MICROBIAL ENCAPSULATION FOR ENHANCING SOY SAUCE AROMA DEVELOPMENT DURING MOROMI FERMENTATION

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Abstract

Moromi fermentation is an essential part of soy sauce production. This thesis aimed to characterize and control the growth and interaction of two predominant microorganisms, *Tetragenococcus halophilus* and *Zygosaccharomyces rouxii* for enhancing the aroma development during moromi fermentation. Antagonism was observed between *T. halophilus* and *Z. rouxii*, regardless of the inoculation sequence. However, sequential inoculation of *Z. rouxii* resulted in more complex aroma profile than simultaneous inoculation. To eliminate antagonism, chitosan-coated alginate and water-oil-water ($W_1/O/W_2$) double emulsions (DEs) were tested for their ability to encapsulate *Z. rouxii* and stability in high NaCl solutions. Alginate was unstable in high NaCl solutions and chitosan exhibited undesirable antimicrobial activity towards *Z. rouxii*. DEs minimized the antagonism between *T. halophilus* and *Z. rouxii*, by segregation in the external W_2 and internal W_1 phase, respectively. Physicochemical changes in the fermentation medium indicated that DEs affected microbial growth and cell physiology, contributing to the elimination of antagonism. The destabilization of DEs over 30-day storage depended on glucose concentration in W_2, which indicated a possibility of sustained release mechanism of *Z. rouxii* into the moromi. Furthermore, the application of DEs was tested in a moromi model, formulated with reduced NaCl and/or substitution with KCl. DEs resulted in moromi with similar microbiological and aroma profile to that of high-salt. Overall, this thesis demonstrates the potential of DE for delivering mixed cultures in moromi fermentation, which could be applicable in any fermentation process where multiple species are required to act sequentially.

Summary

Moromi (brine) fermentation is an important stage in soy sauce production accounting for the majority of aroma compounds found in the final product. *Tetragenococcus halophilus* and *Zygosaccharomyces rouxii* are predominant lactic acid bacteria (LAB) and yeast, respectively, which are essential to the aroma development during moromi fermentation. This thesis aimed to characterize and control the interaction between *T. halophilus* and *Z. rouxii* for enhancing the aroma development during moromi fermentation.

The effect of co-inoculation and sequential inoculation of *Z. rouxii*, was investigated with respect to microbial interactions, physicochemical changes, and formation of aroma compounds. The results revealed antagonism between *T. halophilus* and *Z. rouxii*, regardless of the inoculation sequence. The growth of *Z. rouxii* was suppressed as the population of *T. halophilus* and pH constantly increased. 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, Gas Chromatography-Mass Spectrometry (GC-MS) and Principal Component Analysis (PCA) showed that the sequential culture resulted in more complex aroma profile compared to co-culture.

In order to eliminate antagonism with *T. halophilus*, alginate beads and water-oil-water ($W_1/O/W_2$) double emulsions (DEs) were tested for their ability to encapsulate *Z. rouxii* and stability under high NaCl concentration. The alginate beads were formed by extrusion and coating with chitosan. The alginate beads were found to be unstable in high NaCl solution, while chitosan exhibited antimicrobial effect towards *Z. rouxii*. Therefore, alginate was not investigated further in this thesis. Alternatively, DEs were

used to minimize the antagonism between *T. halophilus* and *Z. rouxii*, by segregation in the external W_2 and internal W_1 phase, respectively. The DE stability and microbial release profile were studied under conditions relevant to moromi fermentation. The results showed that DE destabilized over 30-day storage, regardless the presence and amount of encapsulated cells. DE destabilization resulted in cell release which was proportional to the glucose concentration in W_2. The encapsulated *Z. rouxii* presented higher survival during storage compared to the non-encapsulated cells. Furthermore, the use of DE was found to affect microbial cells growth and physiology, which led to the elimination of antagonism in Tryptic Soy Broth (TSB) medium.

Finally, the feasibility of DE in a moromi model was investigated including formulation of moromi with reduced NaCl and/or substitution with KCl. Moromi samples with DE developed similar microbiological and aroma profile to that of high-salt.

In summary, this thesis demonstrated the potential and applicability of DE for the delivery of mixed starter cultures in moromi fermentation. A similar approach could be applied in any fermentation process where multiple species are required to act sequentially.

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Chapter 1

Introduction

1.1 Context of the study

A great number of foods utilize fermentation in one or more steps of their manufacturing process. Microorganisms play a major role in fermentation due to their capability of transforming the chemical constituents of raw materials to food products with desirable properties, such as enhanced bio-availability of nutrients, sensory perception of food, increased shelf-life and food safety, antioxidant and antimicrobial compounds production, as well as removal of toxic compounds and anti-nutritive factors (Tamang et al., 2016).

Most of food fermentation processes rely on mixed cultures consisting of multiple strains and/or species rather than a single culture (Smid and Lacroix, 2013). All species in mixed microbial cultures act together to perform a complex activities resulting in desired characteristics that could not be achieved individually. Microbes do not only interact with the fermentable substrate, but also with each other. Such interactions may occur directly via physical contact or signaling molecules, and/or indirectly through physicochemical changes in the environment induced by some members of the community, which trigger responses of the others. Interactions between microbes can be classified into five main classes, including amensalism (-/0 interaction), competition (-/- interaction), commensalism (+/0 interaction), parasitism (+/- interaction), and mutualism (+/+ interaction) (Smid and Lacroix, 2013; Sieuwerts

et al., 2008). All these types of interactions play important role in the equilibrium in the mixed microbial community and performance of each species, which eventually determine the product quality as well as the consistency of fermentation.

Soy sauce is a type of condiment which involves a complex microbial community of fungi, yeast, and bacteria. This oriental traditional seasoning has gained more popularity in the Western market, due to its unique flavor and intense umami taste. Soy sauce is made of soybean and wheat mixtures, subjected to a two-step fermentation process, called *koji* and *moromi*. Koji is prepared by growing koji mold, such as *Aspergillus oryzae* or *Aspergillus sojae* on a mixture of steam-cooked soybeans and roasted wheat flour for 3 days (Zhu and Tramper, 2013). The resulting koji is then mixed with brine, typically containing 18 – 22% NaCl, to initiate the moromi stage and left to ferment for approximately 6 months. The aged moromi is then pressed, filtered, pasteurized, and packaged as a final product (Luh, 1995). According its chemical composition, the national standards of soy sauce in China categorizes soy sauce into three different grades as indicated in Table 1.1.

Traditional manufacturing processes of soy sauce rely on spontaneous fermentation by the microflora present in the raw materials. As a consequence, the composition and activity of the microbial community in soy sauce are affected by variations in the microflora of raw materials, resulting in inconsistent final product quality. *Tetragenococcus halophilus* and *Zygosaccharomyces rouxii* have been considered as the most predominant osmophilic lactic acid bacteria (LAB) and yeast, respectively in soy sauce (Röling et al., 1994a). These two microbes are applied as soy sauce starter cultures in the modern production to improve the consistency of product characteristics and quality. However, several studies reported antagonistic interaction between *T.*

halophilus and *Z. rouxii* due to different optimal growth conditions required by each species (Noda et al., 1980, 1982; Kusumegi et al., 1998; Inamori et al., 1984). Such interactions can negatively affect the microbial composition and performance of the mixed culture in flavor development (Noda et al., 1980). Therefore, a formulation is needed for controlling the delivery and activity of these species, thus minimizing the effects of antagonism and maximizing flavor development.

Compared to free cells system in fermentation, microbial encapsulation offers the benefits of enhanced tolerance against adverse conditions. Alginate has been extensively used as an encapsulation material. Encapsulation of two soy sauce fermenting yeasts, *Z. rouxii* and *Candida versatilis* in alginate gel beads has been previously reported in moromi stage of soy sauce fermentation process, in order to shorten the fermentation time (Hamada, Sugishita, Fukushima, Tetsuro, & Motai, 1991). However, this system was found to be unstable over time due to alginate's sensitivity to high NaCl concentration present in the brine added during moromi fermentation (Horitsu et al., 1990; van der Sluis et al., 2000). The ion-exchange between the Na^+ ions present in the moromi and Ca^{2+} ions which are binding with COO^- groups of the polyguluronate unit of the alginate can result in the weakened of bead structure (Bajpai and Sharma, 2004).

Recent studies have shown the potential application of water-oil-water ($W_1/O/W_2$) double emulsion (DE) to encapsulate and release microbial cells in a controlled manner (El Kadri et al., 2015, 2016) as well as to protect probiotic bacteria against adverse environment in human gastrointestinal tract (Shima et al., 2006; Pimentel-González et al., 2009; Rodríguez-Huezo et al., 2014). DE is a type of emulsion that contains two aqueous compartments separated by an oil phase. Its multi-

compartmentalized structure could be used for delivering multiple starter cultures during fermentation, when minimum interference between species is required. This thesis aimed to characterize and control the interaction between *T. halophilus* and *Z. rouxii* during moromi fermentation to enhance aroma quality in soy sauce.

Table 1.1 National standards of soy sauce in China (Xu, 1990)

Item		**Index**		
		1st grade	2nd grade	3rd grade
Special gravity (20 °C)	≥	1.200	1.170	1.140
Total nitrogen (g/100 mL)	≥	1.60	1.20	0.80
Formol nitrogen (g/100 mL)	≥	0.80	0.60	0.40
Reducing sugar (as glucose; g/100 mL)	≥	4.00	3.00	2.00
NaCl (g/100 mL)	≥	19.00	17.00	16.00
Solid except NaCl (g/100 mL)	≥	20.00	15.00	10.00
Total acids (as lactic acid; g/100 mL)	≥	2.50	2.00	1.50
As (mg/l)	≤		0.5	
Pb (mg/l)	≤		1.0	
Sodium benzoate (g/l)	≤		1.0	
Aflatoxin B_1 (μg/l)	≤		5.0	
TM (/mL)	≤		50.000	
Coliforms (/100mL)	≤		30.0	

1.2 Soy sauce and its production process

Soy sauce is a liquid condiment originating from China, which has light brown to black color with salty and intense umami taste (Steinkraus, 1983). Due to its distinctive taste and aroma, soy sauce is used as main seasoning in Japan, China, Korea, and other Asian countries. Moreover, soy sauce has been gaining its popularity in the Western countries with Netherlands (at US $147 million) being the major exporter in a US $600+

million industry (Figure 1.1). However, countries of the Asia-Pacific region, such as China, Japan, Singapore, and Hong Kong still dominate the global trade (Figure 1.2).

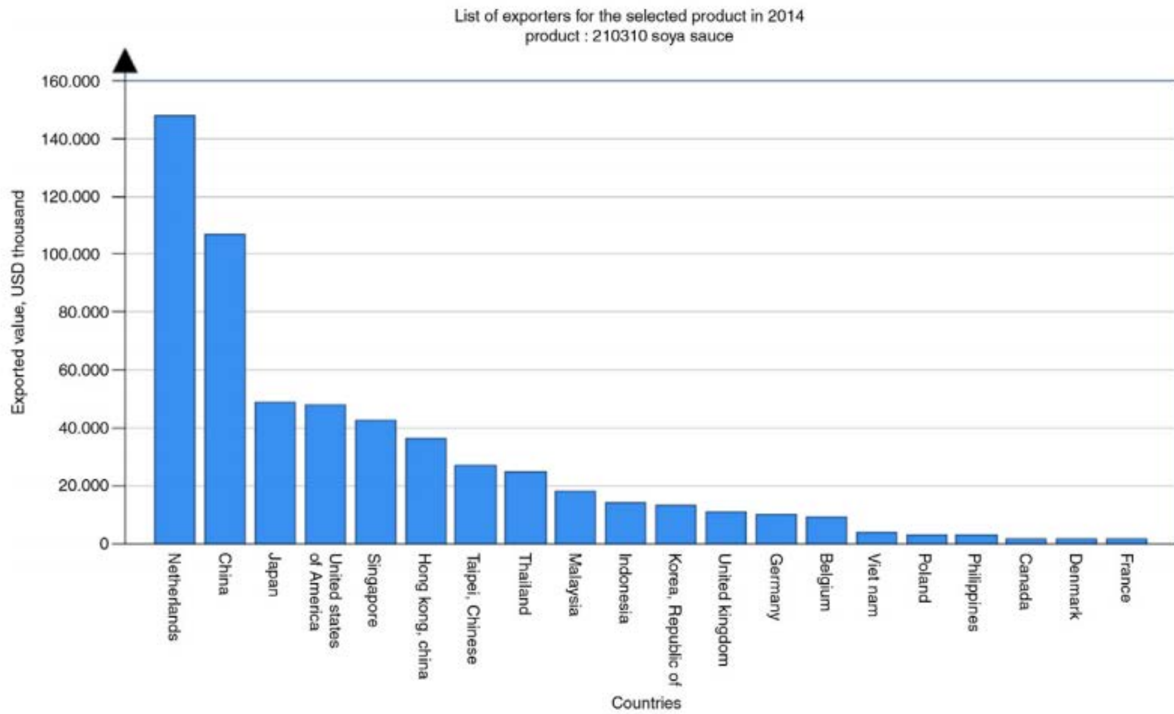


Figure 1.1 Main global exporters of soy sauce (Gordon and Williams, 2017)

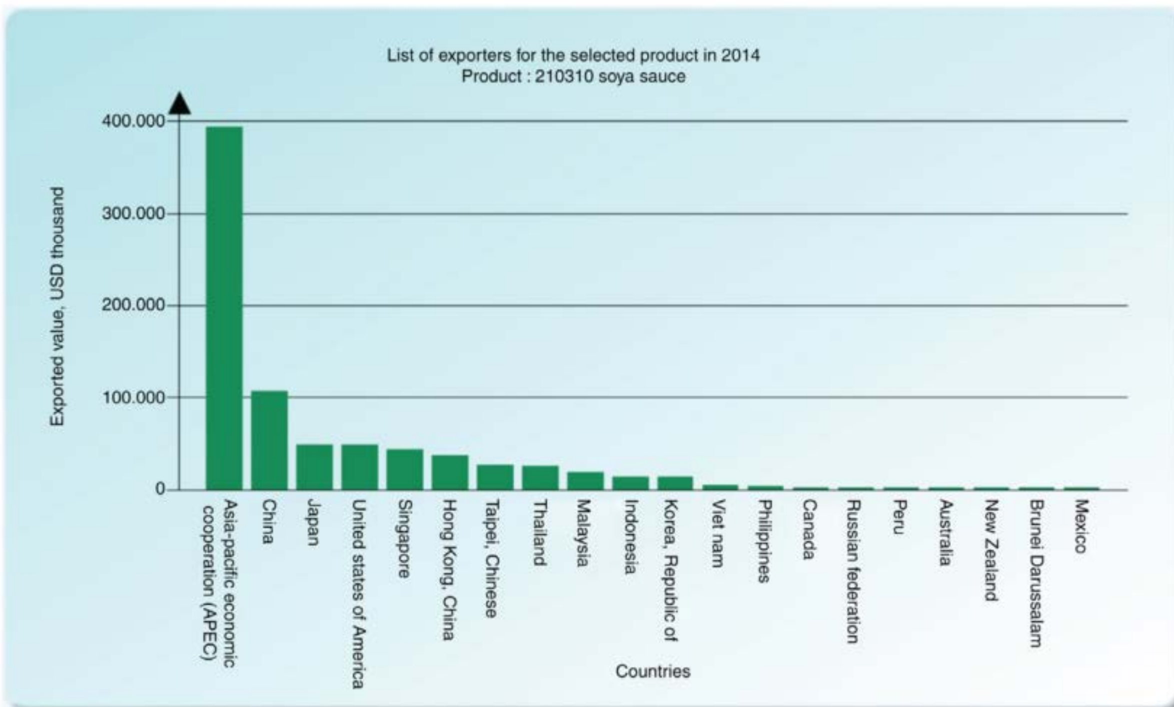


Figure 1.2 Exports of soy sauce from the Asia-Pacific Economic Cooperation Countries (Gordon and Williams, 2017)

Soy sauce is made of four basic ingredients, including soybeans, wheat, salt, and water. According to the amount of wheat used, soy sauce can be distinguished into two types: The Chinese-type produced using predominantly soybeans and wheat, and the Japanese-type made using equal amount of soybeans and wheat (Wanakhachornkrai and 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 and Tramper, 2013).

According to the production process used, soy sauce can be classified into high-salt liquid-state fermentation soy sauce (HLFSS), low-salt solid-state fermentation soy sauce (LSFSS) and Koikuchi soy sauce (KSS). HLFSS and LSFSS are mainly found in China, while KSS dominates both Japanese and global soy sauce market (Feng et al., 2015a). Each production process employs different fermentation conditions, ratios of water, salt, and soybeans used, as well as additional ingredients, as shown in Figure 1.3. However, there are two main fermentation stages involved in every manufacturing process, namely koji and moromi (Figure 1.3). Koji fermentation begins by soaking the soybeans in water to increase its moisture content followed by cooking using steam at high pressure and temperature. Meanwhile, wheat is roasted and cracked in a machine to produce smaller particles of wheat flour (Luh, 1995). The soybeans and wheat flour are then mixed with the spores of koji mold, such as *A. oryzae* or *A. sojae*, which are the most commonly used fungal strains. The mixture is then loaded into tray with 3-5 cm in thickness followed by incubation at 25 °C. The mixture should have water activity (Aw) between 0.8 – 0.9 which is moist enough to enable fungal growth but dry enough to prevent spoilage bacteria growth (Redhead, 1989). During this stage, koji mold produces proteolytic enzymes to break down protein into peptides and amino acids, as

well as amylase to convert starch into simple sugars. Such converted substrates are then utilized by subsequent microorganisms during the moromi stage as nutrients. The enzymatic activity of fungi will cause the pH of koji to increase from around 6.5 to 7.3 as well as heat production. Regular stirring is needed in order to remove the heat, thus maintaining the temperature at 25 – 28°C (Luh, 1995). Fungal mycelium will grow all over the surface of soybean and sporulate, producing a greenish compact mass of soybeans after 3 days. In the modern processing, the koji making process has shifted to an automatic process, including the use of continuous soybeans cooker and wheat roaster, mixer, cooler, automatic inoculator, mechanical mixer, temperature controllers, conveyors, and mechanical devices for stirring the soybean mixtures during incubation (Luh, 1995).

In the second stage, the resulting koji is immersed in brine solution containing 18-22% NaCl, producing a mixture called moromi (van der Sluis et al., 2001b). High salt concentration in the brine is used to limit the growth of microorganism to a number microflora, which are tolerant to high salt environment, and also to suppress the growth of undesirable microorganisms. Since the growth and enzymatic activities of koji mold are terminated due to high salt concentration, moromi stage is mainly driven by halotolerant lactic acid bacteria (LAB) and yeast that grow spontaneously. LAB propagate rapidly in the beginning of the moromi fermentation and produce acids. The pH of moromi gradually decreases due to lactic acid fermentation and other metabolic processes, and when the pH of moromi reaches 4.0 – 5.0, the bacterial cell number starts to decrease while yeast cells start to grow (van der Sluis et al., 2001b). In the modern processing, defined mixed cultures of LAB, *T. halophilus* and yeast, *Z. rouxii* and

Candida species, are utilized to achieve consistent product quality (van der Sluis et al., 2001b).

After 3 – 6 months of fermentation, moromi is subjected to refining process, including pressing, filtration, pasteurization, and packaging (Luh, 1995). The matured moromi is pressed in order to separate moromi solid from the liquid, which is known as raw soy sauce. The raw soy sauce is then pasteurized in order to prolong the shelf life of soy sauce by inactivating undesirable microorganisms and residual enzymes. Furthermore, a number of additional aroma compounds, such as 3-methyl-2(5H)-furanone (caramel-like) and 2-methyl-3-furyl disulfide (meaty), are generated during the heating process (Kaneko et al., 2013). Additional ingredient such as caramel is usually added into the soy sauce to adjust the color. After that, soy sauce is then packaged either in plastic or glass container before being shipped to the market and consumed.

