at 37°C. In the final product, only 4·3% (w/w, dry matter) of FFA were found, hence the difference of 6·5% (w/w, dry matter) of fatty acids were lost. This difference was attributed to assimilation of fatty acids by *R. oligosporus* as a source of carbon. At 25°C the situation was different as the level of FFA was 1·7 times higher than could be explained on the basis of the decrease of GFA. The distribution pattern of GFA showed a slight increase of C18:1 and C18:2 during the fermentation at the expense of C18:3. Similar results were obtained with *R. oryzae*. With this fungus the strongest effects were observed at 30°C which is close to its optimum temperature for growth. More fatty acids were lost than with *R. oligosporus* and a more pronounced shift in GFA towards saturated fatty acids was observed.

INTRODUCTION

Tempe is a traditional Indonesian food in which filamentous fungi, particularly *Rhizopus* spp. play an essential role. Yellow-seeded soya beans are the most common and popular raw material. The resulting 'tempe kedele' is usually referred to as 'tempe' (Nout & Rombouts, 1990). After fermentation, the cake-like mass of soya beans and mould mycelia is sliced and either fried or cooked by other means (Sudarmadji & Markakis, 1978).

Rhizopus spp. produce a variety of enzymes, including carbohydrases, lipases, proteases and phytases. This paper presents the authors' investigations on the fate of soya bean crude lipid and individual fatty acids during the tempe fermentation. Souser and Miller (1977) reported that lipase activity in tempe fermented with *Rhizopus oligosporus* was highest after 24 h of incubation. An enzyme was isolated having a molecular mass of > 100 000 Da and optimum pH 7, optimum temperature 40°C and which was inactivated after heating at 60°C for 10 min. Nahas (1988) reported optimum pH values for *R. oligosporus* growth and lipase production of 5·5 and 6·5, respectively. Temperatures ranging from 35–40°C favoured the growth of *R. oligosporus* whereas its enzyme production was highest at lower temperatures (25°C). The maximum yield of lipase was obtained at 25°C with pH 6·5. In liquid media, maximum *R. oligosporus* lipase activity was obtained after 3 days incubation at 25°C.

Although various aspects of tempe composition have been studied, the knowledge about changes in lipid and fatty acids composition is incomplete. Wagenknecht *et al.* (1961) reported an increase of soya bean free fatty acid (FFA) levels fermented by *Rhizopus oryzae* at 37°C: from 0·3 to 8·2 g/100g tempe dry matter. Sudarmadji and Markakis (1978) monitored changes of FFA levels in tempe fermented with *R.oligosporus* at 32°C which was subsequently fried in oil. Frying resulted in increased crude lipid (CL) from 8·7 to 26·5 g/100g tempe and a decrease of all five FFA present in fresh tempe from 4·45 to 1·72 g FFA/100 g tempe dry matter. Hering *et al.* (1991) compared glyceride-bound fatty acids (GFA) levels in tempe made from various soya bean cultivars with several fungal inocula at different temperatures. They found that the fatty acid composition of tempe is similar to that of soya beans but with higher oleic acid and slightly lower levels of other fatty acids.

The present investigation aims to provide a mass balance of fatty acids occurring in CL, GFA and as FFA in tempe as a function of incubation time and temperature. In addition two strains of the major functional fungi, i.e. *R. oligosporus* and *R. oryzae* are compared.

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MATERIALS AND METHODS

Organisms

Rhizopus microsporus var. *oligosporus* strain LU 575 (NRRL 5905) and *Rhizopus oryzae* strain LU 583 were grown and maintained at 30°C on malt extract agar (CM 59, Oxoid, UK). Sporangiospore suspensions were obtained by scraping off the sporangia from a culture after 7 days incubation at 30°C, and suspending them in sterile distilled water containing 0·1% (v/v) Tween 80 (Merck, Germany). The viable count varied between 5×10^5 and 10^6 cfu/ml, when determined on Rose-Bengal Chloramphenicol Agar (CM 549, Oxoid, UK). The sporangiospore suspensions were used immediately after preparation.

Tempe manufacturing process

Dehulled yellow-seeded soya beans (*Glycine max*) were soaked overnight using the accelerated acidification method (pH of the soak water < 4·2) (Nout *et al.*, 1987). Subsequently, the beans were washed with tap water and boiled for 20 min, cooled, superficially dried (15–30 min, at room temperature) and inoculated using a sporangiospore suspension (1% v/w). Petri dishes (dia. 9 cm; 5 perforations/side, dia. 1 mm) were filled with 60 g inoculated beans, sealed with tape and incubated at 25, 30 or 37°C. Samples were taken at various time intervals and were analysed for moisture content. Remaining sample was frozen at −20°C, lyophilised and stored under vacuum at 4°C in the dark until analysis. Although incubation is stopped after 30–48 h under production conditions, the fermentation was continued for up to 70 h to observe the effects of on-going biochemical processes.

