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Fatty acid profiles of fermented soybean prepared by *Bacillus subtilis* and *Rhizopus oligosporus*

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Abstract

There are various types of fermented soybean products in Asia including *Natto* (Japan), *Jang* (Korea), *Kinema* (India), and *Thua Nao* (Thailand). For these products, bacteria mostly belonging to the genus *Bacillus* (i.e., *Bacillus subtilis*) are used. This study was aimed to use a co-culture between *B. subtilis* and *Rhizopus oligosporus* in preparing the fermented soybean. Initially, the raw soybeans were washed, sterilized by autoclaving, and inoculated with these inocula; for this, three different ratios between *B. subtilis* and *R. oligosporus* were used (100:0, 50:50, and 0:100). The fermentation was carried out at 30 °C for 3 days, and fatty acid concentration was determined. Results showed that palmitic, stearic, oleic, linoleic, and eicosanoic acids were present in the fermented soybeans products. The major fatty acids present in all samples were oleic and linoleic, ranging between 30.49 to 34.07 and 50.35 to 53.72% of total fatty acids, respectively.

Key words: *Bacillus*, fatty acid, fermented soybean, *Rhizopus*.
Abbreviations: CNF, cooked non-fermented soybeans; FAMEs; fatty acid methyl esters.

Introduction

Fermentation of soybean is generally considered as one of the successful methods helping in preserving and prolonging shelf life of soybean. In addition, after the fermentation soybean exhibits distinct characteristics (i.e., flavor and aroma) and, in some cases, becomes popular once accepted by the consumers. *Natto* is probably the best example of this case. Since its origin, *Natto*, which is produced using a pure starter culture of *Bacillus subtilis* (natto strain), has gained popularity and is widely known and consumed by people around the world (Kiuchi, Watanabe 2004). The *Natto* industry is increasing; in 2015, it was estimated that approximately 750 tonnes of *Natto* were produced with a value around 4 million US dollars (FAO 2016). There are other fermented soybean products, traditionally produced and only consumed by local people, which include *Cheonggukjang* of Korea, *Douchi* of China, *Kinema* of Nepal and India, and *Thua Nao* of Thailand. These products are similar in terms of raw materials used and the production process (Sarkar, Nout 2015). It should be noted, however that, unlike *Natto*, these fermented soybean products have not gained much attention and are still produced by artisanal techniques (i.e., produced at the household scale and using natural microbial starter cultures).

During soybean fermentation, it is expected that soy substrates are hydrolysed by many degrading microbial enzymes. Due to the protein-rich nature of soy, proteolysis is typically considered as a key process of soybean fermentation, which has been studied by many researchers (Omafuvbe et al. 2000; Visessanguan et al. 2005; Hu et al. 2010). In contrast, lipolysis has not gained much attention, although the soybean substrate is composed of around 20% of fat. Lipids are degraded into fatty acids resulting in development of some volatiles including alcohols, aldehydes and ketones in some foods (Visessanguan et al. 2006; Gambacorta et al. 2009). Similarly, lipolysis may be one of the key mechanisms occurring during the soybean fermentation. Unfortunately, there are only a few studies dealing with lipid profiles in fermented soybean products (de Reu et al. 1994; Sarkar et al. 1996; Feng et al. 2014). As part of the programme to develop a production process by utilization of fermented soybean, the present study was carried out to determine the lipid composition of the fermented soybeans prepared by a co-culture between the bacterium *B. subtilis* and the fungus *Rhizopus oligosporus*.

Materials and methods

Microbial cultures

The bacterium *Bacillus subtilis* strain TN51 was previously isolated from Thai *Thua Nao* (Dajanta et al. 2009). For inoculum preparation, the bacteria were grown in nutrient broth at 37 °C for 24 h. The cells were then harvested, resuspended in sterile distilled water and adjusted to 10^4 CFU mL^{-1}. The fungus *Rhizopus oligosporus,* which originated from Indonesian *Tempeh*, was obtained from the Agricultural Research Service Culture Collection, Bacterial Foodborne Pathogens and Mycology Research

Unit National Center for Agricultural Utilization Research (USA). For preparation as a starter culture, the fungus was maintained on potato dextrose agar at 30 °C for 3 to 4 days. The spores were then harvested, resuspended in sterile distilled water and adjusted to 10^4 CFU mL^{-1}.