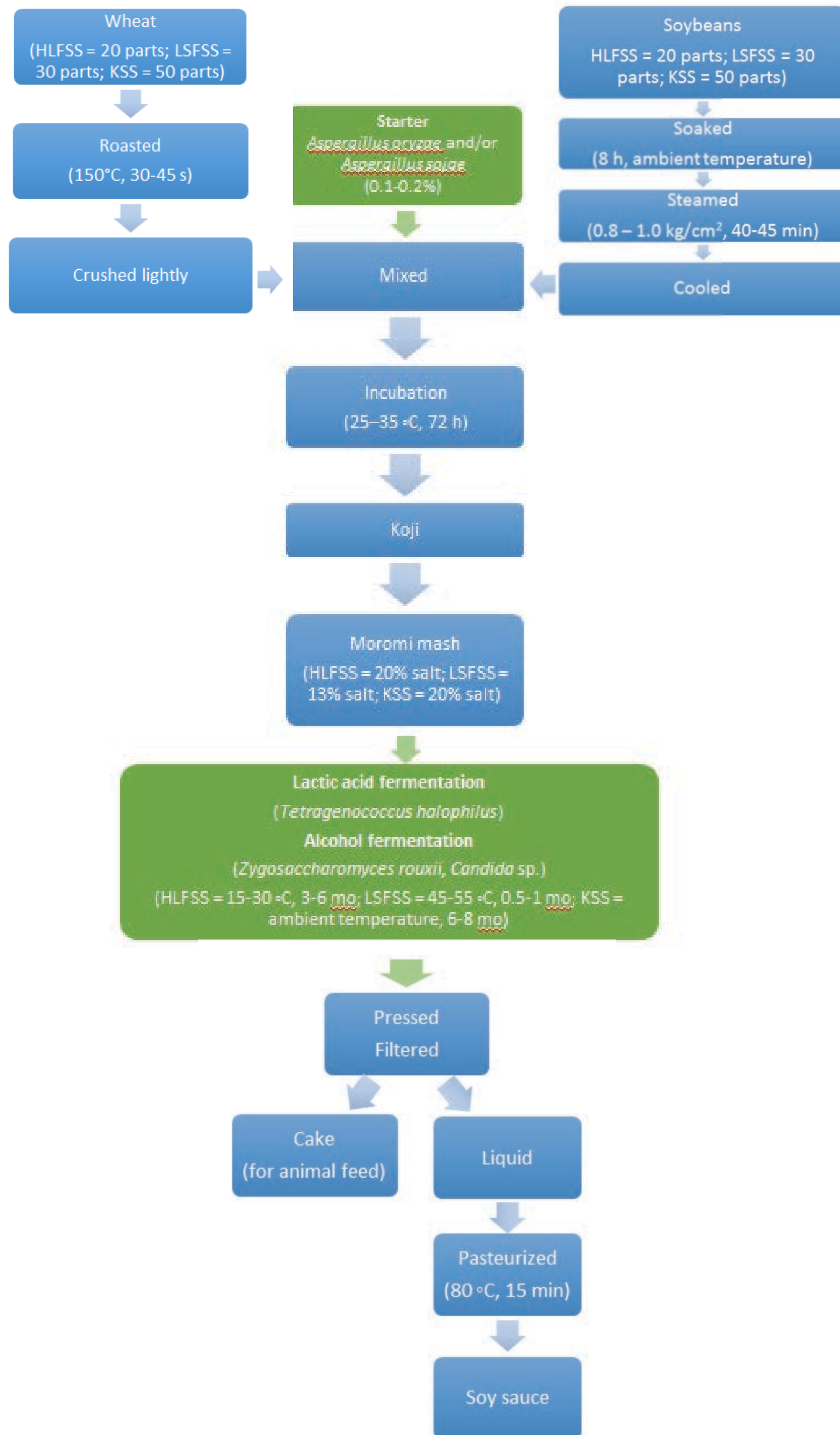


Figure 1.3 Soy sauce manufacturing process flowchart (Luh, 1995; Feng et al., 2015b)

1.3 Soy sauce fermentation process

1.3.1 Microbial diversity during soy sauce fermentation

Soy sauce production is typically performed in non-sterile conditions for a long period, thus allowing the growth of a wide range of microorganisms during the fermentation process that contribute to the flavor and aroma formation in the end product. The diversity and dynamic changes of microbial community during koji and moromi fermentation have been reported in many studies. The diversity and distribution of microbial population in soy sauce vary according to the country of origin, type of raw material, handling process, and fermentation conditions used. Microbial species involved during the two-step fermentation process have been identified using culture-dependent and culture-independent methods (Table 1.2). Culture-dependent methods rely on the ability of microbes to grow on a nutrient medium. In order to extend the study of the microbial diversity, culture-independent methods have been used, based on identification using total DNA and sequencing of phylogenetic markers, without the need of culturing (Wei et al., 2013). Bacteria are identified by the sequence of 16S rDNA, while fungi are identified by the sequence 18S rDNA. Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) is one of the most popular culture-independent techniques used to describe the microbial diversity in soy sauce. Another method has been developed in order to eliminate bias introduced by PCR-DGGE, including whole genome shotgun (WGS) analysis coupled with next-generation sequencing, which provides information on microbial dynamics as well as metabolic and functional diversity of the microbial population during fermentation (Sulaiman et al., 2014).

The microbial community during the fermentation stages for soy sauce production changes. By using a combination of a cultured-dependent method and a cultured-independent method (PCR-DGGE), Wei et al. (2013) found that the microbial diversity in koji was higher than the moromi due to its high salt concentration. Bacteria, especially LAB, were found to dominate koji fermentation and then followed by yeasts and molds. Moreover, the microbial pattern of the moromi also differed from time to time. Groups of bacteria including *Staphylococcus*, *Bacillus*, and *Enterobacter* were found to be dominant during the whole process, while *Klebsiella*, *Paenibacillus*, and *Corynebacterium*, were only detected in koji. *Kurthia* was found in koji but then disappeared in the early stage of moromi. Higher diversity of bacteria in the koji stage was reported by Wei et al., (2013a) by using culture-dependent method during fermentation of *inyu*, a high-temperature fermented soy sauce (Table 1.2).

Table 1.2 The diversity of microbial communities during soy sauce production

Type of soy sauce	Ingredients	Salt concentration	Incubation condition	Fungi/yeast	Bacteria	Method of detection	Reference
Indonesian (*kecap*)	Black soybeans	12.3 - 21.3% NaCl	Koji: room temperature, 3 days Moromi: outdoors under the sun (21-42 ºC)	*Candida parapsilosis*, *C. guilliermondii* var. *membranaefaciens, C. famata, C. pulcherrima, Debaromyces hansenii* var. *fabryi*	*P. halophilus, Staphylococcus, Sterigmatomyces*, *coryneform*	Phenotypic-based technique	Röling, Timotius, Prasetyo, Stouthamer, & Van Verseveld, 1994
Korean	*Meju* (soybeans only)	8% NaCl	Koji: 30ºC for 4 days	*Aspergillus oryzae*	*Bacillus* spp., *B. vallismortis*, *B. subtilis*, *B. amyloliquefacien*, *B. methylotrophicus*, *Staphylococcus* spp., *S. epidermidis*, *S. xylosus*, *Enterococcus* spp., *E. faecium*	Culture independent method (PCR-DGGE)	Song, Jeong, & Baik, 2015a
		11% NaCl	Moromi: 30 ºC		*Bacillus* spp., *B. vallismortis, B. subtilis,B. amyloliquefaciens, B. methylotrophicus*		

		14% NaCl			*Bacillus* spp., *B. vallismortis, B. subtilis, B. amyloliquefaciens, B. methylotrophicus*		
Korean	*Meju* (soybeans and wheat fermented for 3 months)	20% NaCl	n.a	*Wickerhamomyces anomalus, Torulaspora delbrueckii, Tetrapisispora blattae, Rhodotorula* sp., *R. mucilaginosa, R. glutinis, Pichia sorbitophila, P. gulliermondii, P. triangularis, Microbotryum violaceum, Debaryomyces hansenii, Cryptococcus albidus, C. parapsilosis, C. orthopsilosis, C. lusitaniae, Candida* sp., *C. albicans, C. temnochilae, C. versatilis, Zygosaccharomyces* sp., *Penicillium islandicum, P. aurantiogriseum, Aspergillus* sp., *A. flavus*	-	Culture dependent method; Culture independent method (PCR-DGGE)	Song, Jeong, & Baik, 2015a

Chinese	n.a	16.9% NaCl	n.a	*Candida, Starmerella, Wickerhamiella, Aspergillus, Saturnispora*	*Weisella, Bacillus, Lactobacillus, Leuconostoc, Lactococcus, Padiococcus, Enterococcus, Micrococcus, Streptococcus, Xanthomonas, Streptococcus, Staphylococcus, Salmonella, Pseudomonas, Pantoea, Klebsiella, Dechloromonas, Cupriavidus, Arsenophonus, Propionibacteriacea, Acidobacteriaceae*	Whole genome shotgun (WGS); Next generation sequencing (NGS)	Sulaiman, Gan, Yin, & Chan, 2014
Japanese	Yellow soybeans : roasted wheat = 2.67 : 1	20% - 25% NaCl	Koji: 25 - 38 ºC , for 40h	*A. oryzae, Zygosaccharomyces rouxii, Z. pseudorouxii, Candida etchellsii, C. nodaensis, C. versatilis, C. catenulata, Wickerhamomyces anomalus, Pichia anomala,Geotrichum silvicola, Trichosporon jirovecii, T. asahii, T. japonicum, T. inkin, T. insectorum, T. faecale,*	*Weisella cibaria, W. confusa, W. kimchii, W. salipiscis, Lactobacillus fermentum, L. plantarum, L. raffinolactis, L. mesenteroides, Staphylococcus gallinarum, S. xylosus, S. arlettae, S. saprophyticus, S. succinus, S. cohnii, S. caprae, S. kloosii, Pediococcus*	Culture independent method (PCR-DGGE)	Tanaka et al., 2012

				T. ovoides,	*pentosaceus, P. acidilactici, Tetragenococcus halophilus*		
Thai	n.a	n.a	n.a	-	*T. halophilus, L. acidipiscis, L. farciminis, L. pentosus, L. plantarum*	Phenotypic and chemotaxonomic-based technique; DNA-DNA similarity	Tanasupawat, Thongsanit, Okada, & Komagata, 2002
Taiwanese (*inyu*)	Black soybeans : rice bran = 100 : 1	30% - 42% NaCl	Koji: room temperature, 7 days Moromi: outdoors under the sun 21.6ºC - 41ºC, 4 months	-	*Staphylococcus sciuri, S. cohnii, S. condimenti, S. gallinarum, S. kloosii, S. sciuri, Citrobacter farmeri, Enterobacter cloacae, E. hormaechei, E. pulveris, Klebsiella pneumoniae, Pantoea agglomerans, P. dispersa, Salmonella enterica, Serratia marcescens, Weisella confusa, Enterococcus faecium, Brachybacterium rhamnosum, Kurthia gibsonii, Delftia tsuruhatensis, Bacillus amyloliquefaciens, B. licheniformis, B. pumilus, B. subtilis*	Culture dependent method (Randomly amplified polymorphic DNA (RAPD) and 16S rDNA amplification); Culture independent method (nested PCR-DGGE);	Wei et al., 2013a

n.a	Defatted yellow soybean : roasted wheat = 5.5 : 4.5	20% NaCl	Koji: 35ºC for 42h	*Aspergillus sojae, A. parasiticus, Trichosporon ovoides, T. asahii, T. lactis, Zygosaccharomyces rouxii, Saccharomycopsis fibuliger, Millerozyma farinosa, Peronospora farinosa, Pichia farinosa, Candida* sp., *C. rugosa, C. orthopsilosis, C. tropicalis*	*Staphylococcus, Kurthia, Bacillus, Klebsiella, Enterobacter, Paenibacillus, Corynebacterium*	Culture dependent method; Culture independent method (PCR-DGGE)	Wei et al., 2013b
n.a	Defatted yellow soybean : roasted wheat = 5.5 : 4.5	20% NaCl	Koji: 35ºC for 42h Moromi: 30ºC, > 6 months	*Aspergillus sojae, A. parasiticus, Trichosporon ovoides, T. asahii, T. lactis, Zygosaccharomyces rouxii, Saccharomycopsis fibuliger, Millerozyma farinosa, Peronospora farinosa, Pichia farinosa, Candida* sp., *C. rugosa, C. orthopsilosis, C. tropicalis*	*Staphylococcus, Kurthia, Bacillus, Klebsiella, Enterobacter, Paenibacillus, Corynebacterium*	Culture dependent method; Culture independent method (PCR-DGGE)	Wei et al., 2013b

However, most of bacterial species including *Brachybacterium*, *Kurthia*, and *Staphylococcus* were no longer detected in koji after the washing step. Bacterial species from *Enterobacteriaceae* family in koji were also undetectable during moromi fermentation. *Enterococcus faecium* and *Weisella confusa* were the only LAB detected by using PCR-DGGE method during moromi, while no was LAB detected using plate count method, which could be due to very high salt concentration in moromi (30% -42% NaCl). This was in contrast to the study by Tanasupawat et al., (2002), where 14 sphere-shaped and 30 rod-shaped LAB were isolated from Thai soy sauce moromi. These strains were then grouped and identified as *T. halophilus*, *Lactobacillus acidipiscis*, *Lactobacillus farciminis*, *Lactobacillus pentosus*, and *Lactobacillus plantarum*, by DNA similarity. A wide range of bacterial species were detected in Japanese soy sauce during koji step using PCR-DGGE method (Table 1.2). Among these, only *Weissella cibaria* (or *W. confusa, Weissella kimchii, Weissella salipiscis, Lactobacillus fermentum, L. plantarum, Lactobacillus iners*), *Staphylococcus gallinarum* (or *Staphylococcus xylosus), Staphylococcus kloosii* were preserved during moromi stage. *T. halophilus* was detected during lactic acid fermentation in Japanese (Tanaka et al., 2012), Thai (Tanasupawat et al., 2002), as well as Indonesian soy sauce (Röling et al., 1994a). The microbial diversity in soy sauce is affected by the salt concentration of moromi. *Bacillus* spp. was found to be predominant during moromi fermentation of Korean soy sauce regardless of salt concentration of moromi (Song et al., 2015a). Some bacterial species such as *Staphylococcus epidermidis*, *S. xylosus* and *E. faecium* were only detected in moromi with relatively low salt concentration.

A number of fungal species including *A. sojae, Aspergillus parasiticus*, *Trichosporon ovoides*, and *Trichosporon asahii* were found during koji fermentation and

were undetectable in the moromi stage (Wei et al., 2013b). The diversity of yeast species during moromi fermentation changes with time and *Z. rouxii* was found to be the most predominant yeast and was detected during the mid-late period of moromi fermentation. In contrast, Tanaka et al., 2012 found *Z. rouxii* at the early stage, *Candida etchellsii* (or *Candida nodaensis*) and *C. versatilis* at the middle stage, and *C. etchellsii* at the maturation stage. Similarly, *Zygosaccharomyces* sp. was detected only at the early stage of tradition Korean soy sauce fermentation, in addition to *Debaryomyces* and *Torulaspora* (Song, Jeong, & Baik, 2015a). Moreover, *Candida*, *Pichia*, and *Rhodotorula* sp. were found to be the dominant species, while *Cryptococcus*, *Microbotryum*, *Tetrapisispora*, and *Wickerhamomyces* were detected as minor strains.

Although numerous microbial species are involved, there are several microorganisms that are predominant during the moromi stage. *T. halophilus* has been considered as a predominant halotolerant LAB found during lactic acid fermentation stage of moromi. Meanwhile, *Z. rouxii* has been considered as a predominant yeast during alcoholic fermentation (Harada et al., 2016; Singracha et al., 2017).

1.3.2 Properties of *T. halophilus* and *Z. rouxii*

T. halophilus was known as *Pediococcus halophilus* before the reclassification of halophilic LAB (Collins et al., 1990). It has a spherical shaped cell and the size varies from 0.5-0.8 μm. The cells are usually arranged in tetrads, but they also could be found separately or in pairs (Figure 1.4a). This species is able to grow in salt concentrations ranging from 0-26% NaCl, where the optimum growth is observed at 5-10% NaCl. It is also able to grow at pH 9.0 or below but poor growth is shown at pH 5.0 (Holzapfel et al., 2006). *T. halophilus* requires complex nutrients for its growth, including amino acids and vitamins. However, *T. halophilus* is not able to break down protein contained in the

substrate during moromi fermentation. Various strains of *T. halophilus* have been found in moromi, possessing different physiological properties including the ability to ferment carbohydrate and metabolize organic and amino acids (Kobayashi et al., 2000, 2004). *T. halophilus* is able to ferment a number of carbohydrates typically contained in soy sauce, including the polysaccharide dextrin, di- and trisaccharides (maltose, sucrose, melibiose and raffinose), hexoses (glucose, fructose, galactose, and mannose), pentoses (arabinose and xylose), and sugar alcohols (glycerol and mannitol). It metabolizes carbohydrates during homofermentation via the Ember-Meyerhof-Parnas pathway under anaerobic conditions. Carbohydrate metabolism under aerobic conditions can result in the production of acetate from lactate and glucose due to the presence of NADH oxidase.

During its propagation, *T. halophilus* produces organic acids which bring the pH of moromi down to 5.0 – 5.5. The growth of *T. halophilus* is suppressed at such low pH, as it is lack of the ability to maintain the intracellular pH above a value that is favorable for the enzymatic activities required for growth and glycolysis. However, *T. halophilus* is able to counteract this effect by producing alkalizing ammonia from arginine through the arginine deaminase pathway, therefore allowing the fermentation to continue until the pH drops to 4.5 – 4.9. Some strains are also able to neutralize the pH through amino acids decarboxylation to amines and carbon dioxide.

Z. rouxii has been reported as the most predominant yeast species found during moromi fermentation (Figure 1.4b). Optimum growth of yeast occurs at low pH, but under high salt environment, *Z. rouxii* can only grow at pH 4.0 – 5.0 while other yeast species can grow at higher pH. The inability of *Z. rouxii* to grow at high pH might be related to cell's inability to maintain a proton gradient across the membrane which is

important for salt tolerance. Due to low oxygen level in moromi, *Z. rouxii* must be able to ferment sugar. However, the sugar fermentation ability is reduced at higher salt concentration. In a study by Sasaki (1996), it was found that increasing the concentration of NaCl from 6% to 22% decreased ethanol production from 3.15% to 0.26%. Sugar fermentation leads to the production of ethanol and higher alcohols, which are important to soy sauce flavor and aroma characteristic.

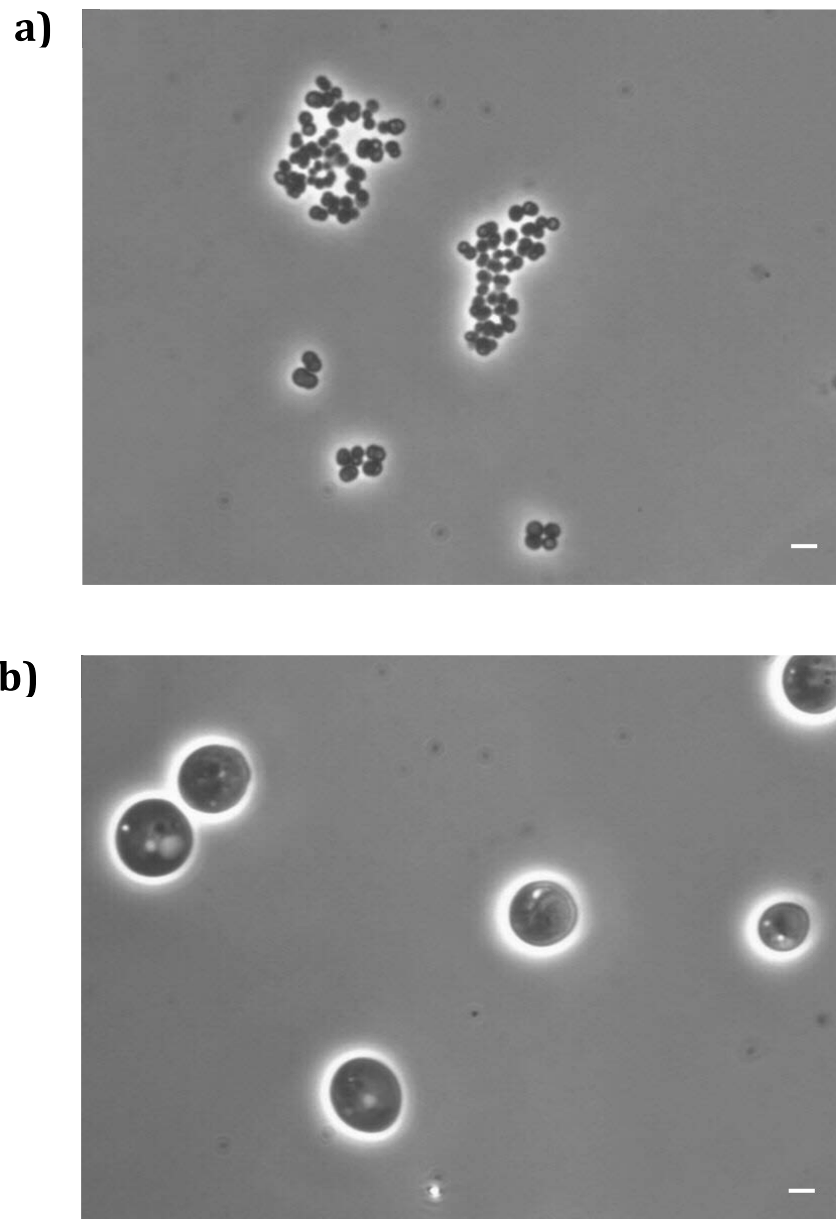


Figure 1.4 Cell morphology under light microscope with 100x magnification. (a) *T. halophilus*. (b) *Z. rouxii*. Scale bar: 2 µm

1.3.3 Interactions between LAB and yeast in moromi fermentation

During mixed culture fermentation, where two or more of microbial species co-exist, interactions such as competition and antagonism are observed. The antagonistic interaction between LAB and yeast during moromi fermentation of soy sauce has been reported in several studies. The pH of moromi has been considered as the main factor affecting microbial interaction during fermentation, since *T. halophilus* and *Z. rouxii*, that are essential to the fermentation process, require different pH for optimum growth. *T. halophilus* grows at higher pH ranging from 5.5 – 9.0, while *Z. rouxii* grows at lower pH within limited range of 4.0 – 5.0 in substrate containing 18% salt (Röling et al., 1994b).