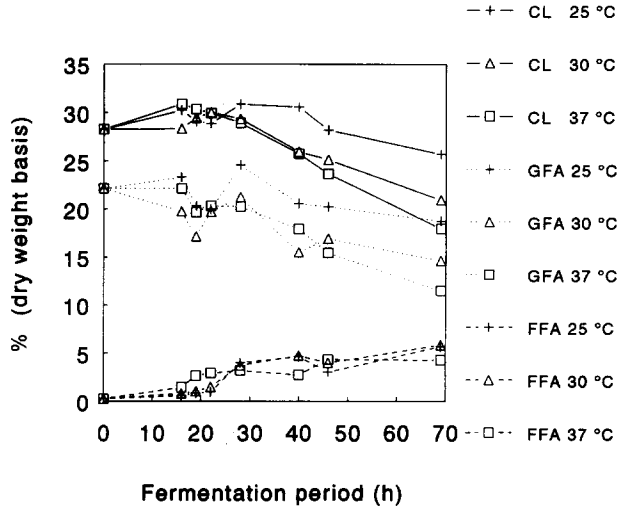
Chemical analyses

The lyophilized tempe samples were ground with a mill (Type 32002, Moulinex, France) to a fine powder, and 7·0 g samples were extracted with petroleum-ether (40:60) in Soxhlet extractors. The ether was evaporated, and quantification of the total CL content was carried out gravimetrically. FFA and GFA were determined by gas-liquid chromatography (GC) of their respective methyl esters according to Metcalfe and Wang (1981), using a methyl ester of heptadecanoic acid (C17:0, margaric acid) (Merck, Darmstadt, Germany) as an internal standard. A Perkin Elmer chromatograph (Sigma 3B, Norwalk, CT, USA) with a flame ionisation detector and an HP3380A integrator (Hewlett Packard, Geneva, Switzerland) was used. Chromatographic conditions were as follows: column, length 2 m, internal diameter 2 mm, external diameter 1/8 inch (Chrompack, Bergen op Zoom, The Netherlands); stationairy phase, 15% CP-Sil 84 on CHROM WHP, 100–200 mesh; injection temperature, 225°C; detector temperature, 250°C; column temperature, 180°C; carrier gas, N_2.

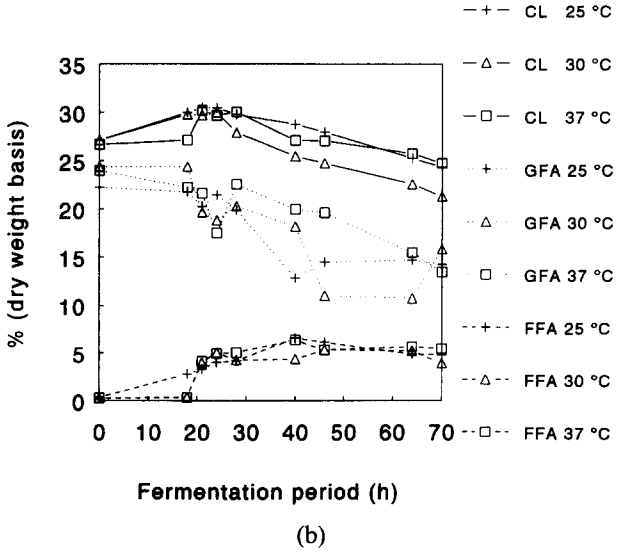
RESULTS

Total crude lipid content (CL)

Changes of the total CL content are shown in Figs 1(a) and 1(b). The data presented for CL are means of duplicate measurements. For each data point the coefficient of variation (CV) was < 4·6%. With *R. oligosporus* (Fig. 1(a)), CL diminished after the period of most active growth (after 40 h at 25°C, 24 h at 30°C or 16 h at 37°C). This was more pronounced at higher incubation temperatures and reached 30% of the initial value after 69h of fermentation at 37°C. *R. oryzae* (Fig. 1(b)) gave a slight increase of CL during most active growth (20–30 h) at all temperatures, which was also followed by a decline. However, with *R. oryzae* the strongest decrease of CL took place at 30°C with 21% of the initial value and was thus less pronounced than with *R. oligosporus*.