Soybean fermentation

Soybeans were washed and soaked in water for 16 h at room temperature. Prior to fermentation, two different methods were employed to prepare cooked non-fermented soybeans (CNF). For conventional fermentation, soaked soybeans were cooked by boiling for 3 h (CNF1) and left to ferment by naturally occurring microbes at 30 °C for 72 h (TNM). In contrast, for inoculum fermentation, soaked soybeans were autoclaved at 121 °C for 40 min (CNF2). The fermentation was then prepared using microbial inocula [10^4 CFU (or conidia) g^{-1} of the autoclaved soybeans]. Three treatments with different ratios between *B. subtilis* and *R. oligosporus* were prepared as follows: (i) 100:0 (TNB), (ii) 50:50 (TNBR), and (iii) 0:100 (TNR). After that, the soybeans were incubated at 30 °C for 72 h.

Fatty acid composition analysis

Initially, forty grams of fermented soybeans were ground into fine powder. For lipid extraction, the sample powder was packed in a cellulose thimble inside the extraction chamber of the Soxhlet unit. Pure n-hexane (280 mL) was used to extract the lipid by refluxing at 70 °C for 2 h. The remaining hexane solvent was then removed under vacuum at 70 °C and the extracted lipid samples were kept at 4 °C until further use.

For fatty acid analysis, fatty acid methyl esters (FAMEs) were prepared as described by Nielsen (2010). In brief, the extracted lipids (30 mg) were mixed with 1 mL of 0.5% NaOH in methanol and incubated at 60 °C for 20 min. After cooling to room temperature, 1 mL of n-hexane was added and vortexed for 1 min. Subsequently, 400 µL of sterile double distilled H_2O were added and mixed by vortexing for 30 s. The hexane layer was then transferred to a vial containing a small amount of sodium sulfate (for at least 15 min). The hexane phase was finally filtered through a 0.25 µm-nylon syringe filter and stored at 4 °C prior to analysis.

An Agilent Technologies 6890N gas chromatograph equipped with an Agilent 5973N mass spectrometer system was used for fatty acid analysis profiling. Operating conditions were as follows: the column was a HP-5 MS Agilent capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness); helium was the carrier gas at a constant flow rate of 1 mL min^{-1}; the GC injector was set up in a splitless mode; the injector and detector temperature was set at 250 °C; the oven temperature was programmed starting at 120 °C for 2 min, then increasing to 250 °C with the rate of 10 °C min^{-1}. To identify individual FAMEs from the samples, an Agilent 5973N mass spectrometer was used in the scan mode with the mass range of 30 to 400 amu and electron impact ionization mode with an ionization voltage of 70 eV. The ion source temperature was 230 °C and the GC-MS transfer line was set at 280 °C. The results obtained were then recorded and expressed as relative area percentage.

Results and discussion

As part of a programme to improve the fermentation process of soybean using co-culture between *B. subtilis* and *R. oligosporus*, this study was focused on fatty acid profiles, which would be useful as food compositional data. In this analysis, total fatty acid content of the samples were extracted, prepared as FAMEs, and subjected to the GC for identification. A representative GC chromatogram of the FAMEs of the fermented samples is depicted in Fig. 1. In total, five fatty acids were identified using the MS analysis. Based on this analysis, there was an additional compound octadecenamide (or oleamide), identified in peak no. 6. This compound is an amide of oleic fatty acid, and thus was excluded from further analysis.