At the beginning of fermentation, moromi has relatively high pH ranging from 6.0 – 7.0 which supports the growth of *T. halophilus*. During its propagation, *T. halophilus* produces organic acids, resulting in moromi acidification. *T. halophilus* is no longer able to grow once the pH drops to below 5.0 and since the pH is suitable for the yeast, *Z. rouxii* begins to grow and produce alcohol by utilizing glucose available in the moromi. Suppression of the alcoholic fermentation by yeast due to lactic acid fermentation by LAB has been observed by Noda, Hayashi, & Mizunuma (1980). The authors suggested that the growth inhibition towards osmophilic yeast, *Saccharomyces rouxii* (later known as *Z. rouxii*) and *Torulopsis versatilis* was due to the production of acetic acid and lactic acid by LAB *P. halophilus* (later known as *T. halophilus*). However, the antagonism was higher with acetic acid compared to lactic acid at comparable pH values. Also, the inhibitory activity of acetic acid on *S. rouxii* was found to increase significantly as the pH of the medium was reduced (Noda et al., 1982).

A study by Kusumegi, Yoshida, & Tomiyama (1998) demonstrated that the acetic acid inhibited the formation of cytochromes and the respiratory activity of *Z. rouxii* R-1.

The growth inhibition by acetic acid was related to the halotolerance properties of *Z. rouxii* as *Z. rouxii* was significantly inhibited by 0.5% acetic acid in a medium containing 18% NaCl. It was suggested that the proton expulsive activity, which is essential for the ability of *Z. rouxii* to grown under high salt environment, significantly decreased when 0.5% acetic acid was present.

Antagonism between *P. halophilus* and *S. rouxii* was also observed in a *shoyu* koji extract supplemented with additions by Inamori, Miyauchi, Uchida, & Yoshino (1984). At an initial pH 6.0. *P. halophilus* was inhibited by *S. rouxii* under aerobic conditions, while *S. rouxii* was inhibited by *P. halophilus* under anaerobic conditions. Poor lactic acid fermentation was also observed when *P. halophilus* was inhibited. Similarly, when *S. rouxii* was inhibited, alcohol fermentation was suppressed. A study on combined effects of temperature and pH on *Z. rouxii* growth in moromi fermentation by Kobayashi & Hayashi (1998) revealed the remarkable effects of pH and temperature on the rate coefficient for *Z. rouxii* growth. During its growth, *T. halophilus* produces organic acids which have a significant impact on pH in the moromi. It was shown that apart from the toxicity of acetic acid and lactic acid, *T. halophilus* could also affect the growth of *Z. rouxii* by changing the pH of moromi.

1.4 Soy sauce flavor

Soy sauce has been consumed as main seasoning in Asian countries to enhance the taste of food during cooking and it has been widely recognized all over the world because of its distinct flavor. The characteristic flavor of soy sauce is a combination of sweet, sour, salty, and umami tastes generated by variety of flavor compounds (Wanshoupeng et al., 2011). Nearly 300 types of aroma compound have been identified in soy sauce. Those aroma compounds include alcohols, aldehydes, acids, acetals,

hydrocarbons, esters, furans, furanones, ketones, lactones, nitrogen-containing compounds, phenols, pyrones, pyrazines, pyridines, sulfur-containing compounds, and thiazole (Nunomura et al., 1980). Among those compounds, alcohols, acids, esters, and aldehydes have been reported as the most abundant aroma compounds found in soy sauce (Feng et al., 2014).

Some important aroma compounds are common in Japanese and Chinese-type soy sauce, including ethanol, 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol, 1-octen-3-ol, 2-phenylethanol, 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, benzeneacetaldehyde, acetic acid, ethyl acetate, guaiacol, 4-ethylguaiacol, 2-methylpyrazine, 2,5-dimethylpyrazine, 2,3,5-trimethylpyrazine, 2,5-dimethylfuran, furfural, 2-furanmethanol, 5-methyl-2-furancarboxaldehyde, 3-phenylfuran, dimethyl disulphide, dimethyl trisulphide, 3-(methylthio)propanal and maltol (Feng et al., 2014). However, the aroma compounds may vary depending on raw materials, salt concentrations, microbial strains, and fermentation time and temperature used during soy sauce manufacturing process. According to Feng et al. (2015), Japanese-type soy sauce contains higher amount of alcohols (ethanol, 2-methyl-1-propanol, 3-methyl-1-butanol and 2-phenylethanol), esters (ethyl acetate, ethyl 2-methylpropanoate and acetic acid 2-phenylethyl ester), benzeneacetaldehyde, 4-ethylguaiacol, 4-vinylguaiacol and HEMF compared to Chinese-type. Meanwhile, Chinese soy sauce made using low-salt solid-state fermentation technique (LSFSS) had higher contents of 3-methylbutanal, 2-methylpropanoic acid, 2-methylbutanoic acid, 3-methylbutanoic acid, 2-methylpyrazine and 2,5-dimethylpyrazine compared to Japanese or Chinese-type made using high-salt liquid-state fermentation technique (HLFSS).

Moromi fermentation is considered as important step that determines the quality of flavor and aroma in soy sauce. The two predominant active microorganisms, *T. halophilus* and *Z. rouxii*, are able to produce various secondary metabolites including important aroma compounds through lactic acid and alcoholic fermentation pathway, respectively (Lee et al., 2013). *T. halophilus* mainly plays role in the production of acetic acid, formic acid, benzaldehyde, methyl acetate, ethyl 2-hydroxypropanoate, 2-hydroxy-3-methyl-2-cyclopenten-1-one, and 4-hydroxy-3-methoxybenzaldehyde. Meanwhile, other important aroma compounds including ethanol, acetaldehyde, ethyl propanoate, 2,3-methylbutanol, 1-butanol, and ethyl 2-methylpropanoate are mainly produced by *Z. rouxii* (Lee et al., 2013). Some important higher alcohols including isobutyl alcohol, isoamyl alcohol, 2-phenyl ethanol, methionol, and polyol and two furan derivatives, 4-hydroxy-2,5-dimethyl-3[2H]-furanone (HDMF) and 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl- 2(3H)-furanone (HEMF), which have been reported as the most important flavor compounds in Japanese-type soy sauce, are also known to be produced by *Z. rouxii* (Sasaki, 1996; Hecquet et al., 1996). Ethanol is produced by *Z. rouxii* from sugar available in the moromi and this only occurs when the pH is lower than 5.0 as it loses its ability to maintain a proton gradient required for salt tolerance at higher pH. However, due to the high salt concentration used in moromi, only some sugars can be fermented into ethanol. At high salt concentration, only glucose can be converted into ethanol while maltose can be fermented in a salt-free medium (van der Sluis, Tramper, et al., 2001). Meanwhile, higher alcohols are produced through α-keto acids pathways, comprising the amino acid biosynthetic and amino catabolic pathway, which is known as Ehrlich pathway (Van Der Sluis et al., 2001; Webb & Ingraham, 1963). The Ehrlich pathway involves deamination or transamination of extracellular amino acids, producing α-keto acids which serves as key intermediates for the formation of higher

alcohols. The biosynthetic pathway for the production of HDMF and HEMF still remains poorly understood. However, it has been reported that both are produced by *Z. rouxii* using Maillard reaction intermediates as precursors (Hayashida et al., 1999).

1.5 Modification of soy sauce manufacturing process

1.5.1 Salt reduction

The demand for low-salt soy sauce has increased due to health concerns related to high sodium intake, such as hypertension and renal problem. The World Health Organization (WHO) recommends a limitation of daily sodium intake to 2 g, which is equivalent to 5 g salts (WHO, 2012). Meanwhile, daily consumption of soy sauce per person in Japan is 30 mL (Kobayashi, 2013), while the per capita daily consumption in Indonesia is 10 – 15 mL (Sasaki & Nunomura, 2003). Due to high NaCl content in soy sauce, the consumption of 1 tablespoon (15 mL) of soy sauce can contribute to 38% of the Recommended Daily Intake (RDI) of sodium. Therefore, the production of soy sauce with low salt content without decreasing its quality has become a challenge for the soy sauce industry. High salt concentration is used in soy sauce production to create anaerobic conditions in moromi, prevent putrefaction of the autolysate, and inhibit the growth of spoilage microorganisms (Luo et al., 2009; Muramatsu et al., 1993). High salt concentration is also important to the organoleptic properties of soy sauce, including texture and aroma (Song, Jeong, & Baik, 2015). Therefore, reducing salt concentration can compromise the quality of the final product.

Manufacturing low-salt soy sauce was previously attempted by performing koji autolysis using high temperature prior to moromi stage (Muramatsu et al., 1993). Such procedure was shown to produce soy sauce with final NaCl concentration of 4.6% with

chemical compositions and flavor characteristics similar to that obtained with the conventional method. Salt reduction was also found to shorten the fermentation time. In order to enhance the perception of saltiness, salt substitutes, such as KCl, glycine ethyl ester hydrochloride, lysine hydrochloride, taurine and glutamic acid were added (Muramatsu et al., 1993). Furthermore, according to Segawa et al. (1995), the amino acid based saltiness enhancers (e.g glycine ethyl ester hydrochloride and taurine) resulted in better taste quality to that of low-salt soy sauce containing conventional saltiness enhancers, such as KCl, $MgCI_2$, and $MnCI_2$. The addition of KCl in soy sauce can cause bitterness, which becomes apparent when added at concentration above 10%. Meanwhile, the addition of $MgCI_2$ and $MnCI_2$ do not add any saltiness in soy sauce. Although the amino acid based saltiness enhancers could produce soy sauce with similar level of saltiness to that of the conventional soy sauce, the balance of the saltiness and umami taste was inferior (Segawa et al., 1995).

Attempts to reduce salt in soy sauce have also been done by replacing salt with osmotically equivalent substitutes, including sugars, polyols, or ethanol (Chiou et al., 1999). Since most pathogenic and spoilage microorganisms are intolerant to ethanol, the combination of ethanol and NaCl can prevent their growth during fermentation. However, adding these substitutes can change the original properties of soy sauce and limit the growth of desirable microorganisms (Baba et al., 1983). Salt reduction in soy sauce were also done by physically removing part of salt using nanofiltration (Luo et al., 2009), ion exchange (Japan Patent No. 52,120,197, 1977), reverse osmosis (Japan Patent No. 4,016,162, 1992), freezing (Watanabe, Tesaki, & Arai, 1996) and extraction (Japan Patent No. 10,295,320, 1998).

Recently, Song et al. (2015) have utilized indigenous yeasts, *Torulaspora delbrueckii* and *Pichia guilliermondii*, that were isolated at different stages during soy sauce fermentation, to produce soy sauce with low salt content. This method was proven to compensate several adverse effects caused by salt reduction such as the growth of spoilage microorganisms and undesirable flavor characteristics. The resulting moromi had a more complex and richer flavor similar to that of high-salt due to high production of fusel alcohols by *T. delbrueckii* and *P. guilliermondii*. Moreover, the application of mixed cultures of *T. halophilus* and *Z. rouxii* (predominant during moromi fermentation) and *P. guilliermondii* (found in Thai soy sauce), was able to produce low-salt soy sauce without undesirable aroma compounds (Singracha et al., 2017). Such combination of LAB and yeast was also reported to reduce the amount of biogenic amines compared to the use of yeast starter cultures only.

1.5.2 Microbial cells and enzymes immobilization

Flavor formation by microorganisms involved in moromi fermentation requires a long period due to low metabolic activity under high salt environment. Slow metabolic activity is also caused by poor availability of substrates for the yeast due to slurry-state of the moromi. Therefore, studies have focused on improving production by shortening the fermentation process. Microbial cells and enzymes immobilization have been reported to be able to shorten the fermentation period and increase the production efficiency due to 10-100-fold higher concentration of yeast cells (van der Sluis et al., 2001a). The use of immobilized glutaminase and cells of *P. halophilus*, *Z. rouxii*, and *C. versatilis* in a bioreactor system was found to shorten the soy sauce fermentation process from six months to two weeks (Hamada et al., 1991). The cells were immobilized using a mixture of colloidal silica and sodium alginate, extruded through

nozzle into calcium chloride solution to form gel beads. Although alginate shows the ability to shorten the fermentation period and is considered to be safe, it appears to be sensitive to heat and therefore it cannot be completely sterilized by steam (Horitsu et al., 1990). Also, the alginate bead structure is weak and can be damaged by high salt concentration (van der Sluis et al., 2000).

A ceramic carrier was developed as an alternative to replace alginate for cell immobilization in soy sauce fermentation *Z. rouxii* and *C.* (Horitsu et al., 1990). The culture separation was performed due to the negative effect of mixed cultures of *Z. rouxii* and *C. versatilis* on the production of 4-ethyl guaiacol by *C. versatilis*. Soy sauce of good quality was obtained within 8 days of fermentation, compared to 6 months using the conventional method. However, the use of ceramic carrier may lower the cell activity and efficiency needed for rapid fermentation. Therefore, a combination of bioreactor and membrane technologies were used in order to increase the productivity. Instead of using immobilized cells, continuous fermentation was carried out using free cells of *Z. rouxii* in a stirred tank reactor with a microfiltration membrane (Iwasaki et al., 1991). The filtrate form moromi was obtained as a permeate through the microfiltration membrane with much higher productivity compared to immobilized cells. Another gel-based cell immobilization was studied by van der Sluis et al., (2000) in order to enhance the gel beads durability under high salt concentration. High cells viability after immobilization process was obtained by avoiding contact between the cross linker and yeast cells. Compared to alginate, polyethylene-oxide gel was not sensitive to abrasion caused by high salt concentration. It also allowed the production of aroma compounds essential to the final product quality, including 4-ethylguaiacol, ethanol, and higher alcohols by *Z. rouxii* and *C. versatilis*. However, the use of polyethylene-oxide gel in

continuous fermentation is difficult as the particles tend to aggregate (van der Sluis et al., 2001a).

1.6 Double emulsions for food application and microbial encapsulation

There has been growing interest on the application of DE in the development of healthy and functional foods. Due to its unique multi-compartmentalized structure, DE is potentially used to encapsulate sensitive water-soluble substances for protection against harsh environment conditions, followed by release of the substances during eating and/or digestion. A wide range of substances have been incorporated in the internal W_1 phase of DE, including bioactive compounds and nutrients, in order to protect them against harsh environmental conditions. The encapsulated substances include resveratrol, anthocyanins, betalain, apigenin, components of saffron, anthocyanins, phytosterol, and various vitamins (e.g. B, C, and D), as well as natural coloring agents. Some other substances such as simple salts (NaCl, $CaCl_2$, $MgCl_2$), aspartame, menthol, xylitol, flavorings, and seasonings are also added for prolonging the release of food components. Moreover, short-chain fatty acids such as butyric acid are often encapsulated within the internal W_1 phase in order to mask the unpleasant aroma. DE has also been considered as a highly attractive option to reduce fat content in food products since the conventional oil-in-water (O/W) emulsion can be replaced by the equivalent $W_1/O/W_2$ DE, which has lower oil content but a similar in-mouth perceived texture (Jiménez-Colmenero, 2013).

In recent years DE has expanded its application in the development of food products enriched in probiotics (Shima et al., 2006; Rodríguez-Huezo et al., 2014; Flores-Andrade et al., 2017; Huerta-Vera et al., 2017). In addition to protecting probiotics from the gastrointestinal tract, DE has also shown the ability to protect the

probiotics in food product from different processing conditions (Rodríguez-Huezo et al., 2014). DE has also shown potential application in encapsulation of probiotics impregnated in fruits, including apple (Flores-Andrade et al., 2017) and banana (Huerta-Vera et al., 2017).

The encapsulated cells can be released from DE in a controlled manner, as previously described by El Kadri et al. (2015) and El Kadri et al. (2016). Release of *E. coli* cells into the W_2 phase was controlled by altering the osmotic pressure balance between the W_1 and W_2 phase of DE using NaCl. The release was suggested to occur due to oil globule bursting independent to water movement between the two aqueous phases. The ability of DE to encapsulate and release microbial cells indicates the possibility of creating a starter culture delivery system for fermentation process, where sequential inoculation of microbes is needed.

1.7 Double emulsion and its preparation

Emulsion is a system formed when there are two immiscible liquids, with one of the liquids being dispersed as small spherical droplets in the other (Dickinson, 2011). There are two types of emulsion, including water-in-oil (W/O) and oil-in-water (O/W) emulsion (Figure 1.5a and b, respectively). A multi-compartmentalized structure called double emulsion is formed when these two types of emulsion co-exist, where the globules of the dispersed phase themselves contain even smaller dispersed droplets (Garti, 1997). There are two major types of double emulsion: water-in-oil-in-water (W_1/O/W_2) DE, which is a water-continuous system containing oil droplets having smaller water droplets dispersed within, and oil-in-water-in-oil (O_1/W/O_2) type, which is an oil-continuous system containing water droplets with smaller oil droplets inside

them (Figure 1.5c and d, respectively; Muschiolik and Dickinson, 2017). Among these two types, $W_1/O/W_2$ DE is the most commonly investigated.

Since DE consists of two different interfaces, it requires two sets of different types of emulsifying agents to formulate DE. The emulsifier used for the W_1/O must have a low Hydrophilic-Lipophilic Balance (HLB) value, while the O/W_2 interface requires emulsifier with high HLB value. Polyglycerol polyricinoleate (PGPR) is the one of the most common low HLB emulsifier used to prepare a food grade DEs (Muschiolik, 2007). PGPR is generally used at concentrations ranging from 4 to 6 wt%, and it can be as low as 1.25 wt% when combined with a similar quantity of hydrophilic emulsifier polyethylene sorbitan monooleate (Tween) dissolved in the external W_2 phase (Lee et al., 2013). According to the European Directive 95/2/EC, PGPR is allowed for use in low-fat formulations with 41% fat or less in a maximum daily intake of 4 g/kg (Bastida-Rodríguez, 2013). Tween80 is one of the most common high HLB emulsifiers used in food production. It is usually added up to 0.5% (v/v) in ice cream to increase its smoothness and make it easier to handle, as well as to increase its resistance to melting (Golf, 1997). Tween80 has been used safely in food products and EU regulations allow its use and consumption with recommended daily intake less than 25 mg/kg per body weigh (Gates, 2010).

DE can be prepared by using one-step emulsification or two-step emulsification method (Garti, 1997). One-step emulsification relies on spontaneous formation of DE, caused by largely excessive amount of relatively hydrophobic emulsifier and small amount of hydrophilic emulsifier. In addition to the right HLB value of the emulsifier, heat treatment is needed to reach suitable temperature in order to form $W_1/O/W_2$ structure. Two-step emulsification is the most commonly used method since a better

controlled preparation can be obtained (Figure 1.6). In this method, two types of emulsifiers are employed, including a low-HLB emulsifier which stabilizes W_1/O emulsion, and a high-HLB emulsifier which stabilizes O/W_2. The preparation of primary W_1/O is done in the first step using high shear conditions (e.g. ultrasonication, homogenization), while the $W_1/O/W_2$ DE is formed during the second step using less shear. Excessive shearing during the second step can lead to rupture of the oil layer between the internal and external water phase, resulting in a single O/W_2 emulsion.

There have been several methods used to prepare DE, including (ultra)sonication, high-pressure homogenization, membrane emulsification, emulsification by microfluidics, high-speed shearing (mainly the rotor–stator apparatus), and other mild dispersion methods. Sonicator and high-pressure homogenizers are typically used during the second step of emulsification at low to moderate intensity. However, due to the generation of high shear forces, these methods can decrease the activity of some of the encapsulated bioactive compounds (Muschiolik, 2007; Shimizu and Nakane, 1995). As an alternative, membranes and microchannels can be used as low-intensity emulsification method to produce very small primary W_1/O droplets size. With these methods, a primary W_1/O emulsion is forced through a microporous membrane or micro-fabricated channel arrays into the external W_2 phase. Since much less shear is used, the resulting droplets are intact and both a high encapsulation efficiency and monodispersity can be obtained (Kumar et al., 2012). Rotor-stator is considered as a conventional homogenizing device used in the two-step emulsification method. Unlike membrane emulsification, rotor-stator produces emulsion with wide droplet size distribution. However, this technique is commonly

used in industry since it is scalable and cost effective compared to other techniques (Hall et al., 2011).

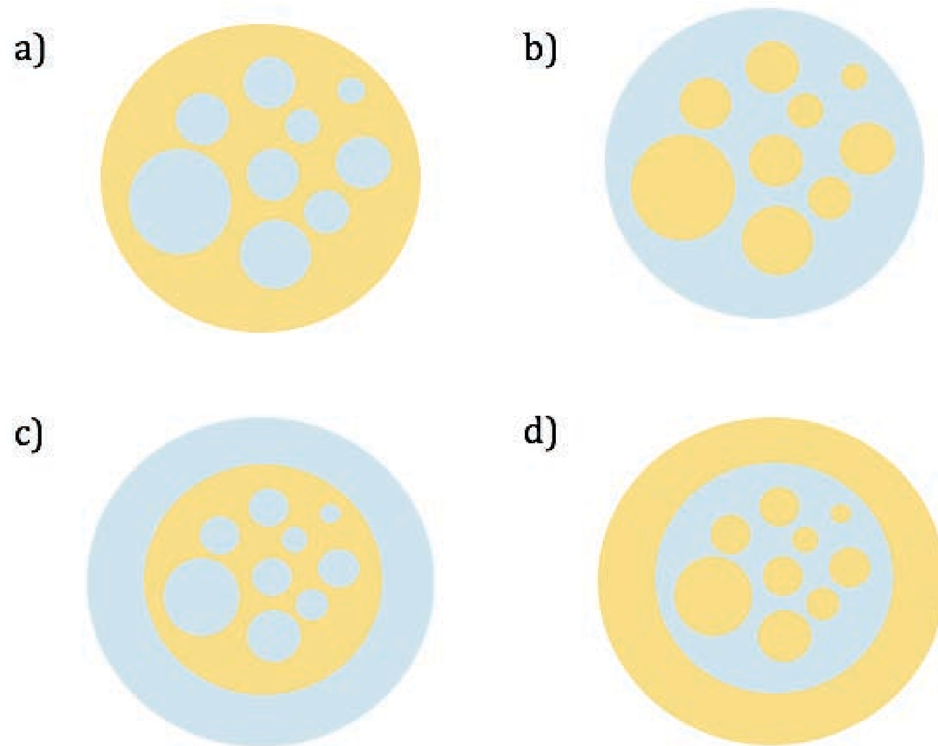
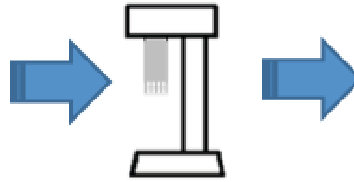
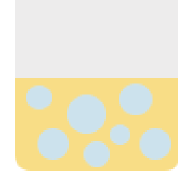


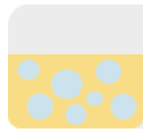
Figure 1.5 Schematic representation of emulsion types. (a) W/O emulsion. (b) O/W emulsion. (c) $W_1/O/W_2$ double emulsion. (d) $O_1/W/O_2$ double emulsion. Blue and yellow represent water and oil phase, respectively.

