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Effects of co-inoculation and sequential inoculation of *Tetragenococcus halophilus* and *Zygosaccharomyces rouxii* on soy sauce fermentation

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ABSTRACT

The use of *Tetragenococcus halophilus* and *Zygosaccharomyces rouxii* as starter cultures is essential for desirable volatiles production during moromi stage of soy sauce fermentation. In this study, the effect of simultaneous and sequential inoculation of cultures in moromi fermentation models, with respect to viability, physicochemical changes, and volatiles formation (using SPME-GC/MS) was investigated. Interestingly, an antagonism was observed as *T. halophilus* only proliferated (3 log increase) in the presence of *Z. rouxii*, while *Z. rouxii* growth was suppressed by 4 log in concurrence with pH increase to 7.31. Final content of reducing sugars, ethanol, acetic acid, and amino nitrogen did not differ significantly ($p < 0.05$) between co-inoculation and sequential inoculation. However, *Z. rouxii* promoted alcohols formation and produced a more complex aroma profile under suppression. According to Principal Component Analysis (PCA), the inoculation sequence (co-inoculation and sequential) has impacts on volatile compound profiles during moromi fermentation.

1. Introduction

Soy sauce is a fermented condiment originating from China, which is popular around the world due to its intense umami taste and distinct aroma. Two types of soy sauce can be distinguished based on the raw materials: The Chinese-type produced using predominantly soybeans and wheat, and the Japanese-type made using equal amounts of soybeans and wheat (Wanakhachornkrai & Lertsiri, 2003). The Chinese-type dominates Asian regions such as China, Indonesia, Malaysia, Philippines, Singapore, Thailand while the Japanese-type is more popular in Japan and western countries (Zhu & Tramper, 2013).

Soy sauce production involves a 2-step fermentation process, *koji* and *moromi*. In Japanese-type, koji is prepared by growing koji mould, such as *Aspergillus oryzae*, on an equal amount of cooked soybean and wheat flour mixture, followed by moromi fermentation by mixing the resulting koji with brine solution containing 18–22% NaCl (Yong & Wood, 1977). Moromi stage is mainly driven by halotolerant lactic acid bacteria (LAB) and yeast that grow spontaneously during conventional brewing. However, in recent years, the amount of NaCl in the final product has been reduced to approximately 8–11%, driven by industry and the World Health Organization (WHO) recommendation on reducing dietary intake of sodium salt. Studies focusing on salt reduction during moromi fermentation have demonstrated that despite a reduced salt concentration, sensory quality and safety of the final product could be preserved with the use of either mixed culture of lactic acid bacteria and yeast (Singracha, Niamsiri, Visessanguan, Lertsiri, & Assavanig, 2017) or mixed culture of indigenous yeast isolated from different stages during traditional moromi fermentation (Song, Jeong, & Baik, 2015a, 2015b).

Moromi stage is very crucial since key volatile compounds, taste active amino acids and peptides, and sugars that contribute to the final flavour of sauce are produced in this stage (Harada et al., 2016; Zhao, Schieber, & Gänzle, 2016; Zhu & Tramper, 2013). Lactic acid bacterium *Tetragenococcus halophilus* and yeast *Zygosaccharomyces rouxii* compose the core microbial complex which drives the moromi fermentation, regardless of soy sauce origin and production procedure (Harada et al., 2016; Singracha et al., 2017) and therefore the sequence of proliferation of microbial species and their equilibria are paramount to the quality of the final product.

There are abundant secondary metabolites produced by *T. halophilus* and *Z. rouxii via* lactic acid and alcoholic fermentation, respectively, which are responsible for the flavour of the final product (Lee, Lee, Choi, Hurh, & Kim, 2013; Tanaka, Watanabe, & Mogi, 2012). Important aroma compounds in soy sauce, such as acetic acid, formic acid, benzaldehyde, methyl acetate, ethyl 2-hydroxypropanoate, 2-hydroxy-3-methyl-2-cyclopenten-1-one, and 4-hydroxy-3-methoxybenzaldehyde are produced by *T. halophilus* (Lee et al., 2013). Moreover, *Z. rouxii* plays an important role in the formation of ethanol, higher alcohols