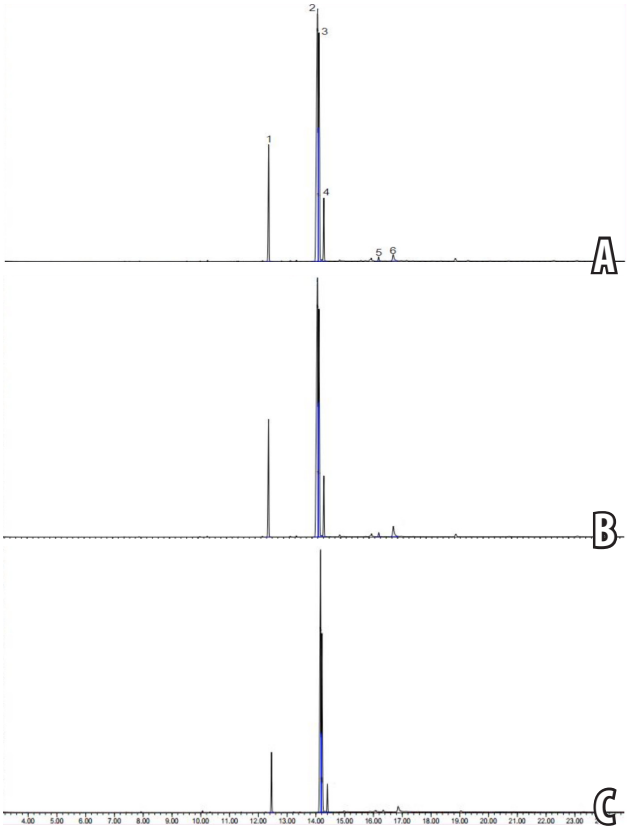

Fig. 1. Representative GC chromatogram of fatty acid methyl esters (FAMEs) from the lipid extracts of the autoclaved soybean (A), the fermented soybean prepared by *B. subtilis* (B), and the fermented soybean prepared by *B. subtilis* and *R. oligosporus* (C). Peak identification: 1, palmitic acid (C16:0); 2, linoleic acid (C18:2, ω6); 3, oleic acid (C18:1, ω9); 4, stearic acid (C18:0); 5, eicosanoic acid (C20:1); 6, 9(Z)-octadecenamide ($C_{18}H_{35}NO$).

Table 1. Fatty acid profiles of cooked non-fermented soybean (CNF) and fermented soybean. The data shown are means and SD. Means in a row with different letters are significantly different ($p < 0.05$). SFA, saturated fatty acid; UNSAT, unsaturated fatty acid; CNF1, boiled soybean; CNF2, autoclaved soybean; TNM, naturally fermented soybean TNB, fermented soybean by *B. subtilis*; TNR, fermented soybean by *R. oligosporus*; TNBR, fermented soybean by a co-culture of *B. subtilis* and *R. oligosporus*

Fatty acids	CNF1	CNF2	TNM	TNB	TNR	TNBR
Palmitic acid (16:0)	12.15 ± 0.10 a	11.96 ± 0.02 b	11.99 ± 0.06 b	11.87 ± 0.08 b	9.67 ± 0.04 c	8.34 ± 0.07 d
Stearic acid (18:0)	5.72 ± 0.07 a	5.78 ± 0.03 a	5.82 ± 0.00 a	5.75 ± 0.06 a	4.62 ± 0.07 b	3.87 ± 0.14 c
Oleic acid (18:1)	31.78 ± 0.10 c	30.61 ± 0.18 d	30.49 ± 0.11 d	30.70 ± 0.25 d	32.76 ± 0.11 b	34.07 ± 0.09 a
Linoleic acid (18:2)	50.35 ± 0.08 d	51.18 ± 0.16 c	51.23 ± 0.10 c	51.22 ± 0.11 c	52.95± 0.14 b	53.72 ± 0.22 a
Eicosanoic acid (20:1)	0.00 ± 0.00 c	0.46 ± 0.01 b	0.47 ± 0.00 a	0.47 ± 0.00 a	0.00 ± 0.00 c	0.00 ± 0.00 c
SFA	17.87 ± 0.03 a	17.74 ± 0.04 ab	17.81 ± 0.05 a	17.62 ± 0.14 b	14.29 ± 0.03 c	12.21 ± 0.14 d
UNSAT	82.13 ± 0.03 d	82.25 ± 0.04 cd	82.19 ± 0.05 d	82.39 ± 0.14 c	85.71 ± 0.03 b	87.79 ± 0.14 a
UNSAT/SFA	4.60 ± 0.01 c	4.63 ± 0.02 c	4.62 ± 0.04 c	4.67 ± 0.04 c	6.00 ± 0.02 b	7.19 ± 0.09 a