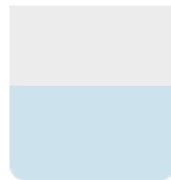
Step 1

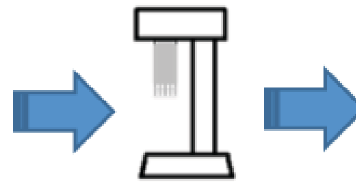




Step 2







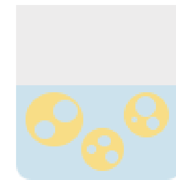


Figure 1.6 Schematic illustration of a $W_1/O/W_2$ double emulsion using a two-step emulsification process.

1.8 DE stability and controlled release mechanism under osmotically imbalance conditions

Compared to single emulsion, DE is considered to have a lower thermodynamic instability due to increased interfacial area. DE also tends to break down into single emulsion during preparation, due to the water movement from the internal W_1 phase (Jafari, 2017). Such water movement could result in the release of the encapsulated materials, which can be used as a method for controlling the delivery of hydrophilic substances into the continuous phase. The water movement from the oil globule may happen through several mechanisms, depending the type and concentration of the surfactant used (Ficheux et al., 1998; Pays et al., 2001; Chávez-Páez et al., 2012). It could also be related to the osmotic pressure gradient between the two aqueous phases.

Oil droplets may either lose or take up water depending on the sign of the water gradient. When the osmotic pressure in the internal W_1 phase is higher than the external W_2 phase, water is preferentially transported into the W_1 phase, causing the oil globule to swell due to the increased phase volume of encapsulated water. On the other hand, when the osmotic pressure in the internal W_1 phase is lower than the external W_2 phase, water is preferentially transported to the W_2 phase, causing the oil globule to be empty and shrinking (Frasch-Melnik et al., 2010; Wen and Papadopoulos, 2001). These osmotic pressure-related instability mechanisms could be utilized to control the release of encapsulated substances under hyper- or hypo-osmotic pressure. Soy sauce fermentation is performed in the presence of high concentration of salt, which creates hyper-osmotic condition for DE. Such condition will allow transport of the internal W_1 phase together with the encapsulated cells (*Z. rouxii*) to the W_2 external phase/moromi.

1.9 Aim and objectives

The ultimate goal of this research was to improve the fermentation process in moromi stage of soy sauce fermentation to obtain final product with enhanced flavor formation and consistent quality. To achieve this goal, the following objectives were set:

- Investigate the interaction between co-inoculated and sequentially inoculated *T. halophilus* and *Z. rouxii* during moromi fermentation.
- Investigate the utilization of formulations based on alginate and DEs for encapsulation of *Z. rouxii* in a high salt environment and delivery during fermentation.
- Evaluate the effect of DEs on minimizing antagonistic interactions between *Z. rouxii* and *T. halophilus* under conditions relevant to moromi fermentation.
- Investigate the effect of DEs in reduced-salt moromi on aroma formation.

Chapter 2

Effects of inoculation sequence of *Tetragenococcus halophilus* and *Zygosaccharomyces rouxii* on moromi fermentation

2.1 Background

Moromi stage is crucial for soy sauce flavor development since a bulk of key aroma compounds, taste active amino acids and peptides, and sugars that contribute to the final flavor of sauce are produced in this stage (Zhao et al., 2016; Zhu and Tramper, 2013; Harada et al., 2016). Lactic acid bacterium *T. halophilus* and yeast *Z. rouxii* compose the core microflora 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 these 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 flavor of the final product (Tanaka et al., 2012; Lee et al., 2013). 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 important role in the formation of ethanol, higher alcohols (isobutyl alcohol, isoamyl alcohol, 2-phenylethanol) (van der Sluis et al., 2001b; Jansen et al., 2003), 4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF) (Hauck et al., 2003; Hecquet et al., 1996), and 4-hydroxy-P(or 5)-ethyl-5(or 2)-methyl-3(2N)-furanone (HEMF) (Sasaki, 1996) during moromi stage, which are essential for the characteristic flavor in the final product. Studies showed that despite salt reduction, productions of essential aroma compounds such as ethanol, 2-methyl-1-propanol, HDMF, and maltol were significantly higher than traditional moromi when 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 utilization of both cultures in the manufacturing process is important. *T. halophilus*, *Z. rouxii*, and their co-culture were reported to cause physicochemical changes and affect aroma formation during moromi fermentation (Cui et al., 2014b; Lee et al., 2013; Harada et al., 2016). 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 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 (Yong and Wood, 1976; Van Der Sluis et al., 2001b; Röling et al., 1994). The inoculation method is important for production of desirable aroma compounds achieving full complexity.

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 aroma 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 aroma compound profile of soy sauce.

2.2 Materials and methods

2.2.1 Materials

Soy flour, wheat flour, and sodium chloride (NaCl, extra pure) were purchased from Real Foods (UK), Gilchesters Organics (UK), and Acros Organics (New Jersey, USA), respectively. *Aspergillus oryzae* 126842 was purchased from Centre for Agriculture and Biosciences International (UK). *Tetragenococcus halophilus* 9477 was purchased from National Collection of Industrial Food and Marine Bacteria Ltd. (UK), which was previously isolated from Swedish *tidbits*, a salt-sugar preserved fish product. *Zygosaccharomyces rouxii* 1682 was purchased from National Collection of Yeast Cultures (UK), which was previously isolated from Japanese *miso*, a high-salt fermented soybean. Microbiological growth media used were Czapex Dox Agar (CDA, Oxoid Ltd., UK), Brain Heart Infusion Agar (BHI, Oxoid Ltd., UK), de Man, Rogosa, and Sharpe broth (MRS broth, Oxoid Ltd., UK), Yeast Malt agar (YM agar, Sigma-Aldrich, UK), Yeast Malt broth (YM broth, Sigma-Aldrich, UK). Bacteria and yeast growth were controlled using chloramphenicol (Oxoid Ltd., UK) and natamycin (Sigma-Aldrich, UK), respectively. 1-octen-3-ol (purity ≥98%) was purchased from Sigma Aldrich (UK).

2.2.2 Microbial culture preparation

Microbial cell cultivation:

A. oryzae was maintained on CDA at 25 °C. The spore suspension of *A. oryzae* was prepared according to the method described by Chou & 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, UK) 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. *T. halophilus* was maintained on BHI with 10% (w/v) NaCl and incubated at 37 °C for 72 h and then kept in 4 °C until further use. *T. halophilus* was grown in MRS broth with 7% NaCl for 36 h at 30 °C and the cell concentration was adjusted to a final concentration of 10^6 cells/mL. *Z. rouxii* was maintained on YM agar with 5% (w/v) NaCl and incubated at 25 °C for 48 h then kept in 4 °C until further use. The inoculum was prepared by growing *Z. rouxii* in YM broth containing 5% (w/v) NaCl in 30 °C shaker incubator for 24 h and cell concentration was adjusted to 10^6 cells/mL.

Microbial growth curve:

T. halophilus from solid medium was subcultured into MRS broth supplemented with 7% (w/v) NaCl and incubated statically at 30°C for 5 days. The optical density (OD) was measured using spectrophotometer at 660 nm (Roling and Verseveld, 1996). *T. halophilus* (10% v/v) was inoculated into the same fresh media and incubated at 30°C in a static incubator for 5 days. OD, pH, and viable cell counts (BHI Agar) were monitored every 12 h.

Z. rouxii was inoculated in YM broth containing 5% (w/v) NaCl and kept in a shaker incubator at 30°C for 2 days. After 2 days, the OD was measured at 660 nm (Kobayashi and Hayashi, 1998) and 1% (v/v) culture was added into fresh media followed by incubation for 2 days. Samples were taken every 4 hours and subjected to OD, pH, and viable cell counts (YM Agar) measurements.

2.2.3 Assessment of *T. halophilus* and *Z. rouxii* for salt-tolerance

The ability of *T. halophilus* and *Z. rouxii* to grow in different concentrations of salt was monitored. *T. halophilus* was subcultured into MRS Broth supplemented with 0%, 5%, 7%, 10%, 15%, and 18% (w/v) NaCl followed by incubation at 37°C in a static incubator for 5 days. *Z. rouxii* was grown in YM broth and the salt concentrations used were 0%, 5%, 10%, and 18% w/v followed by incubation at 25°C in a static incubator for 3 days. Cell viable counts of *T. halophilus* and *Z. rouxii* were conducted on BHI Agar and YM Agar, respectively.

2.2.4 Koji fermentation

Koji was prepared using modified method of Su et al. (2005). Soy flour and wheat flour were sterilized at 121 °C for 15 min in an LTE Series 300 autoclave (LTE Scientific Ltd, England). In order to prevent soy flour from drying during autoclaving, 100 g of soy flour was mixed with 120 mL of sterile distilled water. The cooked soy flour was cooled to room temperature and then mixed thoroughly with 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 & Ling (1998). The inoculated substrates were transferred into sterile petri dishes (d:140 mm) and incubated at 30 °C for 3 days.

2.2.5 Physical characteristics of koji

pH measurement: pH values of koji were measured periodically at 0, 24, 48, and 72 h according to the method of Chancharoonpong et al. (2012). Koji samples were weighed (2 g) and mixed with deionised water with ratio of 1:4 w/v using pestle. pH measurement was carried out directly using pH meter.

Moisture content measurement: Koji samples were weighed (2 g) and dried in an oven for 24 h at 100°C, removed, and then cooled in a desiccator. Weighing, drying, and cooling procedures were repeated until the weight was constant (Yong and Wood, 1977).

2.2.6 Effect of inoculation time on *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 moromi (Wan et al., 2013; Wu et al., 2010). The relatively low salt concentration was selected to allow faster fermentation (Muramatsu et al., 1993; van der Sluis et al., 2001b) and to reflect the reduction of salt in soy sauce industry.

Five sets of soy moromi were prepared for two separate experiments as follows: (i) uninoculated moromi as control, (ii) inoculated with *T. halophilus*, (iii) inoculated with *Z. rouxii*, (iv) co-inoculated with *T. halophilus* and *Z. rouxii*, and (v) sequentially inoculated with *T. halophilus* followed by *Z. rouxii* when the pH decreased to 5.0 (SevenCompact S220 pH meter, Mettler Toledo, Switzerland). After inoculation, the moromi was homogenized by vortexing and incubated at 30 °C for 30 days. Samples of 1 mL were taken at day 0, 5, 10, 15, 20, 25, and 30, and serially diluted in PBS followed by plate counting. *T. halophilus* was counted on BHI agar supplemented with 7% (w/v) NaCl and natamycin while *Z. rouxii* was counted on YM agar with the addition of 5% (w/v) NaCl, and 100 mg/L chloramphenicol.

2.2.7 Physicochemical characteristics of moromi

Prior to analysis, moromi samples were treated at 100 °C for 2 min, to prevent the interference of enzymes produced during moromi fermentation with the assay. Then samples were centrifuged at 10000 g for 10 min at 4 °C. The supernatant regarded as raw soy sauce was transferred to microtubes and kept in -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 analyzed using an enzymatic assay kit (Megazyme, International Ireland LTd., Ireland) according to the manufacturer instructions. Changes in pH were monitored using pH meter (SevenCompact S220, Mettler Toledo, Germany).

2.2.8 Aroma compounds analysis of moromi

Automated headspace solid phase microextraction (SPME) followed by GC-MS analysis were used for evaluating the *in vitro* production of aroma compounds due to microbial activity. Moromi samples (1.5 g) were transferred into 20 mL headspace vials (22.5 mm x 75.5 mm, Grace Alltech, UK) and the vials were sealed with magnetic cap (20 mm diameter, 5 mm centre, PTFE / Silicone Liner; Grace Alltech). Samples were allowed to equilibrate at 22 °C for 30 min before analysis. Three replicates were prepared for all samples.

The aroma compounds extraction was performed using a 1-cm Stableflex fiber coated with 50/30 μm divinylbenzene-carboxen on polydimethylsiloxane bonded to a flexible fused silica core (Supelco, Bellefonte, PA, USA). It was conditioned for 90 min at 300 °C in the injection port. The fiber was pushed out of the housing and inserted to the vials through the center of the vial cap. The penetration depth was fixed at 22 mm. The

extraction was carried out by exposing the fiber to the headspace for 10 min at 40 °C. For all analyses, desorption time was set to 10 min at 230 °C.

Chromatography was carried out using a Trace GC Ultra gas chromatography (Thermo Electron Corporation, Hemel Hempstead, UK) equipped with a polar column ZB-Wax (30 m x 0.25 mm I.D.; film thickness: 1 μm) from Phenomenex. Mass spectrometry (MS) was performed with a DSQ mass spectrometer (Thermo Electron Corporation, Hemel Hempstead, UK)). GC-MS was set according to the previous study (Gkatzionis et al., 2009): The temperature of the injection port was 230 °C. Helium was employed as the carrier gas, at a constant pressure of 17 psi. The oven temperature program was as follows: an initial temperature of 40 °C was maintained for 2 min, increasing at a rate of 8 °C /min to a final temperature of 220 °C. The transfer line from the gas chromatograph to the mass spectrometer was held at 250 °C. The mass spectrometer was operated under positive ionization electron impact mode (EI+) at 70 eV. The detector was operated in scan mode (2 scans/s) scanning from m/z 20 to 250. Source temperature was 200 °C.

Compounds were identified by comparing their retention times and mass spectra with those of standards or their retention indexes (RI) with those published in databases and their mass spectra with the National Institute of Standards and Technology (NIST) mass spectral library using XCalibur Software (Thermo Electron Corporation, UK). The signal intensity for each compound was expressed relative to the signal observed when the headspace above a 0.1 μg/mL 1-octen-3-ol solution was sampled.

2.2.9 Statistical analysis

Microbial cell enumeration, physicochemical tests, and aroma compounds analysis were conducted in triplicate and repeated in two independent experiments. The results were presented as means ± standard deviation. Significant differences among means were tested by one-way analysis of variances (ANOVA) using IBM SPSS Statistics Software version 21 at $p<0.05$ and Tukey's test was applied for means comparison. Principal component analysis (PCA) was performed using XLSTAT™ version 2015.6.01.24027 (Addinsoft, USA) to reduce the dimensionality of the dataset and show the differences in aroma compounds among the soy sauce samples. Observations/variables was chosen as data format and Pearson's correlation matrix was used as PCA type.

2.3 Results and discussion

2.3.1 Microbial growth curve

The growth phases of *T. halophilus* and *Z. rouxii* were studied in a batch culture. Figure 2.1a illustrates three distinct phases of *T. halophilus* growth including lag phase, exponential phase, and stationary phase, as well as pH changes. The cell density (OD) and viable cell counts remained constant during the first 24 h of incubation indicating the lag phase and there was no significant change in pH within this period. The lag phase during batch culture was due to cell adaptation to the new environment. During this period, the cells need to produce new enzymes, which are necessary for metabolizing different nutrients contained in the new media. The cells also synthesize other new components essential for growth, such as ATP, cofactors, and ribosomes

during the lag phase. After adaptation to the new growth conditions, the cell started growing and dividing at maximal and constant rate, called the exponential phase.

T. halophilus started its exponential growth after 36 h of incubation and reached maximum population at 72 h (8.28 log CFU/mL). The exponential phase was accompanied with significant drop in pH from 5.82 to 4.42. The pH decrease was more likely to be caused by the lactic acid produced by *T. halophilus*. During growth, the enzymatic activities of LAB break down the sugar into organic acids, such as lactic acid, which can lower the pH (Yong and Wood, 1976). The population of *T. halophilus* remained constant thereafter until the end of incubation period, showing that the cells were in their stationary phase. Stationary phase occurs as the medium is depleted of nutrients, causing the cell population to become constant due to balance between cell division and death. During this period, pH continued to decrease slowly to a final value of 4.09.

A similar growth trend was observed in *Z. rouxii* batch culture (Figure 2.1b). Lag phase occurred during the first 8 h of incubation as no significant change in cell density and viable cell counts were shown. However, the pH was reduced within the first 4 h of incubation from 5.26 to 4.63, and then remained constant throughout the incubation period. Similar pH trend was also reported in a study by Onishi (1957) in a medium containing 18% NaCl (pH 4-5), while larger pH range was observed in the same medium in the absence of NaCl. *Z. rouxii* started to multiply exponentially after 8 h to a maximum level of 7.50 log CFU/mL (OD 3.53) at 44 h. The cell population decreased slightly towards the end of incubation period to 7.17 log CFU/mL (OD 3.23).

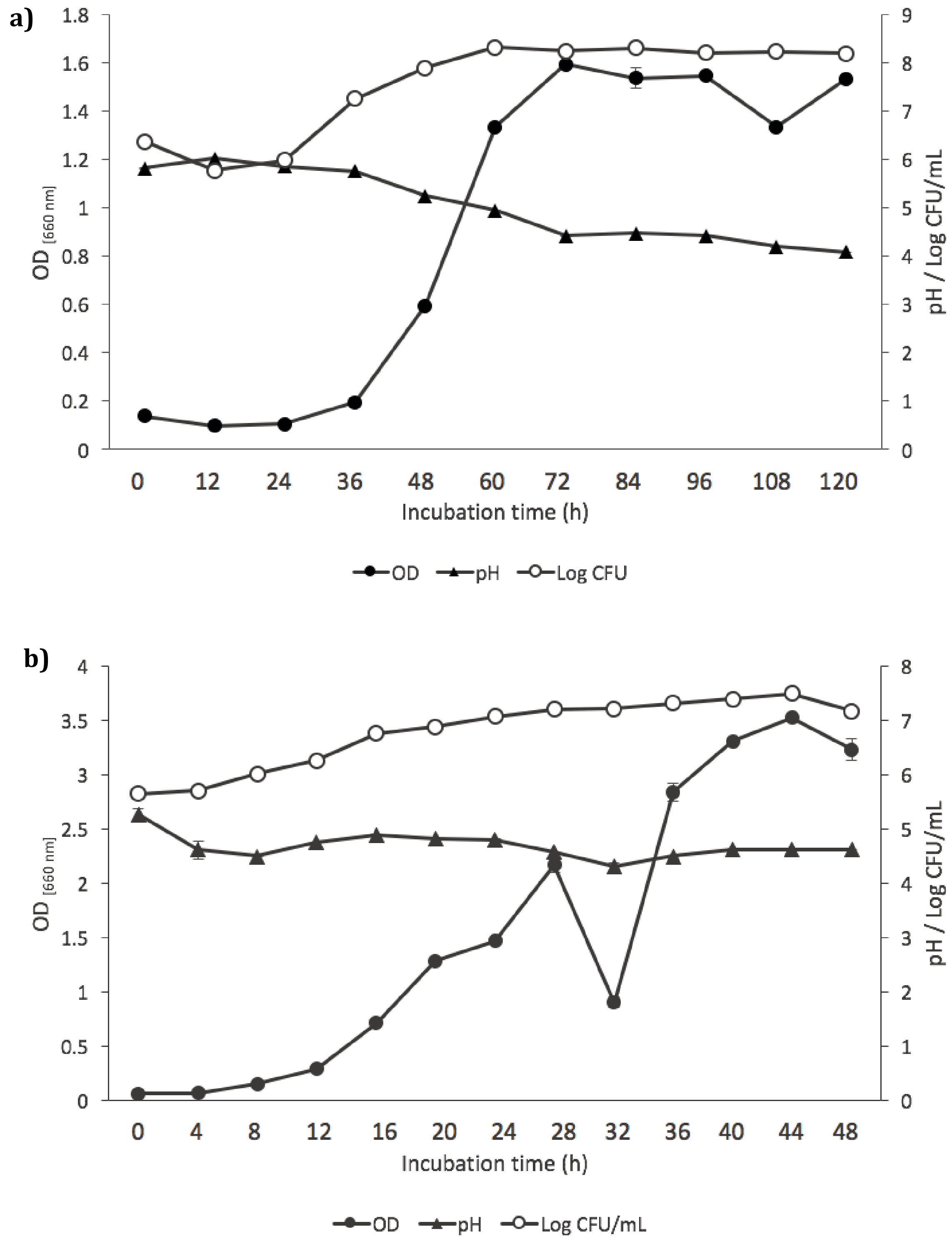
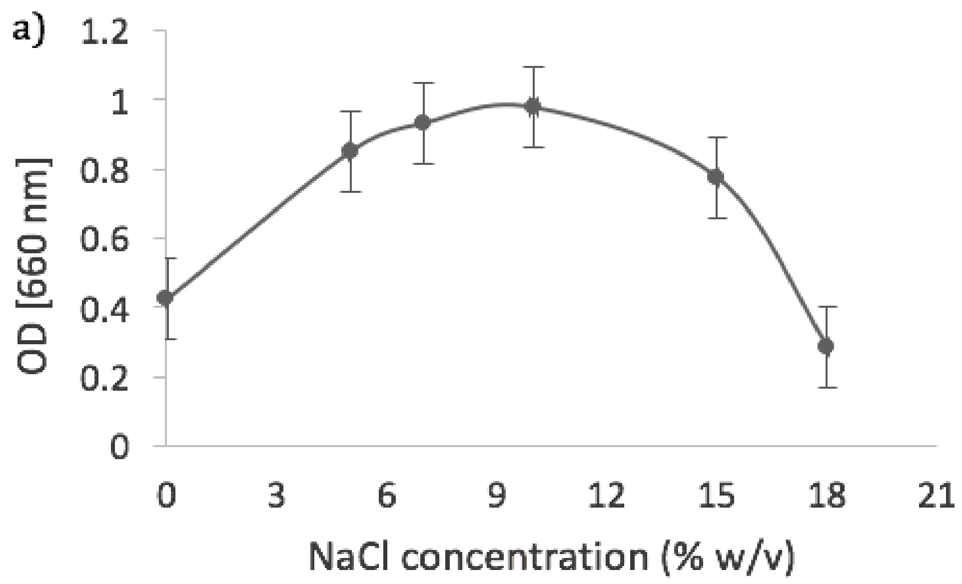


Figure 2.1 Microbial growth curve. (a) *T. halophilus.* (b) *Z. rouxii.*

2.3.2 Assessment of *T. halophilus* and *Z. rouxii* for salt-tolerance

The ability of *T. halophilus* and *Z. rouxii* to grow in medium containing different salt concentrations was investigated. *T. halophilus* exhibited better growth in the presence of NaCl and maximum growth was observed in 10% NaCl (OD 0.979; Figure 2.2a). The growth of *T. halophilus* decreased as the concentration of NaCl was increased. In the medium containing 15% and 18% NaCl, the OD decreased to 0.775 and 0.288, respectively.