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(isobutyl alcohol, isoamyl alcohol, 2-phenylethanol) (Jansen, Veurink, Euverink, & Dijkhuizen, 2003; Van Der Sluis, Tramper, & Wijffels, 2001), 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (HDMF) (Hauck, Brühlmann, & Schwab, 2003; Hecquet, Sancelme, Bolte, & Demuynck, 1996), and 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone (HEMF) (Sasaki, 1996) during moromi stage, which are essential for the characteristic flavour in the final product. Studies showed that despite salt reduction, production of essential volatiles, such as ethanol, 2-methyl-1-propanol, HDMF, and maltol, was significantly higher than traditional moromi when a combination of *T. halophilus* and *Z. rouxii* was used (Singracha et al., 2017).

Since the activity of *T. halophilus* and *Z. rouxii* contributes to the aroma profiles of soy sauce, the utilisation of both cultures in the manufacturing process is important. *T. halophilus*, *Z. rouxii*, and co-culture of both were reported to cause physicochemical changes and affect aroma formation during moromi fermentation (Cui, Zheng, Wu, & Zhou, 2014; Harada et al., 2016; Lee et al., 2013). Metabolomics analysis demonstrated different aroma profiles of moromi according to the types of microorganism added (Harada et al., 2016). *T. halophilus* TS71 and *Z. rouxii* A22 were also reported to enhance the aroma profile of moromi under a reduced-salt environment (Singracha et al., 2017). However, these studies did not investigate the impact of inoculation sequence of *T. halophilus* and *Z. rouxii*. Sequential growth of *Z. rouxii* occurs naturally during spontaneous fermentation of moromi because of lactic acid and acetic acid production by *T. halophilus* that reduces the pH. As the pH drops to < 5.0, *Z. rouxii* starts to grow and begins the alcoholic fermentation (Röling, Timotius, Prasetyo, Stouthamer, & Van Verseveld, 1994; Van Der Sluis et al., 2001; Yong & Wood, 1976). The inoculation method is important for production of desirable volatile compounds allowing full complexity to be achieved. In this study the effect of simultaneous and sequential inoculation of *Z. rouxii* as the pH drops to 5.0 was investigated for the first time in moromi models, with respect to microbial interactions, physicochemical changes, and formation of volatile compounds. Principal component analysis (PCA) was used to evaluate the influence of inoculum type (single culture or mixed culture) as well as the inoculation method on the volatile compound profile of soy sauce.

2. Materials and methods

2.1. Materials

Soy flour, wheat flour, and sodium chloride (NaCl, extra pure) were purchased from Real Foods (Edinburgh, UK), Gilchesters Organics (Stamfordham, UK), and Acros Organics (Fairfield, NJ), respectively. *Aspergillus oryzae* 126842 was purchased from Centre for Agriculture and Biosciences International (Wallingford, UK). *Tetragenococcus halophilus* 9477 and *Zygosaccharomyces rouxii* 1682 were purchased from National Collection of Industrial Food and Marine Bacteria Ltd. (Aberdeen, UK) and National Collection of Yeast Cultures (Norwich, UK), respectively. Microbiological growth media used were Czapex Dox agar (CDA; Oxoid Ltd., Basingstoke, UK), brain heart infusion agar (BHI; Oxoid Ltd), de Man, Rogosa, and Sharpe broth (MRS broth; Oxoid Ltd.), yeast malt agar (YM agar, Sigma-Aldrich, Gillingham, Dorset, UK), yeast malt broth (YM broth, Sigma-Aldrich). Bacteria and yeast growth were controlled using chloramphenicol (Oxoid Ltd.) and natamycin (Sigma-Aldrich), respectively. 1-octen-3-ol (purity ≥98%) was purchased from Sigma Aldrich.