(a)



(b)

Fig. 1. Changes of total crude lipid (CL), glyceride fatty acids (GFA) and free fatty acids (FFA) during fermentation of soya beans in perforated petri dishes at 25, 30 and 37°C, with (a)*Rhizopus oligosporus*, and (b)*Rhizopus oryzae*.

Table 1. Levels of glyceride fatty acids (GFA) and free fatty acids (FFA) during soya bean fermentation at 30°C

Incubation time (h)	C16:0		C18:0		C18:1		C18:2		C18:3		TOTAL		
	GFA[a]	FFA[b]	GFA	FFA	GFA	FFA	GFA	FFA	GFA	FFA	GFA	FFA	FA[c]
Rhizopus oligosporus													
0	2·5	0·1	0·7	0·0	5·1	0·1	12·3	0·0	1·6	0·0	22·2	0·3	22·4
16	2·2	0·1	0·6	0·0	4·5	0·1	11·0	0·6	1·4	0·0	19·8	0·8	20·6
19	1·9	0·2	0·6	0·0	3·9	0·1	9·6	0·8	1·2	0·0	17·2	1·0	18·2
22	2·1	0·3	0·6	0·0	4·3	0·2	11·2	1·0	1·4	0·0	19·7	1·5	21·2
28	2·3	0·7	0·6	0·2	5·9	0·7	12·1	2·0	1·4	0·2	21·3	3·8	25·0
40	1·6	0·8	0·5	0·2	3·8	1·0	8·8	2·4	0·9	0·2	15·5	4·8	20·3
46	1·7	0·7	0·5	0·2	4·2	0·9	9·6	2·1	0·9	0·2	16·9	4·0	21·0
69	1·4	0·9	0·4	0·3	3·7	1·4	8·3	3·0	0·9	0·3	14·7	5·9	20·5
Rhizopus oryzae													
0	2·7	0·1	0·8	0·0	5·4	0·1	13·9	0·0	1·8	0·0	24·4	0·3	24·7
18	2·6	0·1	0·8	0·0	5·5	0·1	13·7	0·1	1·8	0·0	24·4	0·4	24·8
21	1·9	0·9	0·6	0·2	4·5	0·8	11·3	1·9	1·3	0·2	19·6	4·0	23·6
24	1·9	1·1	0·5	0·3	4·3	0·9	10·9	2·4	1·3	0·3	18·8	5·1	23·9
28	1·9	1·0	0·5	0·3	4·7	0·8	11·8	2·0	1·4	0·2	20·3	4·4	24·7
40	1·6	1·0	0·5	0·3	4·2	0·9	10·6	2·0	1·2	0·2	18·2	4·4	22·5
46	1·1	1·0	0·3	0·3	2·6	1·3	6·4	2·5	0·6	0·2	11·0	5·3	16·3
64	1·1	0·9	0·3	0·3	2·6	1·4	6·2	2·5	0·6	0·2	10·7	5·2	16·0
70	1·7	0·6	0·4	0·2	3·7	1·1	9·1	2·0	0·9	0·1	15·8	4·0	19·8

[a,b] Expressed as % of dry matter.
[c] FA, fatty acid.

GFA versus FFA

Total GFA and total FFA are shown in Figs 1(a) and 1(b). The data points for GFA and FFA were based on single results, after it had been verified that the CV of the GC analyses was < 1·9%. In Figure 1(a) it is shown that *R. oligosporus* reduced the GFA with 15·4, 35·4 or 48% after 69 h of fermentation at 25, 30 or 37°C, respectively. During the same period the FFA increased by 5·7, 5·9 or 4·3% of dry matter at 25, 30 or 37°C, respectively. When the GFA decreases were compared with FFA increases, an overall loss of fatty acids of 54% of the initial level at 37°C was observed. At 30°C, an overall loss of 21% took place, whereas at 25°C there was an apparent increase of fatty acids of 70% compared to the initial level. With *R. oryzae* a GFA decrease of 7·9, 8·6 and 10·2% of the dry matter at 25, 30 and 37°C, respectively, was found. During this period the FFA levels increased with 4·8, 3·9 and 5.5% at 25, 30 and 37°C, respectively. Here again, losses of 39, 54 and 46% of the initial level of fatty acids occurred at 25, 30 and 37°C, respectively.