Higher OD values were observed in *Z. rouxii* cultures in every NaCl concentration tested (Figure 2.2b). Compared to *T. halophilus*, the effect of NaCl concentration on *Z. rouxii* growth was not as significant. In the absence of salt, *Z. rouxii* was able to grow to an OD of 1.502 and the maximum value (OD 1.709) was reached when 5% NaCl was present in the medium. Increasing NaCl concentration up to 18% caused slight reduction in OD to 1.252. The results suggested that *Z. rouxii* is highly tolerant to NaCl.



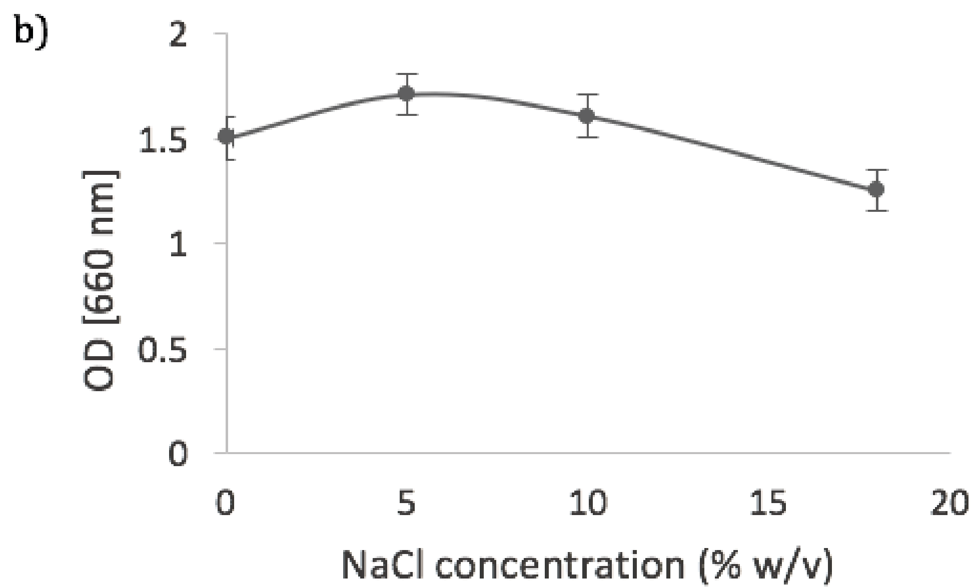


Figure 2.2 Optical density of cells after incubation in varying concentrations of NaCl. (a) *T. halophilus*. (b) *Z. rouxii*.

2.3.3 Physical characteristic of fermented koji

The growth of *A. oryzae* mycelium was observed during koji incubation period. Its mycelium grew covering the mixture of koji and wheat all over the surface and turned it into a compact mass. The koji turned to be greenish in color due to the production of *A. oryzae* spores (Figure 2.3).

The pH of koji decreased from 6.40 to 5.98 during the first 48 h of incubation, but then increased to 6.61 at the end of incubation period (72 h; Figure 2.4a). Similar pattern was also reported by Chancharoonpong et al. (2012). The authors suggested that the pH of koji decreased from 6.32 to 6.12 during the first 24 h of fermentation and then followed by an increase to 6.97 at 72 h. Such changes could occur due to secretion of various extracellular protein and ammonia by *A. oryzae* during fermentation (Liang et al., 2009).

Moisture content is an important physical factor, which determines the growth of mold during fermentation. The amount of water needed in the substrate to support maximum growth and nutrient intake ranges from 40 to 70% (Prior et al., 1992). Figure 2.4b shows the recorded changes in moisture content during koji fermentation. The initial moisture content of koji was 37.13% and continuously decreased to reach 27.28% by the end of incubation period.

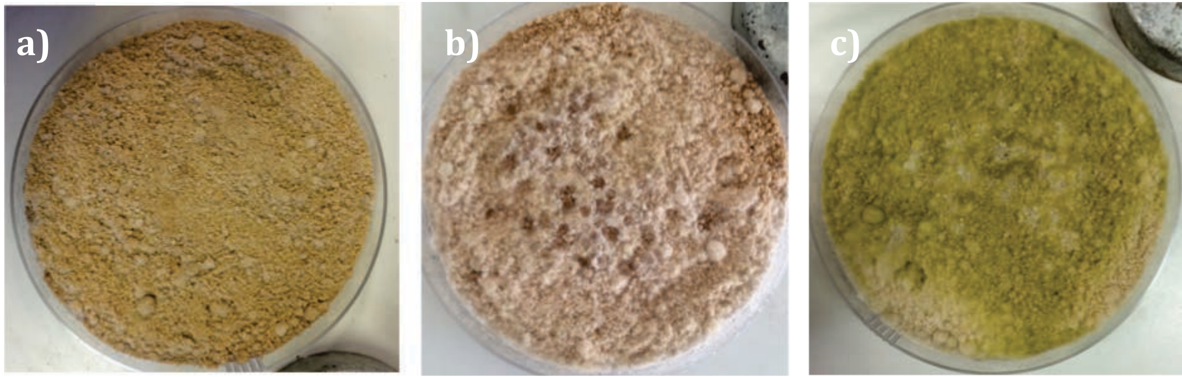


Figure 2.3 Koji characteristic after (a) 24 h, (b) 48 h, and (c) 72 h of fermentation at 30°C.

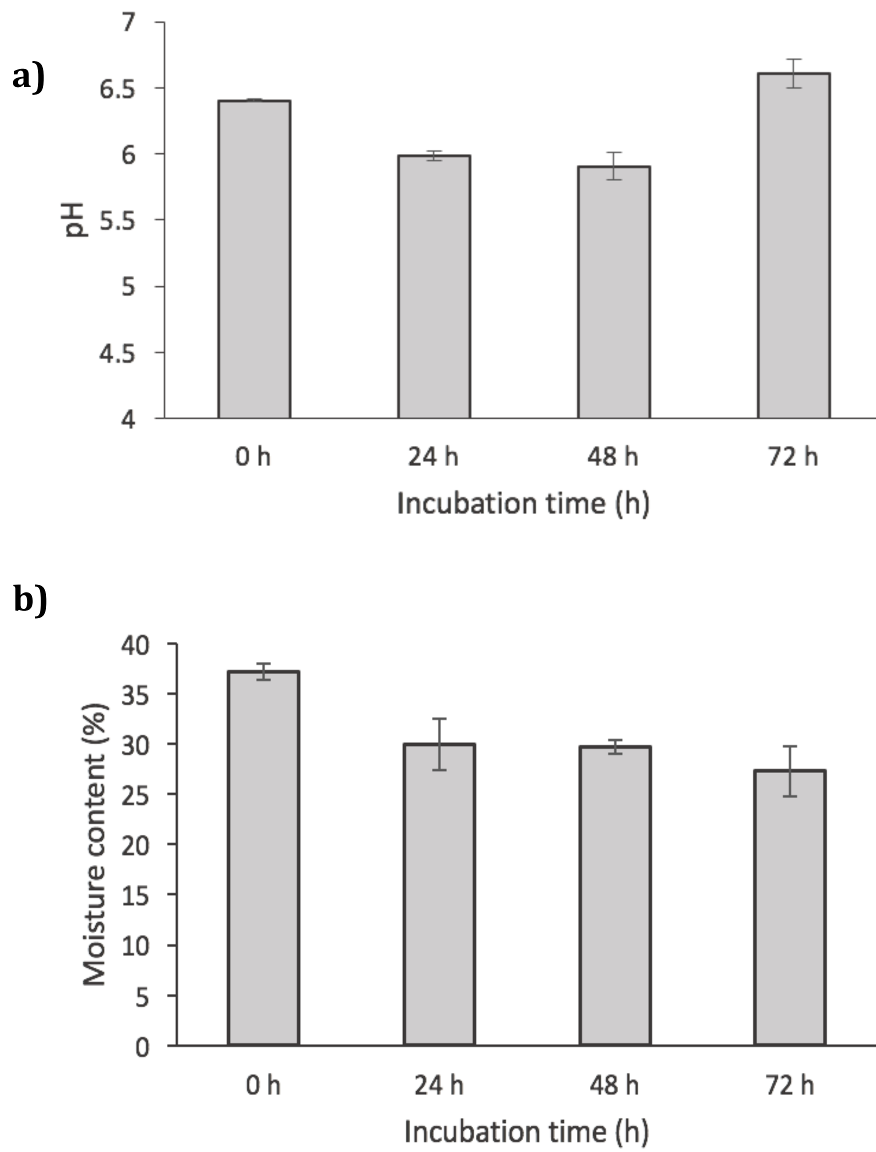


Figure 2.4 Changes in (a) pH and (b) moisture content during koji fermentation.

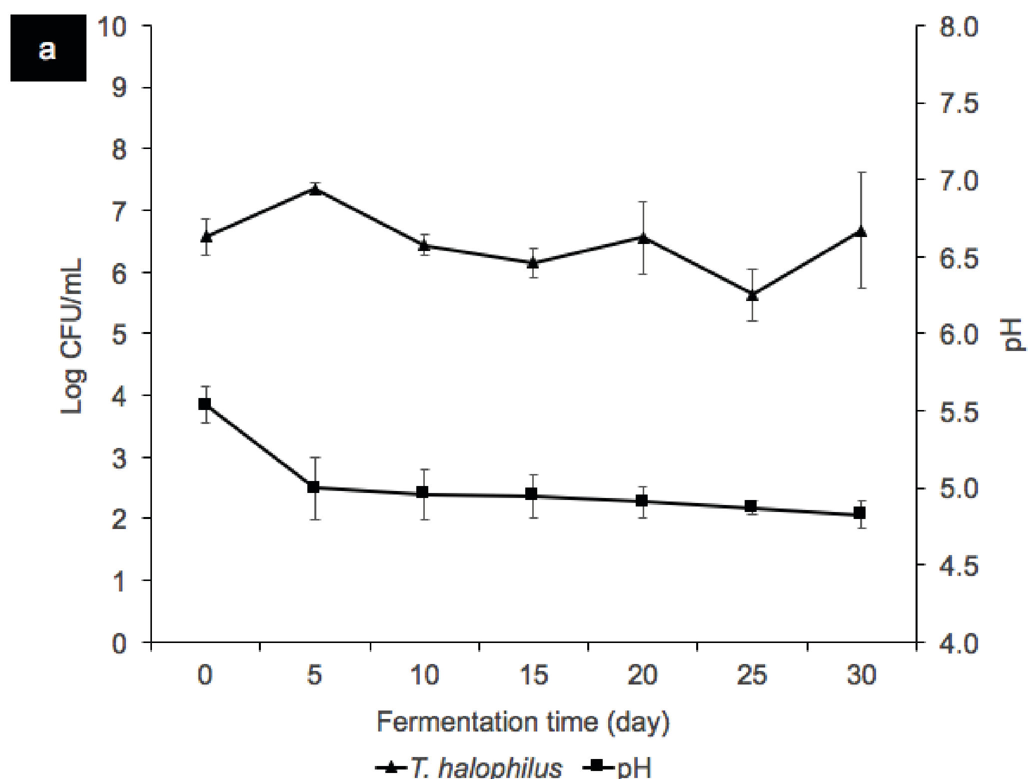
2.3.4 The effect of *T. halophilus* and *Z. rouxii* interaction on growth during moromi fermentation

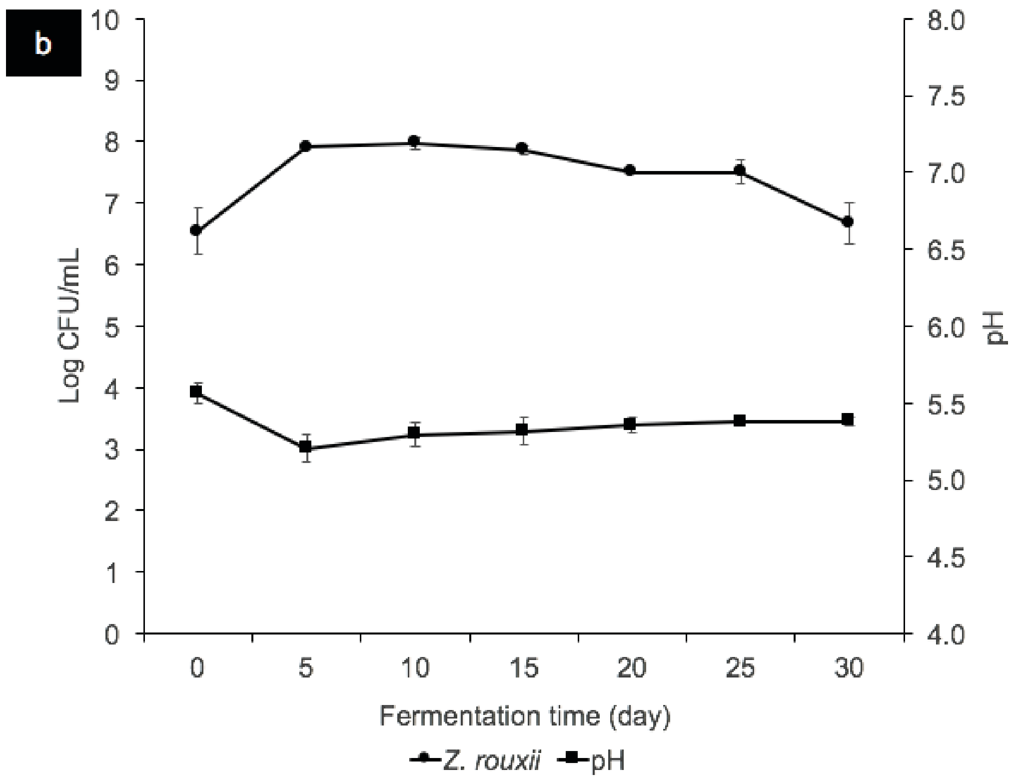
An antagonistic interaction between *T. halophilus* and *Z. rouxii* was evident in mixed culture fermentation regardless of the inoculation sequence. *Z. rouxii* viable cell counts markedly decreased to less than 2 log CFU/mL by the end of fermentation period while counts in single culture remained >6 log CFU/mL (Figure 2.5c and d). On the other hand, *T. halophilus* growth was stimulated by co-inoculation and sequential inoculation, to final counts of 8.62 log CFU/mL (Figure 2.5c) and 8.47 log CFU/mL (Figure 2.5d) respectively, compared to growth in single culture, which was constant (6.67 log CFU/mL; Figure 2.5a).

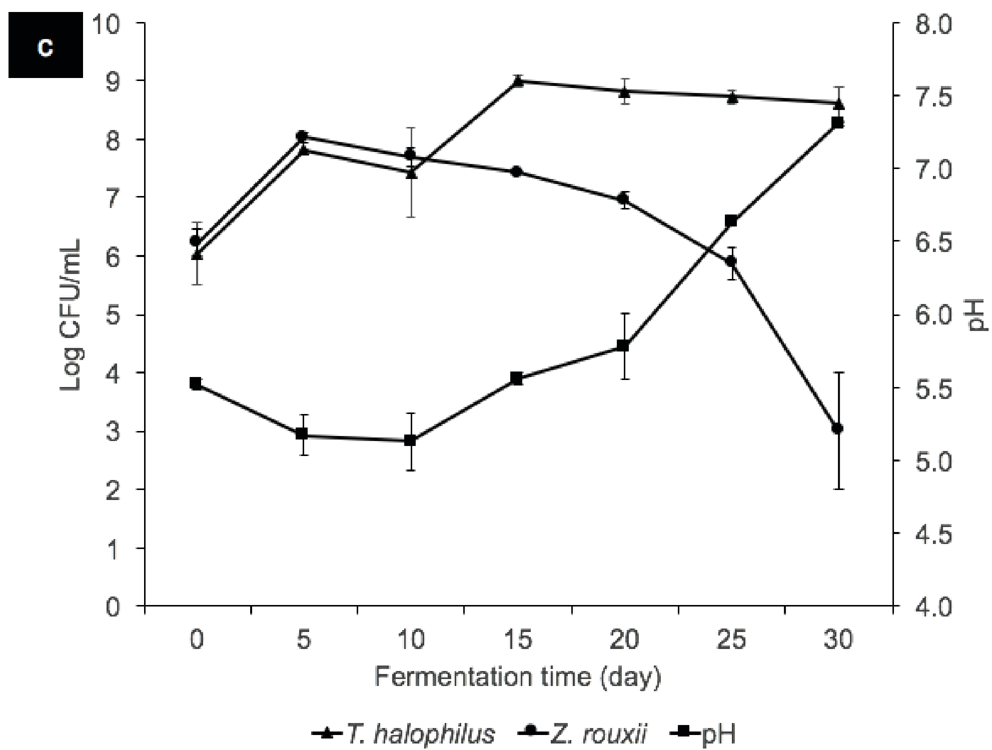
A different pattern was observed for *T. halophilus* growth between co-inoculation and sequential inoculation. *T. halophilus* in co-inoculation reached its stationary phase (~9 log CFU/mL) at day 15, while in sequential inoculation its stationary phase was reached at day 25 (Figure 2.5c and d). These results indicate that delaying *Z. rouxii* inoculation could slow down the growth of *T. halophilus*.