As shown in Table 1, five fatty acids were identified in the CNF and fermented samples. These included two saturated fatty acids [palmitic (16:0) and stearic (18:0)], and three unsaturated fatty acids [oleic (18:1), linoleic (18:2), and eicosanoic (20:1)]. In general, the fatty acid composition profile was very similar between CNF samples. These amounts of fatty acids can be considered as the starting composition of soybean raw material prior to fermentation. It should be noted, however, that there were some slight differences in amounts of the three fatty acids: palimitic, oleic, and linoleic. Also the presence of eicosanoic acid was detected in the CNF2 sample, but not in the CNF1 sample. These differences may have arisen from the cooking process used (i.e., boiling and autoclaving for the CNF1 and CNF2, respectively), as heat treatment significantly affects fatty acid levels (Zilic et al. 2010; Ouazib et al. 2015).

The CNF soybean samples were then fermented with different starter cultures. For this, the CNF1 was fermented by naturally occurring microorganisms yielding the TNM sample. The CNF2 samples were inoculated with *B. subtilis, R. oligosporus*, and a co-culture of *B. subtilis* and *R. oligosporus*, resulting in the TNB, TNR, and TNBR samples, respectively. As illustrated in Table 1, the fermentation by different starter cultures seemed to affect the fatty acid contents. TNB and TNM samples had a similar fatty acid composition profile. Similarity was also observed in fatty acid concentrations with the starter soybean substrates (CNF1 and TNM; CNF2 and TNB). This suggests that the bacterial starter culture might have little (or no) effect on fatty acid concentration. This is in agreement with previous research describing that most *Bacillus* species do not have (or slightly have) lipase enzyme activity (Aderibigbe, Odunfa, 1990; Omafuvbe et al. 2000; Chukeatirote et al. 2006).

In contrast, the use of the fungus *R. oligosporus* in preparing fermented soybean products (TNR and TNBR) resulted in a dramatic change of fatty acid composition. In general, there was a shift from the saturated fatty acids (16:0 and 18:0) to the unsaturated fatty acids (18:1 and 18:2) in the composition. The fungal inoculation also caused disappearance of the eicosanoic acid (20:1) which was initially present in the starter CNF2 substrate. Interestingly, our data showed that the fungal inoculation as a (co–) starter culture in soybean fermentation contributed to an increase in concentration of unsaturated fatty acids. Also, the ratio between the total unsaturated and saturated fatty acids was higher in the TNR and TNBR samples. The fungus *R. oligosporus* is well known for its ability to produce the lipase enzymes (ul–Haq et al. 2002; Iftikhar et al. 2010). It is therefore obvious that a significant shift in fatty acid contents found in the fermented soybean products was due to effect of fungal lipase enzymes.

Fermentation of soybeans led to a shift in fatty acid profiles. For example, there was an increase of the levels of the unsaturated fatty acids, as shown in the present study (Table 1). Fermented soybean products especially those prepared by the fungus *R. oligosporus*, are rich in oleic and linoleic fatty acids. Many previous studies have shown that fatty acid contents play an important role in the flavor of the food products (Larick, Turner, 1990; Ha, Lindsay, 1991; Toldra, Flores 1998). Soybeans in particular are known to contain fair amounts of lipids, and in general are considered 'undesirable' by most consumers in the western countries. These undesirable features include characters like as beany, green, grassy, painty, astringent, and bitter, which have been shown to be associated with flavours arising from the oxidation of polyunsaturated lipids by lipoxygenases of soybeans (Yang et al 2015). Further work is needed to identify the key components (including the fatty acid components) to ensure optimal sensory characteristics.

Conclusions

This present study described the fatty acid composition of fermented soybean products prepared by selected starter cultures of (i) *B. subtilis*, (ii) *R. oligosporus*, and (iii) a co-culture *B. subtilis* and *R. oligosporus*. In general, it was found that there was a change in fatty acid content of the fermented products studied. Interestingly, the use of the fungal starter culture (i.e., *Rhizopus* as shown in this study)

appeared to be a key factor causing a shift in the fatty acid profiles. Our result is preliminary and can be useful as a reference for food compositional data, and further work on understanding of lipolysis of fermented soybeans should be undertaken.

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