GFA patterns

In dehulled soya beans, the following distribution of GFA was found after soaking and cooking: palmitic acid C16:0 (11·2% of total GFA), stearic acid C18:0 (3·3%), oleic acid C18:1 (23·0%), linoleic acid C18:2 (55·6%) and linolenic acid C18:3 (7·0%). In Figure 1(a), total GFA decreased with time and with increasing incubation temperature for *R. oligosporus*. In Table 1, the individual levels of GFA during fermentation at 30°C are presented. These follow a similar trend as that of total GFA shown in Fig. 1(a). There was a slight decrease until 19 h followed by a slight increase during the period of most active growth. Beyond 28 h of fermentation, the levels of all GFA decreased. Compared with the initial levels of individual GFA (at time = 0), the minority fatty acids (C16:0, C18:0 and C18:3) decreased faster than the majority fatty acids (C18:1 and C18:2). With *R. oryzae* at 30°C, GFA decreased until 64h and slightly increased again. As can be seen in Fig. 1(b), this did not occur at 25 or 37°C. The distribution of the individual GFA was similar as with *R. oligosporus*. The effect of temperature on the GFA pattern is shown in Figs 2(a) and 2(b). With both *Rhizopus* spp. the distribution of GFA developed in a similar pattern at all temperatures, i.e. C18:3 tended to decrease more than the other fatty acids. With *R. oligosporus* this was most pronounced at 37°C and with *R. oryzae* at 30°C.

FFA patterns

As was shown in Figs 1(a) and 1(b), the levels of FFA increased during fermentation. With *R. oligosporus* at 25°C, more FFA was retrieved than expected on the basis of GFA decrease. At 30 and 37°C considerably less FFA was found than expected. Figures 3(a) and 3(b) show the level and distribution pattern of individual FFA expressed as a percentage of the expected level. The expected level of FFA equals the decrease of GFA between time = 0 and 46 h of fermentation. With *R. oligosporus* at 25°C (Fig. 3(a)), about twice as much C18:1 and C18:2 but less C18:3 were found than expected on the basis of GFA decrease. At the higher temperatures, the pattern of FFA shifted towards a higher degree of saturation, but FFA levels were generally lower. With *R. oryzae* (Fig 1(b)), total FFA levels

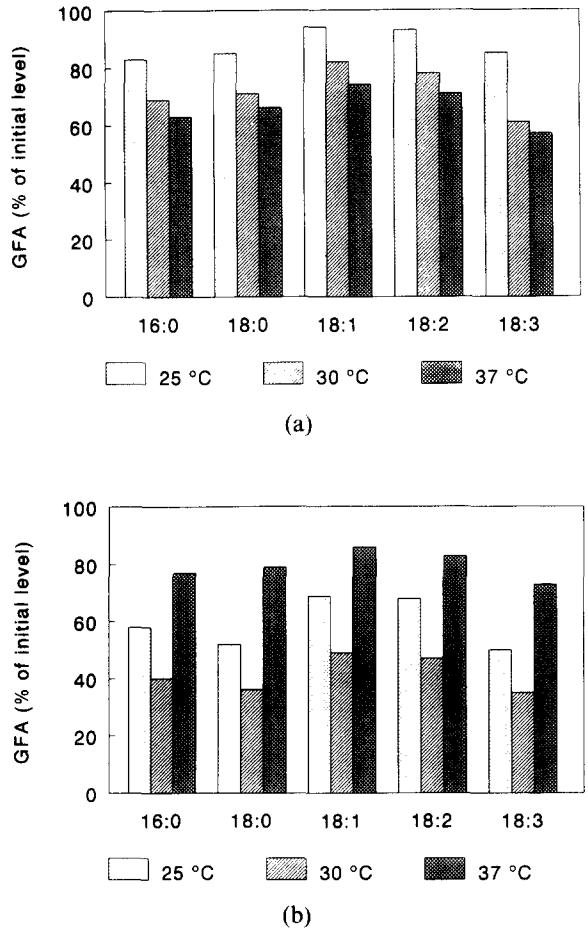


Fig. 2. Relative changes of GFA distribution after 46 h of fermentation of soya beans in perforated petri dishes at 25, 30 and 37°C, with (a)*Rhizopus oligosporus* and (b)*Rhizopus oryzae*.