The increase in *T. halophilus* growth in mixed culture could possibly be due to metabolites production such as pyruvate, amino acids, and vitamin by *Z. rouxii* which are essential for the bacterial growth (Sudun et al., 2013). Moreover, the dynamics of *T. halophilus* and *Z. rouxii* viable cells were influenced by moromi pH values during moromi fermentation. Initially, the pH values of all moromi samples were similar (~5.5). When the pH was constantly low (~5.0) throughout the fermentation period, the viable cell counts of *T. halophilus* and *Z. rouxii* in single culture remained constant (Figure 2.5a and b). The final pH values of moromi inoculated with pure culture of *T. halophilus* and

Z. rouxii were 4.83 and 5.38, respectively. In contrast, the mixed cultures caused moromi pH to increase to >6.0, which enhanced the growth of *T. halophilus*, while causing gradual decrease of *Z. rouxii* population (Figure 2.5c and d). The increase in pH could stimulate *T. halophilus* growth since the optimal pH for its growth is around 7.0 (Justé et al., 2008; Wilred F. M. Röling & van Verseveld, 1997). On the other hand, since the optimal pH range for *Z. rouxii* is 3.5-5.0, the pH increase in moromi could inhibit *Z. rouxii* growth (Membré et al., 1999). Moreover, *Z. rouxii* cannot maintain its salt tolerance when the extracellular pH reaches above 5.5 due to loss of the proton gradient across the plasma membrane (Watanabe & Tamai, 1992). In addition, the growth retardation of *Z. rouxii* might also be influenced by the inoculum size and ratio in the mixed culture. According to the finding of Kedia, Wang, Patel, & Pandiella (2007), in a mixed culture with *Lactobacillus reuteri* (LAB), yeast growth was inhibited at LAB : yeast ratio of 1 : 1 and 2 : 1, but enhanced at the ratio of 1 : 2.




b
Log CFU/mL
pH
Fermentation time (day)
Z. rouxii
pH


c
Log CFU/mL
pH
Fermentation time (day)
T. halophilus
Z. rouxii
pH

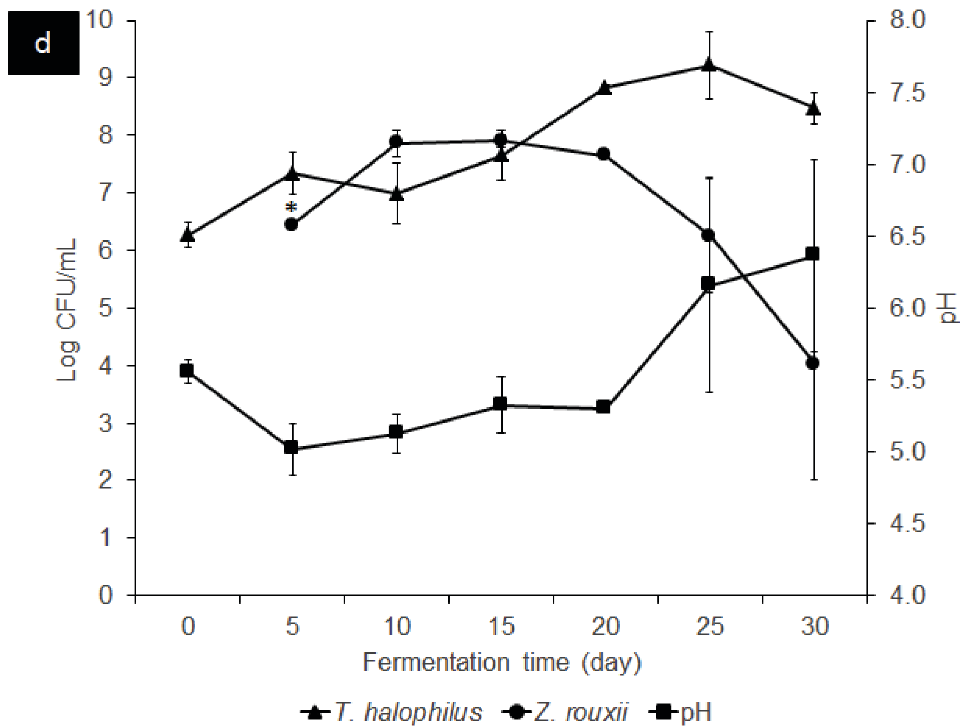


Figure 2.5 Changes in pH and growth (cfu) of *T. halophilus* and *Z. rouxii* growth during moromi fermentation with (a) pure culture of *T. halophilus*, (b) pure culture of *Z. rouxii*, (c) co-inoculation, and (d) sequential inoculation.

2.3.5 Physicochemical changes in moromi fermentation

The reducing sugar content in all *Z. rouxii* containing models decreased to undetectable level 5 days after inoculation (Figure 2.6a). This reducing sugar depletion was due to sugar utilization by yeast for propagation as well as ethanol conversion. In contrast to the present study, the reducing sugar content in both traditional and industrial practice normally undergoes rapid increase during the first month of moromi fermentation (Cui et al., 2014a; Singracha et al., 2017). The reason could be due to the heat-inactivation of *A. oryzae* prior to moromi fermentation, therefore it could no longer perform starch hydrolysis resulting in lower availability of reducing sugar for *Z. rouxii*.

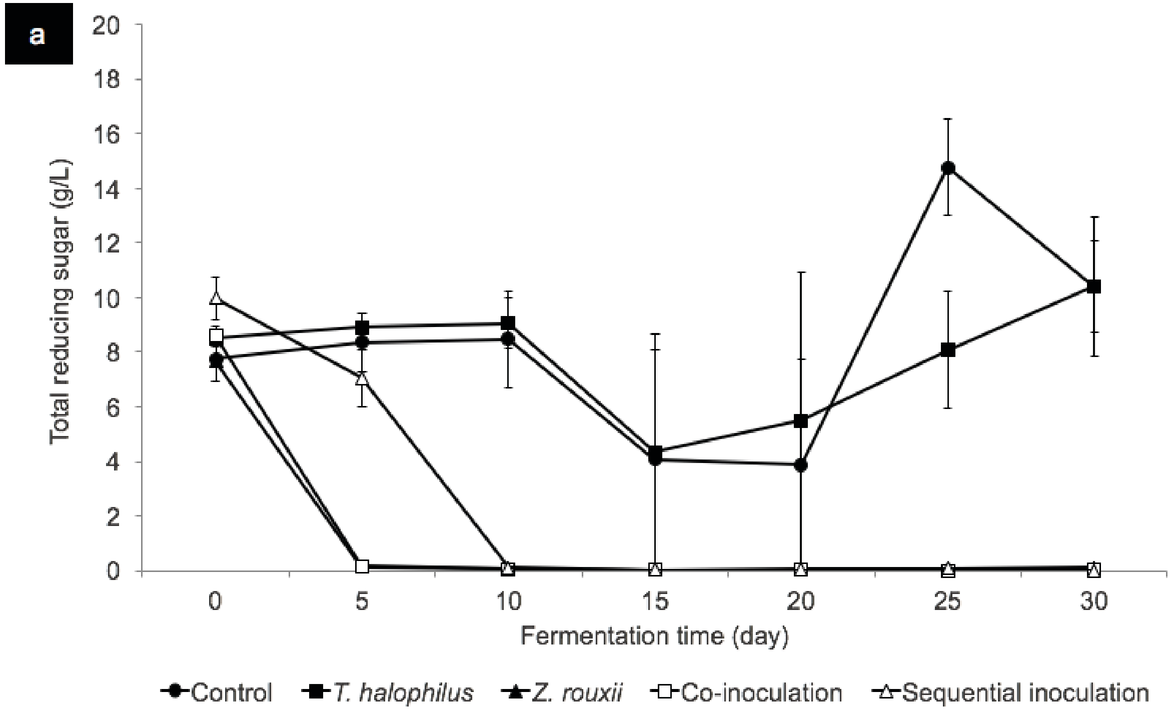
In contrast, the amount of reducing sugar in control and single culture of *T. halophilus* remained high throughout the incubation period. By the end of fermentation, 10.39 g/L and 10.42 g/L of reducing sugar were found in control and single culture of *T. halophilus*, respectively, which were relatively higher than the initial amount (Figure 2.6a). Such increase could be resulting from the hydrolyzing activity of amylase produced by *A. oryzae* which remains active during moromi fermentation (Chou and Ling, 1998; Cui et al., 2014b). The higher amount of reducing sugar in the sample treated with *T. halophilus* indicated limited ability of *T. halophilus* in utilizing D-glucose and D-fructose.

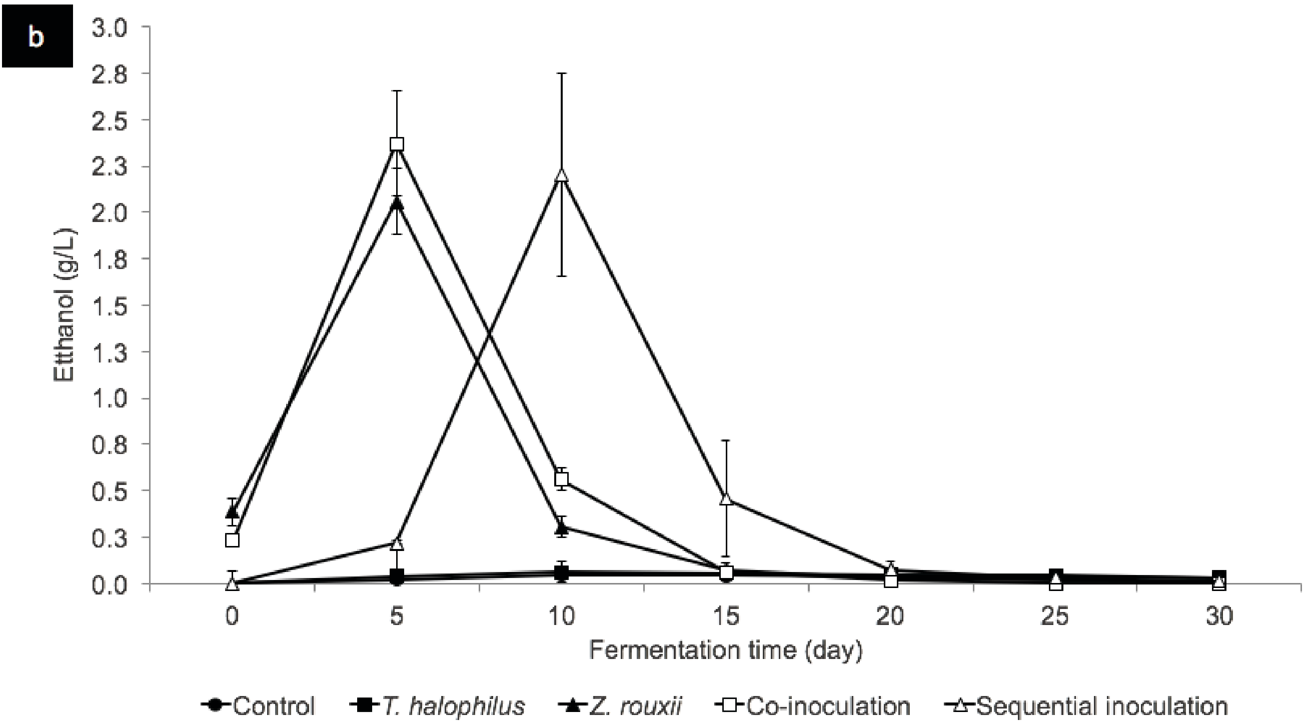
The changes in the reducing sugar content were associated with the ethanol production during moromi fermentation (Figure 2.6b). Ethanol was monitored since it is one of the main alcohols produced by *Z. rouxii* during brine fermentation via sugars conversion (Van Der Sluis, Tramper & Wijffels, 2001). Reducing sugar depletion in all *Z. rouxii*-containing samples occurred in parallel with maximum ethanol production 5 days since *Z. rouxii* was inoculated. As expected, ethanol was not detected in the sample treated with single culture of *T. halophilus* and control (Figure 2.6b) since the reducing sugar content remained stable. Once sugar in all *Z. rouxii*-containing samples was exhausted, ethanol content dropped to below detection level.

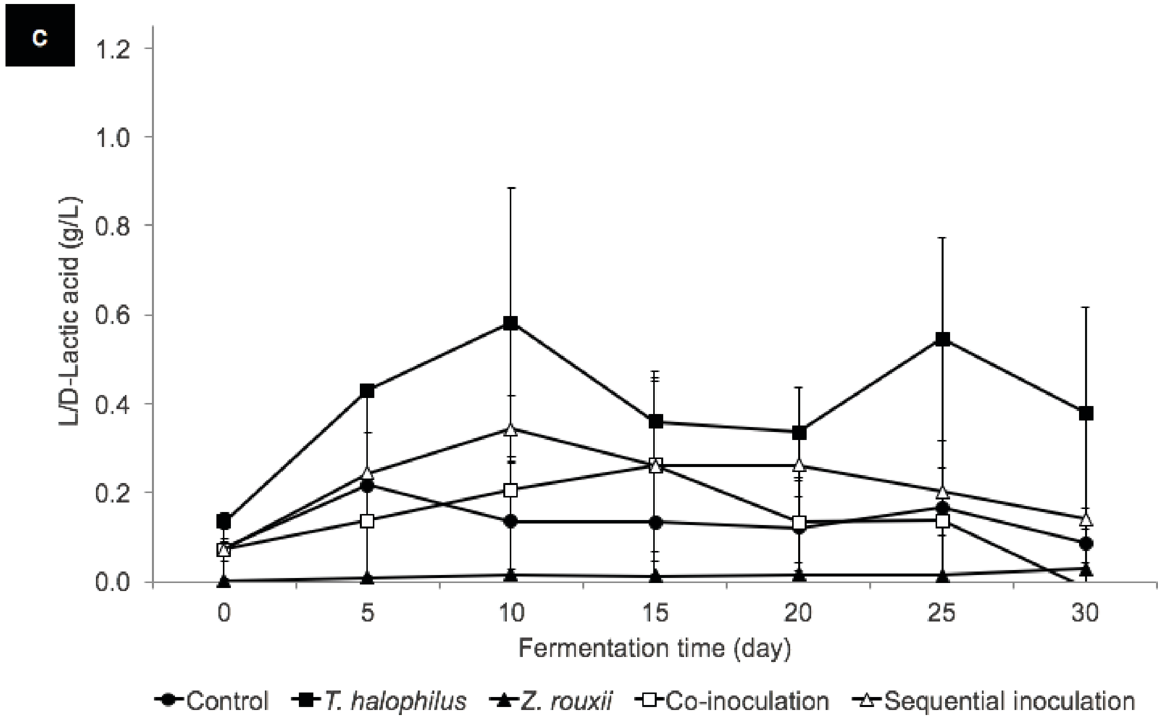
As expected, *T. halophilus* was found to play a major role in both lactic acid and acetic acid production (Figure 2.6c and d). However, constant increase in acid productions as previously reported by Cui et al. (2014a) and Singracha et al. (2017) was not observed in this study. This could be related to the availability of residual reducing sugars for organic acids production by *T. halophilus*. Surprisingly, even though *T. halophilus* growth was enhanced, both acid productions were suppressed when *Z. rouxii*

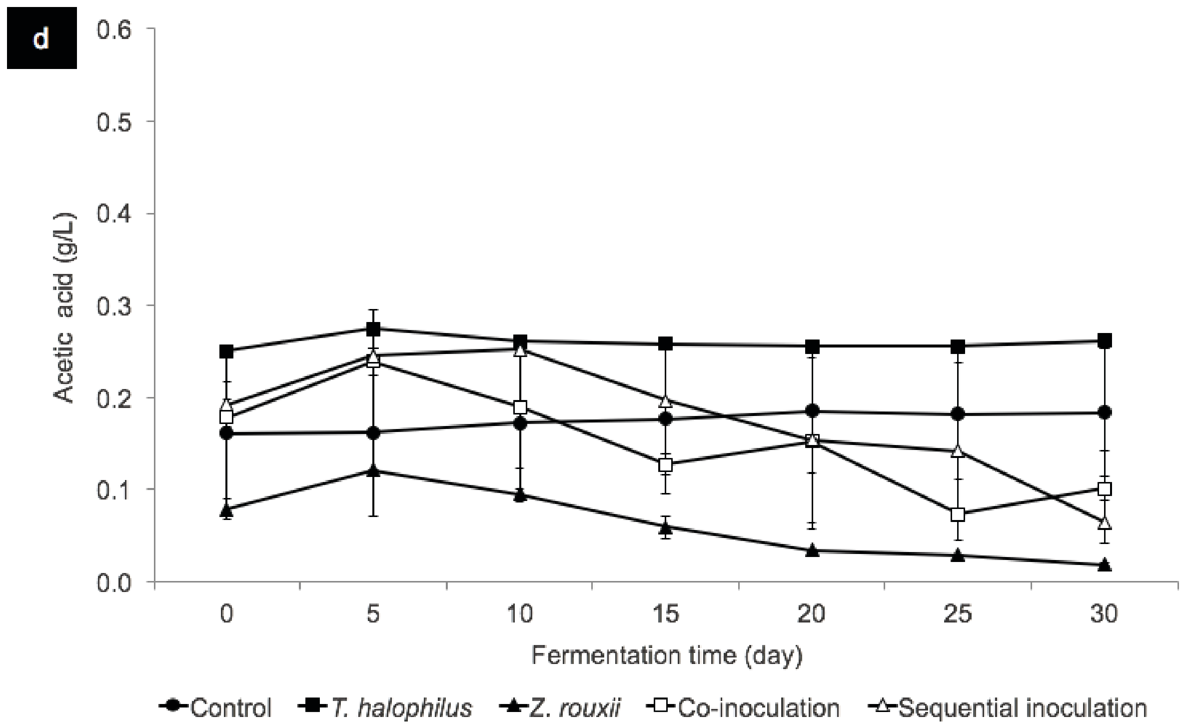
was present. This could be due to consumption of lactic acid by the yeast as previously reported in *Saccharomyces cerevisiae* when co-cultured with LAB in kefiran fermentation (Cheirsilp et al., 2003). In single culture, *T. halophilus* was able to produce lactic acid and acetic acid to a maximum level of 0.58 g/L and 0.26 g/L, respectively, which were the highest among all samples.

The amino nitrogen content generally undergoes rapid increase during the first month of moromi fermentation (Cui et al., 2014b, 2014a). In the present study, the amino nitrogen contents in all soy sauce samples were generally constant with some fluctuation throughout the fermentation process. This was expected since *A. oryzae* was heat-inactivated prior to moromi fermentation, resulting in lower protease activity. The fluctuation in amino nitrogen content observed in this study might be due to the metabolic activity of *T. halophilus* or Maillard reaction (Cui et al., 2014) which is responsible for deep brown color formation in soy sauce. This reaction involves condensation of carbonyls such as reducing sugars and aldehydes and compound possessing a free amino group (Martins et al., 2000; Lertsiri et al., 2001).


a
Total reducing sugar (g/L)
20
18
16
14
12
10
8
6
4
2
0
0
5
10
15
20
25
30
Fermentation time (day)
Control
T. halophilus
Z. rouxii
Co-inoculation
Sequential inoculation


b
Etthanol (g/L)
3.0
2.8
2.5
2.3
2.0
1.8
1.5
1.3
1.0
0.8
0.5
0.3
0.0
0
5
10
15
20
25
30
Fermentation time (day)
Control
T. halophilus
Z. rouxii
Co-inoculation
Sequential inoculation


c
1.2
1.0
0.8
0.6
0.4
0.2
0.0
L/D-Lactic acid (g/L)
0
5
10
15
20
25
30
Fermentation time (day)
Control
T. halophilus
Z. rouxii
Co-inoculation
Sequential inoculation


d
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Acetic acid (g/L)
0
5
10
15
20
25
30
Fermentation time (day)
Control
T. halophilus
Z. rouxii
Co-inoculation
Sequential inoculation

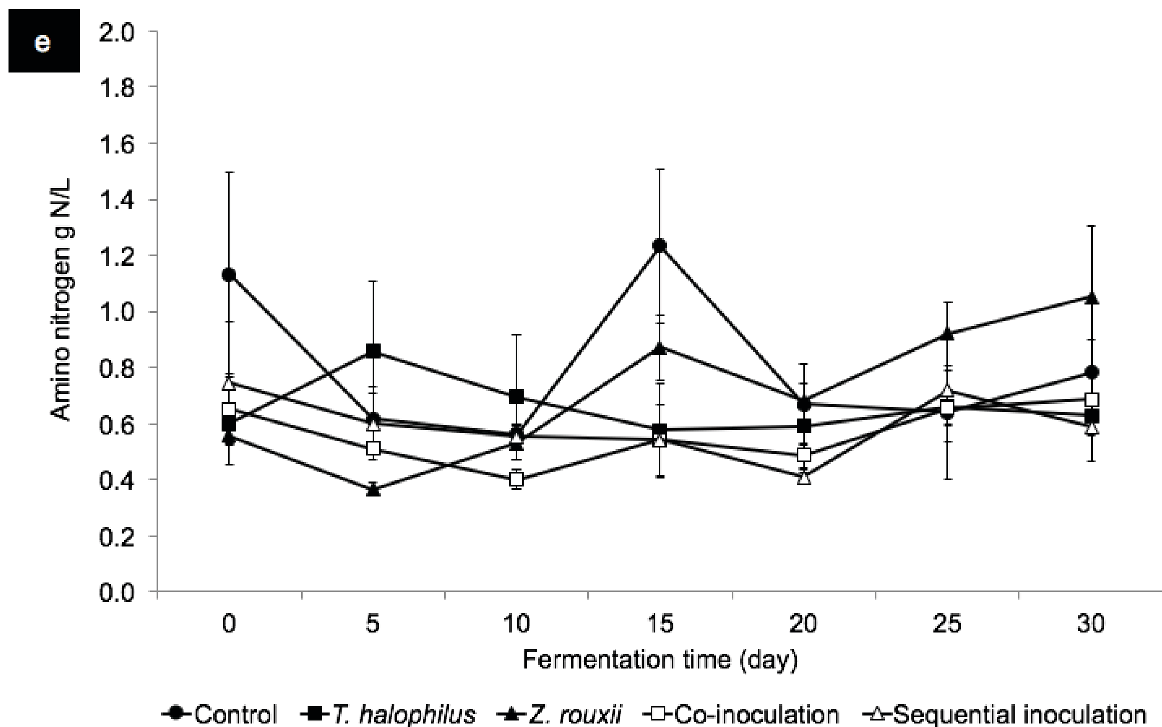


Figure 2.6 The changes of (a) total reducing sugar, (b) ethanol, (c) L/D-lactic acid, (d) acetic acid, and (e) amino nitrogen during 30-day period of moromi fermentation at 30°C with different combinations of microorganisms.

2.3.6 The effect of co-inoculation and sequential inoculation of *T. halophilus* and *Z. rouxii* on formation of aroma compounds

A total of 23 major aroma compounds were identified in the moromi samples, including eight alcohols, three acids, five aldehydes, two esters, two pyrazines, one furan, and two ketones (Table 2.1). Alcohols were found to be the most abundant compounds detected in all soy sauce samples which is in agreement with previous study conducted under high (Feng et al., 2015) and low salt concentration (Singracha et al., 2017). However, the ultimate contribution of a particular compound to the overall aroma of soy sauce is not only determined by its concentration, but also its odour threshold. The odour threshold is the minimum concentration of a compound in the air that can be detected by smell.