2.2. Culture preparation

Aspergillus oryzae was maintained on CDA at 25 °C. The spore suspension of *A. oryzae* was prepared according to the method described by Chou and Ling (1998) with slight modification. Spores were obtained by growing *A. oryzae* on CDA at 25 °C for 7 days. NaCl solution (0.85%, *w/v*) solution containing 0.01% of Tween 80 (Sigma-Aldrich) was added into the agar slant bottle followed by vigorous mixing to collect the spores. The number of spores were counted using an improved Neubauer haemocytometer and adjusted to 10^6 spores/mL. *Tetragenococcus halophilus* was maintained on BHI with 10% (*w/v*) NaCl and incubated at 37 °C. *T. halophilus* was grown in MRS broth with 7% NaCl for 36 h and the cell concentration was adjusted to a final concentration of 10^6 cells/mL. *Zygosaccharomyces rouxii* was maintained on YM agar with 5% (*w/v*) NaCl and incubated at 25 °C. The inoculum was prepared by growing *Z. rouxii* in YM broth containing 5% (*w/v*) NaCl in a 30 °C shaker incubator for 24 h and cell concentration was adjusted to 10^6 cells/mL.

2.3. Koji fermentation

Koji was prepared using the modified method of Su, Wang, Kwok, and Lee (2005). Soy flour and wheat flour were sterilised at 121 °C for 15 min in an LTE Series 300 autoclave (LTE Scientific Ltd, Oldham, UK). Soy flour moisture was maintained by mixing 100 g of soy flour with 120 mL of sterile distilled water. The cooked soy flour was cooled to room temperature and then mixed thoroughly with the wheat flour (1:1 *w/w*). The mixture was inoculated with *A. oryzae* spores to a final concentration of 10^5 spores/g substrate Chou and Ling (1998). The inoculated substrates were transferred into sterile Petri dishes (d: 140 mm) and incubated at 30 °C for 3 days.

2.4. Study of T. halophilus and Z. rouxii growth in moromi fermentation

Koji was transferred aseptically into flasks. Brine solution (10% *w/v* NaCl) was added to the koji with ratio 3:1 (brine:koji) to create mash (Wan, Wu, Wang, Wang, & Hou, 2013; Wu, Kan, Siow, & Palniandy, 2010). The relatively low salt concentration allows faster fermentation (Muramatsu, Sano, Uzuka, & Company, 1993; Van Der Sluis et al., 2001) and reflects the reduction of salt in the soy sauce industry.

Five types of soy moromi were prepared as follows: (i) uninoculated as control, (ii) inoculated with *T. halophilus*, (iii) inoculated with *Z. rouxii*, (iv) co-inoculated with *T. halophilus* and *Z. rouxii*, and (v) inoculated with *T. halophilus*, followed by sequential inoculation of *Z. rouxii* when the pH dropped to 5.0 (SevenCompact S220 pH meter; Mettler Toledo, Switzerland). After inoculation, the mash was homogenised with a vortex and incubated at 30 °C for 30 days. Samples were taken at Day 0, 5, 10, 15, 20, 25, and 30. *T. halophilus* was grown on BHI agar supplemented with 7% (*w/v*) NaCl and natamycin while the cell count of *Z. rouxii* was done on YM agar with the addition of 5% (*w/v*) NaCl, and 100 mg/L chloramphenicol.

2.5. Physicochemical analysis

Before analysis, soy mash samples were treated at 100 °C for 2 min, to prevent assay interference by enzymes produced during moromi fermentation. Then samples were centrifuged at 10,000*g* for 10 min at 4 °C. The supernatant regarded as raw soy sauce was transferred to microtubes and kept at −20 °C until analysis. Total reducing sugar (D-glucose and D-fructose), total lactic acid (L-lactic acid and D-lactic acid), acetic acid, primary amino nitrogen, and ethanol were analysed using an enzymatic assay kit (Megazyme, International Ireland Ltd., Bray, Ireland) according to the manufacturer's instructions. Changes in pH were monitored using a pH meter (SevenCompact S220; Mettler Toledo, Germany).

2.6. Flavour analysis (SPME/GC-MS)

An automated headspace solid-phase microextraction method (SPME) followed by GC–MS analysis was used for evaluating the *in vitro* production of microbial volatile organic compounds. Soy sauce mash samples (1.5 g) were transferred into 20-mL headspace vials (22.5 mm × 75.5 mm; Grace Alltech, UK) and the vials were sealed