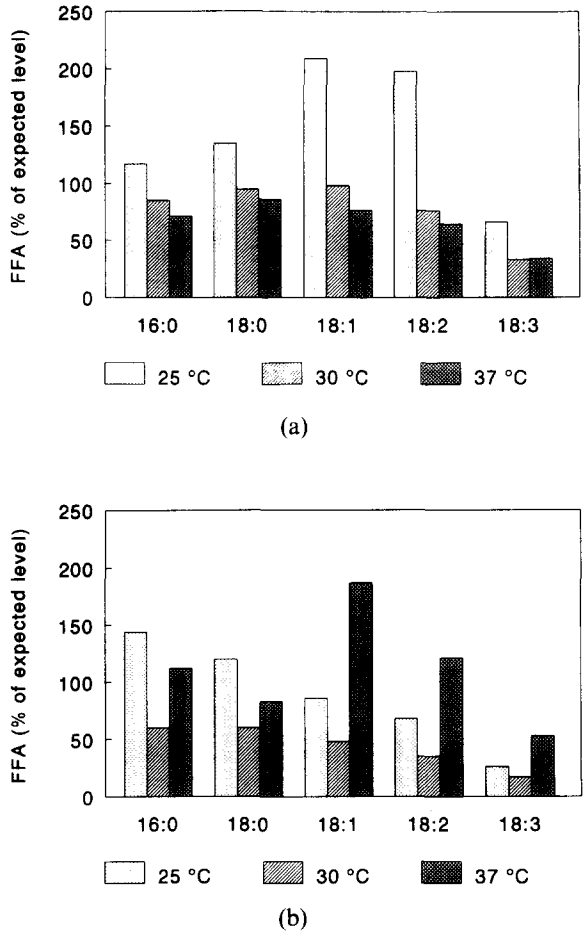


Fig. 3. Changes of FFA distribution after 46 h of fermentation of soya beans in perforated petri dishes at 25, 30 and 37°C, with (a)*Rhizopus oligosporus* and (b)*Rhizopus oryzae*. The expected FFA level corresponds with the quantity of GFA which disappeared during 46 h of fermentation at 25, 30 or 37°C, respectively.

were lower than expected from the GFA decrease. However, for the individual FFAs there were some remarkable differences between the respective incubation temperatures. At 25°C, there was an increase in C16:0 and C18:0. At 37°C, C18:1 and C18:2 exceeded 100%, and at 30°C the residual FFAs showed a general tendency towards a higher degree of saturation.

DISCUSSION

During the tempe fermentation, a temperature-related decrease of total CL content was observed. With *R. oligosporus*, strongest CL reduction was 39% of the initial level after 69 h of fermentation at 37°C. *R. oligosporus* has an optimum growth temperature between 35 and 37°C. *R. oryzae* has its optimum growth temperature at approximately 30°C. In the experiment with *R. oryzae* at 25 and 37°C we obtained a less pronounced decrease of CL than at 30°C. The data presented here suggest that the decrease of CL is related to the growth rate.

Earlier investigations of CL changes during tempe fermentation did not include the effect of incubation temperature or microbial strain. Van Buren *et al.* (1972) reported a CL decrease of 18% after 72 h of fermentation at 38°C using a mixed culture of *Rhizopus* spp. Van Veen and Schaefer (1950) found a CL decrease of 30% with *R. oryzae* at 30°C. Wagenknecht *et al.* (1961) reported that tempe fermented at 37°C with *R. oryzae* had a fairly constant CL level varying from 22·3 to 26·7%. Hering *et al.* (1991) reported that a significant CL loss could not be detected with different *Rhizopus* spp. at 24, 32 and 36°C. The difference between the present results and those obtained by Hering *et al.* (1991) are probably caused by the different methods of acidification. During the accelerated acidification method (Nout *et al.*, 1987) profuse growth of lactic acid bacteria occurs. By consequence, a decrease of fermentable carbohydrates takes place. This can cause a quicker depletion of assimilable carbohydrates and, hence, a shift towards the use of lipids as a source of energy and carbon during the stage of fungal fermentation. With the chemical acidification procedure used by Hering *et al.* (1991), the fermentable sugars remain available for the fungal fermentation stage. Consequently, less CL will be assimilated during the fermentation.

With *R. oligosporus* grown at 25°C, the apparent increase of CL during the period of most active growth is

due to active assimilation of carbohydrates and limited consumption of lipids, causing a shift in the dry matter composition resembling an enrichment of CL.

The results for *R. oligosporus* show that at 30°C and 37°C, the GFA decrease is larger than the FFA increase. This difference is created after the period of most active mould growth (20–30h). The disappearance of the liberated FFA indicates that the fungus metabolised the latter as a carbon source. Indeed, *R. oligosporus* was shown to grow on soya oil as a carbon source (Nahas, 1988). The fact that fatty acids disappear after the most active growth period implies that fatty acids are not the preferred carbon source.

Although tempe production usually takes place under uncontrolled conditions, the results presented indicate that it will be possible to influence the fatty acid pattern by varying the incubation temperature. In order to ensure maximum nutritional value of the product, it will be of interest to optimise bean pretreatments, incubation temperatures and periods in order to avoid undesirable losses of fatty acids.

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