Higher amount of total alcoholic compounds was detected in the moromi treated with *Z. rouxii* either as single or mixed cultures, suggesting that *Z. rouxii* was mainly responsible for the alcohols formation. Fusel alcohols such as phenyl ethyl alcohol (floral, sweet), 3-methyl-1-butanol (malty, rancid, and pungent), and 2-methyl-1-propanol (bitter) were found to be the major alcohol components of the final moromi. Song et al. (2015a) also reported high production of the above fusel alcohols in reduced-salt moromi by adding indigenous yeast isolated from different stages during traditional moromi fermentation. These fusel alcohols could have been generated from their corresponding amino acids by *Z. rouxii* through the Ehrlich pathway (Jansen et al., 2003). Extracellular amino acids present during moromi fermentation can undergo deamination or transamination through the Ehrlich pathway, producing α-keto acids. The α-keto acids serve as the main key intermediates in the formation of fusel alcohols. Fusel alcohols are formed through decarboxylation and subsequent reduction of the corresponding α-keto acids (van Der Sluis et al., 2001).

Combination of *Z. rouxii* and *T. halophilus* significantly increased 3-octanol (mushroom-like) production compared with the single cultures. Ethanol was obtained in significantly lower amount ($P<0.05$) in single culture and co-culture compared to those treated with sequential inoculation. Ethanol is generally produced by *Z. rouxii* from glucose through glycolytic pathway and anaerobic fermentation (Benitez & Codon, 2004).

Other alcohols such as 2-furanmethanol (burnt sugar) and methanol were detected in all samples before fermentation started. All samples contained 1-octen-3-ol (mushroom-like), but its amount decreased due to microbial activity during fermentation. Although most alcohols in soy sauce are originated from sugars and

amino acids metabolism, small amounts of alcohols are also produced by yeast through metabolism of related aldehyde compounds (Sun et al., 2010).

Aldehydes (furfural, benzaldehyde, 2-methylpropanal, 3-methylpropanal, 3-methylbutanal, and 2-methylbutanal) were the second main group of aroma compounds found in the moromi. They were present in moromi since the beginning of fermentation. However, their amount decreased significantly due to the activity of *Z. rouxii* especially in mixed culture. The decrease might be due to aldehyde conversion to its corresponding alcohol (Song et al., 2015a). This hypothesis is supported by the fact that the amount of alcohols obtained in the samples treated with *Z. rouxii* is higher than control and single culture of *T. halophilus*. These aldehydes are responsible for soy sauce malty and nutty aroma (Feng et al., 2015).

Acetic acid was found to be the major aroma in single culture of *T. halophilus*. Its amount was significantly ($P<0.05$) higher compared to other samples since *T. halophilus* is known to produce acetic acid (Justé et al., 2012; Tanasupawat et al., 2002). Acetic acid can give sour odor to the final product. Production of 3-methylbutanoic acid was favored in single cultures of *T. halophilus* and *Z. rouxii*. It has been reported to be the major aroma compound in Chinese soy sauce and it is responsible for strong pungent, sweaty, and cheese-like odor notes (Sun et al., 2010). This branched-chain acid might be produced by *Z. rouxii* from branched-chain amino acids contained in soybean via the Ehrlich pathway (Lee et al., 2013). The presence of mixed culture seemed to suppress the production of 3-mehylbutanoic acid.

Acetate esters comprising isoamyl acetate (banana aroma) and acetic acid, 2-pehnylethyl ester (honey, rosy) were detected in soy sauce samples fermented with *Z. rouxii*. Mixed cultures significantly enhanced the production of isoamyl acetate

(P<0.05). The production of 2-phenylethyl acetate was higher in samples treated with *Z. rouxii* either alone or in mixed cultures. Interestingly, sequential inoculation produces significantly higher amount of 2-phenylethyl acetate than co-inoculation. Higher esters production in the presence of yeast could happen since yeast is capable of esterifying alcohols with fatty acid (Van Der Sluis et al., 2001). Fatty acids can be produced through fungal lipase activity on the lipid contained in the soybean (Chung, 1999).

Pyrazines were detected in all samples and they might be produced through Maillard reaction between saccharide and amino residues (Sun et al., 2010). Therefore, trimethylpyrazine (burnt) and 2, 5-dimethyl pyrazine (roasted nuts) were found in all samples although 2,5-dimethylpyrazine was found significantly higher in the mixed culture samples. The main furan compound detected was 2-pentylfuran and its levels were similar among samples. It is known as singlet oxidation compound produced from linoleic acid (Lee et al., 2013).

Acetone seemed to be major ketone found in all samples which is in agreement with the finding of Lee et al. (2013) and Wanakhachornkrai & Lertsiri (2003). The formation of acetone is related to metabolism activity of microorganisms, especially yeast, via lipid oxidation (Lee et al., 2013; Song, Jeong, & Baik, 2015a). However, its amount was significantly higher at day 0, suggesting that the acetone might have originated from koji. According to Feng et al. (2013), acetone found in koji could be derived from raw materials used in the koji making. Co-inoculation was found to significantly reduce the amount of acetone after 30 day-fermentation. Acetoin contributing to buttery odor was detected in all samples and its amount was not significantly different from each other. Therefore, in this case acetoin might be derived from koji fermentation (Lee et al., 2013).

Table 2.1 Aroma compounds found in moromi before and after 30 days of fermentation with different types of inoculation.

Compound	LRI[1]	Day 0			Day 30														
					Control			*T. halophilus*			*Z. rouxii*			Co-inoculation			Sequential inoculation[2]		
		Mean (n=3)	SD	%CV	Mean (n=3)	SD	%CV	Mean (n=3)	SD	%CV	Mean (n=3)	SD	%CV	Mean (n=3)	SD	%CV	Mean (n=3)	SD	%CV
Alcohols																			
Methanol	915.22	0.67 a	0.12	17.44	0.82 a	0.14	16.81	0.78 a	0.08	10.35	0.75 a	0.07	8.79	1.01 a	0.07	7.20	0.55 a	0.06	10.61
Ethanol	951.72	0.48 a	0.10	21.56	4.86 b	0.95	19.47	4.70 b	0.40	8.53	1.51 ac	0.19	12.38	0.29 a	0.03	9.75	3.51 bc	0.37	10.46
2-Methylpropanol	1117.26	0.00 a	0.00	62.35	0.01 a	0.00	22.05	0.01 a	0.00	21.21	0.06 b	0.01	16.63	0.08 b	0.00	6.45	0.07 b	0.02	33.15
3-Methylbutanol	1226.73	0.03 a	0.00	11.60	0.87 a	0.17	19.33	0.88 a	0.06	7.02	14.93 b	1.03	6.88	13.35 b	0.49	3.65	11.68 b	2.05	17.52
3-Octanol	1408.49	0.01 a	0.00	11.65	0.00 bc	0.00	14.35	0.00 b	0.00	12.84	0.07 b	0.02	21.42	0.04 c	0.01	15.25	0.04 c	0.00	12.12
1-Octen-3-ol	1467.60	0.65 a	0.06	9.59	0.28 b	0.07	25.80	0.16 b	0.02	9.87	0.18 b	0.03	18.59	0.23 b	0.04	16.42	0.21 b	0.02	10.66
2-Furanmethanol	1692.95	0.26 a	0.03	13.42	0.23 a	0.03	11.95	0.25 a	0.03	13.18	0.01 a	0.00	21.18	0.04 a	0.00	8.87	0.02 a	0.00	9.52
Phenylethyl alcohol	1960.59	0.06 a	0.05	80.68	0.06 a	0.04	55.45	0.07 a	0.06	90.85	19.50 b	1.96	10.06	16.45 b	1.85	11.22	10.38 c	0.59	5.70
Acids																			
Acetic Acid	1485.56	0.12 a	0.09	75.95	0.02 a	0.04	166.27	14.34 b	0.64	4.46	0.39 a	0.31	79.10	0.09 a	0.09	104.26	0.10 a	0.12	121.71
2-Methylpropanoic acid	1598.71	0.01 a	0.01	116.55	0.00 a	0.00	73.30	0.17 a	0.01	5.52	0.42 a	0.01	2.92	0.04 a	0.02	53.98	0.16 a	0.05	28.99
3-Methylbutanoic acid	1699.57	0.05 a	0.06	116.13	0.04 a	0.04	109.89	2.03 b	0.11	5.54	1.71 b	0.14	8.41	0.14 a	0.06	42.87	1.18 ab	0.34	28.78
Aldehydes																			
2-Methylpropanal	825.43	0.31 a	0.06	18.53	0.06 bcd	0.03	49.35	0.09 b	0.01	16.11	0.11 bc	0.01	11.55	0.00 d	0.00	69.25	0.00 d	0.00	18.60
2-Methylbutanal	930.15	0.18 a	0.05	28.93	0.02 b	0.01	54.55	0.03 b	0.00	14.55	0.05 b	0.01	13.07	0.00 b	0.00	23.90	0.01 b	0.00	31.10
3-Methylbutanal	934.02	0.14 a	0.02	15.11	0.06 b	0.03	49.57	0.07 b	0.01	9.94	0.05 b	0.00	4.41	0.00 c	0.00	23.73	0.02 c	0.01	40.01
Furfural	1502.16	1.11 a	0.18	15.94	0.23 b	0.04	18.87	0.14 b	0.01	5.37	0.01 b	0.00	18.16	0.01 b	0.00	17.66	0.01 b	0.00	8.94
Benzaldehyde	1569.97	0.31 abc	0.05	17.60	0.44 a	0.09	20.61	0.17 bd	0.03	18.16	0.14 d	0.02	14.26	0.03 d	0.01	35.83	0.02 d	0.00	18.36
Esters																			

Isoamylacetate	1140.42	0.00	a	0.00	48.90	0.01	a	0.00	43.31	0.03	a	0.00	19.07	0.05	a	0.01	14.65	0.27	b	0.03	11.42	0.45	b	0.16	36.46
Acetic acid, 2-phenylethyl ester	1860.34	0.01	a	0.00	42.47	0.00	a	0.00	13.61	0.00	a	0.00	67.25	0.17	b	0.03	15.47	0.15	b	0.04	30.79	0.43	c	0.02	3.95
Pyrazines																									
2,5-Dimethyl pyrazine	1352.73	0.03	a	0.00	9.18	0.06	a	0.01	16.74	0.17	a	0.02	9.31	0.05	a	0.01	12.33	2.00	b	0.38	19.06	0.10	ac	0.00	4.26
Trimethyl pyrazine	1434.11	0.05	a	0.01	13.26	0.05	a	0.01	18.38	0.05	a	0.01	11.05	0.05	a	0.01	20.16	0.15	a	0.02	14.52	0.05	a	0.00	1.72
Furans																									
2-Pentyl furan	1250.68	0.07	a	0.02	25.03	0.05	a	0.04	76.96	0.08	a	0.01	17.30	0.08	a	0.01	10.67	0.07	a	0.02	35.44	0.08	a	0.03	34.88
Ketone																									
Acetone	830.37	1.47	a	0.23	15.64	0.62	b	0.16	26.52	0.63	b	0.09	14.18	0.59	b	0.01	1.09	0.15	c	0.02	16.25	0.63	b	0.04	6.10
Acetoin	1320.84	0.15	a	0.02	13.61	0.16	a	0.02	13.46	1.23	a	0.09	7.32	0.14	a	0.01	6.57	0.00	a	0.00	38.52	0.01	a	0.00	20.31

The values are relative to the peak area observed when the headspace above a 0.1 μg/mL 1-octen-3-ol solution was analyzed.
[1]Linear retention indices of the compounds relative to an alkane series.
[2]*Z. rouxii* was inoculated at day 5.
Means within the same row with different letters (a, b, c) are significantly different ($p<0.05$).

2.3.7 Principal Component Analysis (PCA)

PCA was performed in order to understand the relationship between formation of aroma compounds, microbial species, and their type of inoculation. According to the PCA score plot (Figure 2.7a), all moromi samples were clearly differentiated indicating that the microbial species and their order of inoculation contributed to different aroma profiles in each sample. Single culture of *Z. rouxii*, co-inoculation, and sequential inoculation were positioned in positive PC1 region. On the other hand, control and single culture of *T. halophilus* were located in the negative PC1 suggesting that *T. halophilus* alone is not adequate to develop complex aroma profiles. The PCA loading plot (Figure 2.7b) shows this separation to be driven by a group of compounds dominated by alcohols (3-octanol, 3-methyl-1-butanol, 2-phenylethanol, 2-methyl-1-propanol, 2-phenylelthyl acetate, isoamyl acetate, trimethylpyrazine and 2,5-dimethylpyrazine) while aldehydes are positioned on the left side of the plot (PC1; 44.95%). This is in agreement with the observed physicochemical changes discussed in section 3.3.5, where higher amount of alcohols detected in all moromi containing *Z. rouxii*.

Interestingly, sequential inoculation was distinguished from co-inoculation on PC2 (23.60%). Co-inoculation was distinguished by pyrazines and random groups of compounds. However, sequential inoculation and the top of PC2 accounted for significantly higher number of aroma compounds (13 out of 21) indicating production of more complex flavor profiles. PCA results demonstrated that simultaneous or subsequent inoculation of *Z. rouxii* matters to the flavor characteristics development in moromi fermentation.

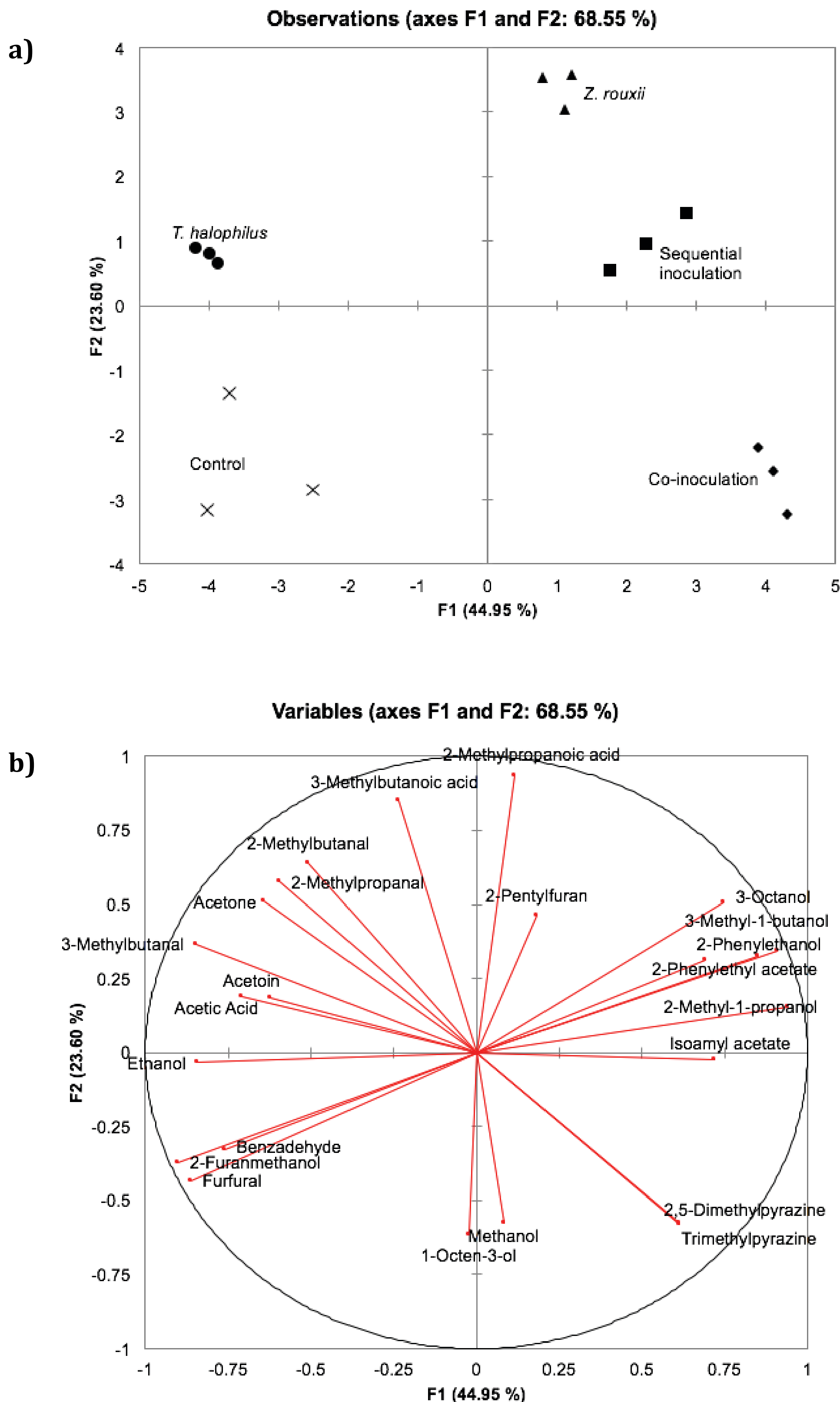


Figure 2.7 (a) PCA score plot of five moromi samples after 30-day fermentation. The scores are based on three replicates of each sample. The identical symbols represent triplicate measurements. (b) PCA loading plot of the aroma compounds detected in moromi after 30-day fermentation with either single culture of *T. halophilus* and *Z. rouxii*, co-inoculation, and sequential inoculation.

2.4 Conclusion

The inoculation sequence demonstrated the antagonistic relationship between *T. halophilus* and *Z. rouxii* which also affected the physicochemical and aroma profile changes during reduced-salt moromi fermentation. Although the antagonistic interaction occurred in both co-inoculation and sequential inoculation, it resulted in different physicochemical changes which seemed to favor the aroma profiles in the case of sequential inoculation. Since the microbial proliferation and activity is very important to the development of moromi, understanding microbial interactions can assist strategies for controlling microbial release and activity (e.g. encapsulation) or modulation of the inoculation time. Furthermore, varying the inoculum size and ratio between *T. halophilus* and *Z. rouxii* should also be investigated in order to fully explore the interaction between the two microbes.

Chapter 3

Encapsulation of *Zygosaccharomyces rouxii* in alginate beads

3.1 Background

As described in Chapter 2, *T. halophilus* and *Z. rouxii* exhibited antagonistic interactions during moromi fermentation, which resulted in compromising flavor development due to the rapid growth of *T. halophilus* producing lactic acid and acetic acid, suppressing the alcoholic fermentation by yeast. According to Kusumegi, Yoshida, & Tomiyama (1998), acetic acid can inhibit the growth of *Z. rouxii* by interfering with the proton expulsive activity which is important for its halotolerance. Therefore, it was necessary to develop a formulation that can minimize such antagonism.

Microbial encapsulation has been used as a method to protect microbial cells from harsh environmental conditions during fermentation (Bilenler et al., 2017; Pourbafrani et al., 2007), food processing (Abbaszadeh et al., 2014), as well as during digestion process (Chávarri et al., 2012). Alginate is one of the most common materials used for encapsulating microbial cells, due its low cost, simplicity, and biocompatibility. It is suitable for encapsulating microbial cells since the gel formation process can be performed under mild conditions. Moreover, the porous structure of alginate beads allows the diffusion of nutrients, oxygen, metabolites, and wastes for the encapsulated cells.

Alginate is a naturally derived polysaccharide extracted from various species of algae. Alginate is a linear heteropolysaccharide, composed of β-D-mannuronic (M) and α-L-guluronic acids (G) which are linked by 1-4 glycosidic bonds (Figure 3.1a; Pistone et al., 2015; Simó et al., 2017). It is able to form gel through ionic cross-linking of its negatively charged carboxyl groups with the divalent cations such as such as Ca^{2+}, Ba^{2+}, and Sr^{2+}. Such interaction results in a three-dimensional network of alginate filaments that are held together described as the "egg-box model" (Figure 3.1b; Lee and Mooney, 2013; Bajpai and Sharma, 2004).

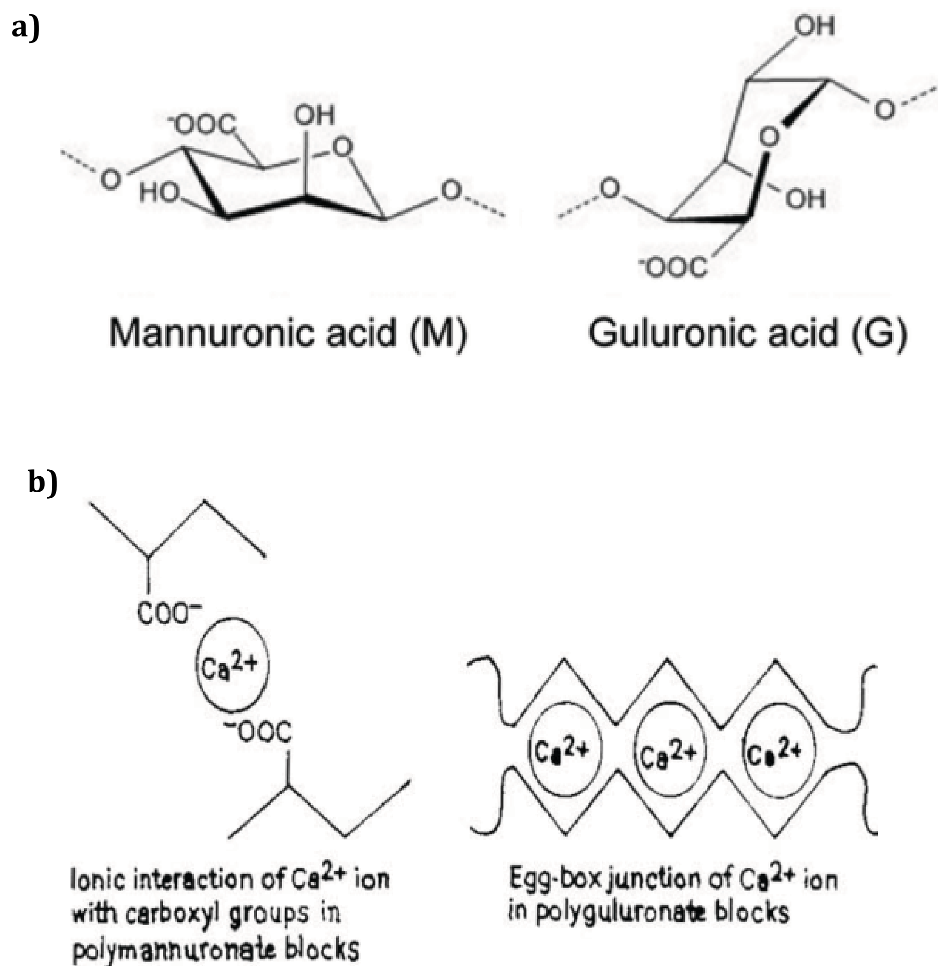


Figure 3.1 (a) Chemical structure of alginate monomers: β–D-mannuronic acid (left) and α-L-guluronic acid (Pistone et al., 2015; Simó et al., 2017). (b) The binding of Ca^{2+}ions to $–COO^{-}$ group in the calcium alginate beads (Bajpai and Sharma, 2004).

Previously, encapsulation of *Z. rouxii* and *C. versatilis* in alginate beads has been investigated during moromi fermentation, in order to shorten the fermentation time (Hamada et al., 1991). However, this system was found to be unstable over time due to alginate's sensitivity to high NaCl concentration present in the brine added during moromi fermentation. The ion-exchange between the Na^{+} ions present in the medium and Ca^{2+} ions which are binding with COO^{-} groups of the polyguluronate unit can result in the weakened of bead structure (Bajpai and Sharma, 2004). Once the Ca^{2+} ions diffuse out into the medium, alginate beads start to disintegrate and dissolve completely. Weakening of beads followed by dissolution can also occur due to the production of lactic acid during fermentation, since lactic acid can compete with alginate for the binding of Ca^{2+} (Voo et al., 2011). As an alternative, encapsulation of *Z. rouxii* in polyethylene-oxide gel was found to be more stable in high salt concentration compared to alginate gel (van der Sluis et al., 2000). However, the production of these polymers is time consuming, costly and it introduces ingredients which are not compatible with the composition of soy sauce.

Alginate concentration has been reported to highly affect the physical stability of alginate beads (Bhujbal et al., 2014). Furthermore, coating the alginate beads with a polycation layer, such as chitosan (1,4-linked-2-amino-2-deoxy-β-d-glucan; Figure 3.2), can also enhance its physical stability against chelator (e.g. phosphate, lactate, or citrate) or non-gelling cations (e.g. Na^{+} or Mg^{+} ions) (Gåserød et al., 1999). Since alginate is anionic and chitosan is cationic, the negatively charged carboxylic acid groups of alginate can ionically bind with positively charged amino groups of chitosan, allowing polyelectrolyte complex of alginate and chitosan to be formed (Liouni et al., 2008; Takahashi et al.1990). Thus, this study aimed to investigate the effect of different

concentrations of alginate and chitosan as a coating agent, as well as multilayered beads, on the overall stability of alginate beads in the presence of high NaCl

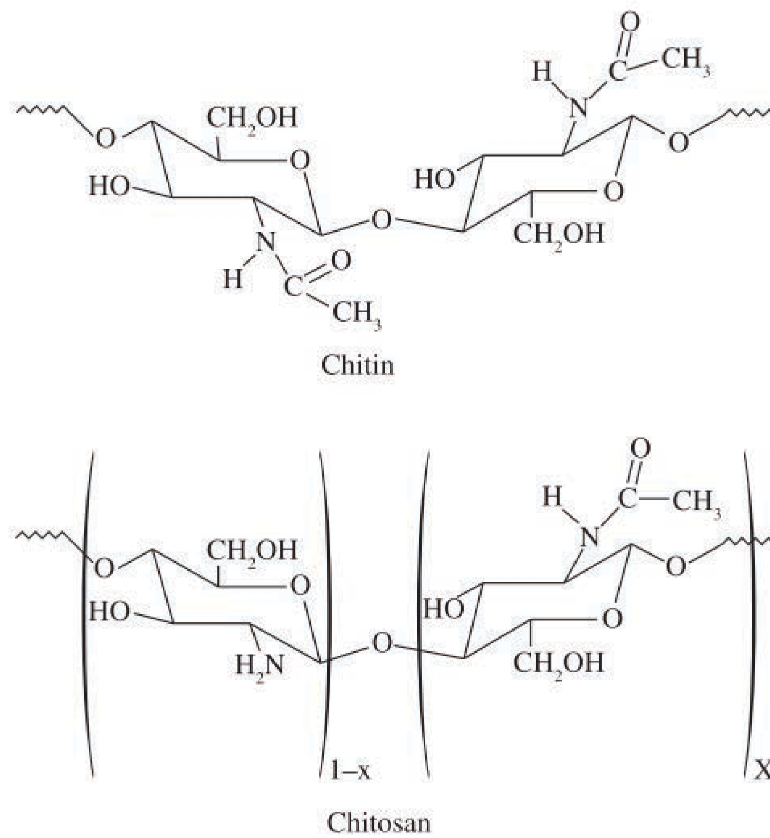


Figure 3.2 Chemical structure of chitin and chitosan (Goy, de Britto, & Assis, 2009).

concentration. The effect of encapsulation on *Z. rouxii* growth and glucose uptake was also assayed.

3.2 Materials and methods

3.2.1 Materials

In addition to materials that have been described in section 2.2.1, sodium alginate (high in d-mannuronic acid content, Sigma-Aldrich, UK), calcium chloride ($CaCl_2$, Oxoid Ltd., UK), chitosan (medium molecular weight, 75 – 85% deacetylated, Sigma-Aldrich, UK), citric acid (Sigma-Aldrich, UK), dibasic sodium phosphate (Sigma-Aldrich, UK), and acetic acid (Sigma-Aldrich, UK) have been used.

3.2.2 *Z. rouxii* encapsulation in alginate

Z. rouxii cells were incubated in YM broth for 24 h at 30 °C with gentle shaking at 100 rpm. The cell suspension (10 mL) was centrifuged at 1122 rcf for 10 min to separate the cells from the broth. After washing twice, the cells were suspended in 1 mL of sterile deionized water and transferred into 9 mL of sterile sodium alginate solutions of varying concentration: 4% w/v and 8% w/v (Table 3.1). The mixture was drawn using a sterile 1 mL syringe and then added dropwise into 50 mL of $CaCl_2$ solution (2% w/v and 4% w/v; Table 3.1) through a 0.11 mm needle under constant stirring at room temperature. Then, the beads were left to solidify in the $CaCl_2$ solution for 30 min with gentle stirring.

Table 3.1 Formulations of alginate beads

Sample	Concentrations of sodium alginate (%w/v)	Concentrations of $CaCl_2$ solution (%w/v)
C2A4	4.0	2.0
C4A4	4.0	4.0
C2A8	8.0	2.0
C4A8	8.0	4.0

3.2.3 Alginate beads coating with chitosan

Chitosan solution was prepared by dissolving chitosan in 1% w/v acetic acid at a concentration of 0.5% and 2% w/v, followed by overnight stirring. Then, the chitosan solution was sterilized by autoclaving at 121°C for 15 minutes. Alginate beads prepared with 4% w/v sodium alginate cross-linked in 2% w/v $CaCl_2$ solution were used as control. Chitosan-coated alginate beads were prepared by immersing the alginate beads in chitosan solutions at different concentrations (0.5% and 2% w/v; Table 4.2), with constant stirring for 1 h. The chitosan-coated beads were washed with sterile distilled water before use. Alginate-chitosan-alginate (ACA) multilayered beads were prepared using the same technique, except that the chitosan concentration used was 0.15% w/v (Table 3.2). After coating with chitosan, the resulting beads were then immersed in 0.15% w/v sodium alginate solution for 30 min to counteract charges on the membranes.

Table 3.2 Formulations of chitosan-coated alginate beads

Sample	Concentrations of sodium alginate (%w/v)		Concentrations of $CaCl_2$ solution (%w/v)	Concentrations of chitosan solution (%w/v)
	1st layer	2nd layer		
AC0	4	n.a	2	n.a
AC0.5				0.5
AC2				2
ACA		0.15		0.5

3.2.4 Antimicrobial activity of chitosan

The effect of chitosan on *Z. rouxii* cell viability was tested by transferring 1 mL of cell suspension into 9 mL of chitosan solution at concentrations of 0%, 0.1%, 0.2% and 0.5% w/v. The effect of chitosan on the encapsulated cell viability was also investigated by incubating the encapsulated cells in 0.1% and 0.5% w/v chitosan solution. Samples were taken at 0, 10, and 30 min and the viable cell count was conducted as described in section 3.2.6. In the case of encapsulated cells, 1 g of alginate beads were dissolved in 9 ml of citrate-phosphate buffer (0.1 M, pH 7.0) prior to plate counting.

3.2.5 Measurement of beads size and encapsulation efficiency of alginate beads

Beads size measurement: The size of ten beads from every formulation were measured using a micrometer screw gauge with an accuracy of ±0.01 mm.

Encapsulation efficiency: In order to determine the encapsulation efficiency (%), the encapsulated *Z. rouxii* cells were first released from the beads. One gram of beads was dissolved in 9 ml of citrate-phosphate buffer (0.1 M, pH 7.0) with gentle shaking at room temperature. The number of yeast cells was determined using the plate count method

as previously described in section 2.2.6, and expressed as CFU/mL. The encapsulation efficiency (%) was calculated using the formula:

$$Encapsulation\ efficiency\ (\%) = \frac{N}{N_0} \times 100 \qquad (1)$$

where N is the number of viable encapsulated cells released from the beads and N_0 is the number of free cells initially loaded into the beads.

3.2.6 Measurement of cell release under high salt conditions

Beads prepared with different formulations were incubated in NaCl solution at various concentrations (0%, 5%, 10% w/v) for 72 h at 30 °C with agitation. Samples were taken at 1, 2, 4, 6, 24, and 72 h and subjected to cell counting using a Nageotte cell counting chamber under optical microscope (20x magnification). The concentration of released cells (cell/mL) was calculated using the formula:

Cell concentration (cell/mL) = (Total number of cells x 25 x 10^4)/Number of squares (2)

3.2.7 Statistical analysis

Each measurement was conducted in two independent experiments. The data were subjected to one-way analysis of variance (ANOVA) as previously described in section 2.2.9.

3.3 Results and discussion

3.3.1 Bead size

The beads produced based on different formulations were characterized by measuring their size and encapsulation efficiency. The concentration of alginate has been reported as the most important factor that determines the size, shape, and encapsulation efficiency of the beads (Lotfipour et al., 2012). In this study, the bead size was significantly ($p<0.05$) affected by the concentration of alginate. However, there was no significant difference by varying the concentration of $CaCl_2$ solution ($p>0.05$). The size ranged from 1.89 ± 0.03 to 2.12 ± 0.01 mm as shown in Figure 3.3. The bead size increased with increasing the concentration of alginate which was in agreement with a study by Lotfipour et al. (2012). It was suggested that increasing the concentration of alginate resulted in an increase of the alginate solution viscosity, which led to the formation of bigger beads by extrusion. Meanwhile, $CaCl_2$ had no effect on the bead size which could be due to the type of alginate used in this study, which was rich in d-mannuronic acid (M) content. Generally, divalent cations such as Ca^{2+} bind preferentially to the monomer of l-guluronic (G). Therefore, beads made of polymer with low G content are less affected by the concentration of $CaCl_2$ (Lotfipour et al., 2012).

In the case of chitosan-coated beads, the bead size was significantly affected by the concentration of chitosan. The increasing amount of chitosan present during coating caused the alginate beads to shrink (Figure 3.4a). When another layer of alginate was applied on the chitosan surface, larger size of beads was obtained (Figure 3.4b), which was in agreement with Nualkaekul et al. (2012). The size of beads was also influenced by the coating time during the first 20 min of incubation in chitosan solution. Within

this period, the beads size decreased significantly ($p<0.05$) and then stabilized throughout the incubation period. The size reduction was higher with increasing the concentration of chitosan. There has been much dispute over the effect of chitosan on the size of alginate beads. Abbaszadeh et al. (2014) suggested that chitosan did not have significant effect on the size of alginate beads, while Krasaekoopt et al. (2004) reported significantly larger beads after additing a chitosan layer. These contrary results may result from the variation in the preparation procedure of alginate beads such as the alginate used (G/M content), needle size, distance between syringe and gelling bath, and flow rate. The molecular weight, deacetylation degree, concentration, and pH of chitosan used could also affect the beads properties. In the present study, low pH of the chitosan solution might have caused the alginate to shrink during coating process. Unlike in other studies, the pH of chitosan solution was not adjusted to 5.7 – 6 with NaOH after the addition of 1% (w/v) acetic acid solution. According to Takka and Gürel (2010), alginate shrinks in low pH and dissolves in high pH.

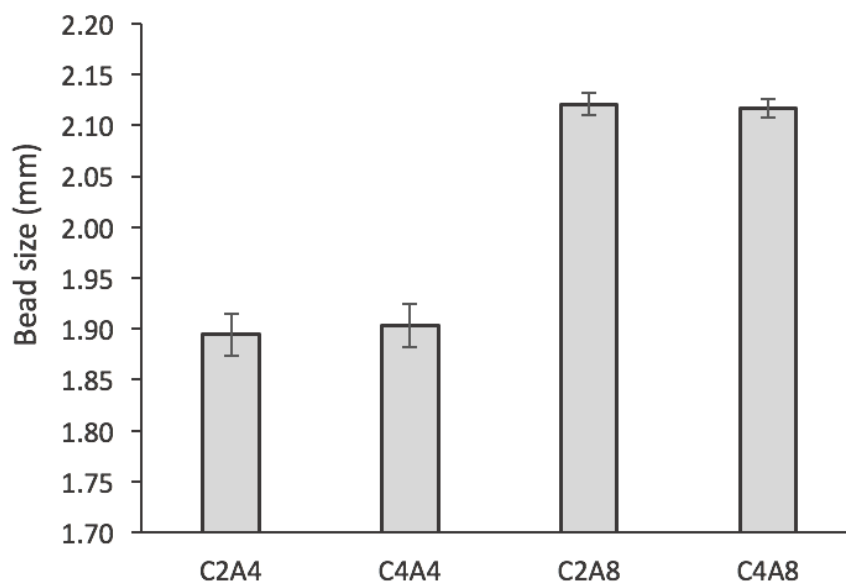


Figure 3.3 Size of beads prepared with varying concentrations of alginate and $CaCl_2$.

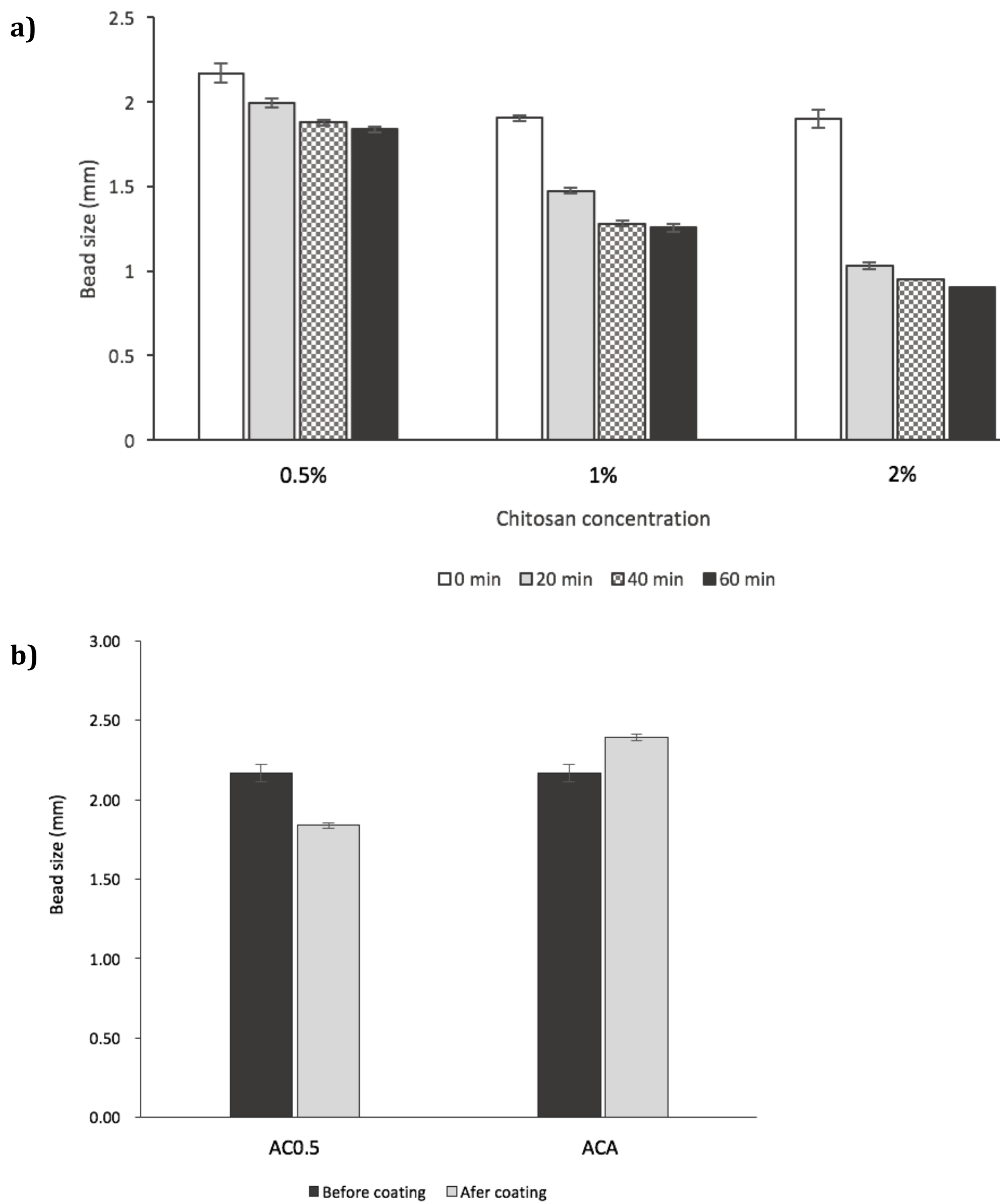


Figure 3.4 Size of alginate beads. (a) Beads coated with varying concentrations of chitosan with different coating time. (b) Beads prepared with multilayer (alginate-chitosan-alginate).

3.3.2 Encapsulation efficiency

The encapsulation efficiency was not significantly ($p<0.05$) affected by the concentration of alginate and $CaCl_2$. As shown in Figure 3.5a, more than 98% cells were successfully encapsulated within the beads prepared from all formulations. Since these numbers were obtained by counting the viable cells number, it was also indicated the ability to encapsulate cells without considerable loss in cells viability due to mild conditions used during preparation. In contrast to our study, Lotfipour et al., (2012) have reported that alginate concentration is the most important factor that affects the encapsulation efficiency of alginate beads with direct effect. Encapsulation efficiency increases by increasing the concentration of alginate as the structure becomes stiffer.

The addition of chitosan layer on the surface of alginate beads was found to have negative impact on *Z. rouxii* cells viability. After the coating process, no growth was observed on the agar plate. The antimicrobial activity of chitosan has been reported in many studies. It is influenced by many factors which can be classified into 4 categories as follow: (i) microbial factors, such as microbial species and cell age, (ii) intrinsic factors of chitosan, such as positive charge density, molecular weight, concentration, hydrophilicity/hydrophobicity, and chelatic capacity, (iii) physical state of chitosan, whether it is in water-soluble or solid state, (iv) environmental factors, such as ionic strength of the medium, pH, temperature, and reaction time (Kong et al., 2010). In the present study, chitosan might have killed or only hindered the growth of *Z. rouxii* cells. According to a review article by Goy et al. (2009), chitosan is more likely to be considered as bacteriostatic (inhibit bacterial growth, but does not imply whether the bacteria are killed or not) rather than bactericidal (kill the live bacteria). Although the exact mechanism is unknown, the most commonly proposed mechanism of

antimicrobial activity is the interaction between positively charged chitosan molecules and negatively charged microbial cell membranes (Goy et al., 2009; Zheng and Zhu, 2003). This could lead to the formation of a coating over the cell which prevents nutrients from entering the cell. Such interaction could also lead to cell death due to the leakage of proteinaceious and other intracellular constituents (Rabea et al., 2003). Since various factors and modes of action could play role in the antimicrobial activity of chitosan, further investigation is needed in order to gain better understanding of the chitosan effect on *Z. rouxii* cells.

Since no growth was observed on the agar plate, the encapsulation efficiency was measured by counting the encapsulated cells using haemocytometer, instead of using total plate count method. The results showed that chitosan had a greater influence on the encapsulation efficiency compared to alginate. The encapsulation efficiency dramatically decreased to < 35% after the coating process, regardless the concentration of chitosan solution used (Figure 3.5b). The loss in the number of entrapped cells might have resulted from the beads shrinkage during immersion in chitosan solution. Due to loss in cells viability and encapsulation efficiency, the chitosan-alginate formulations were not investigated further in subsequent studies.

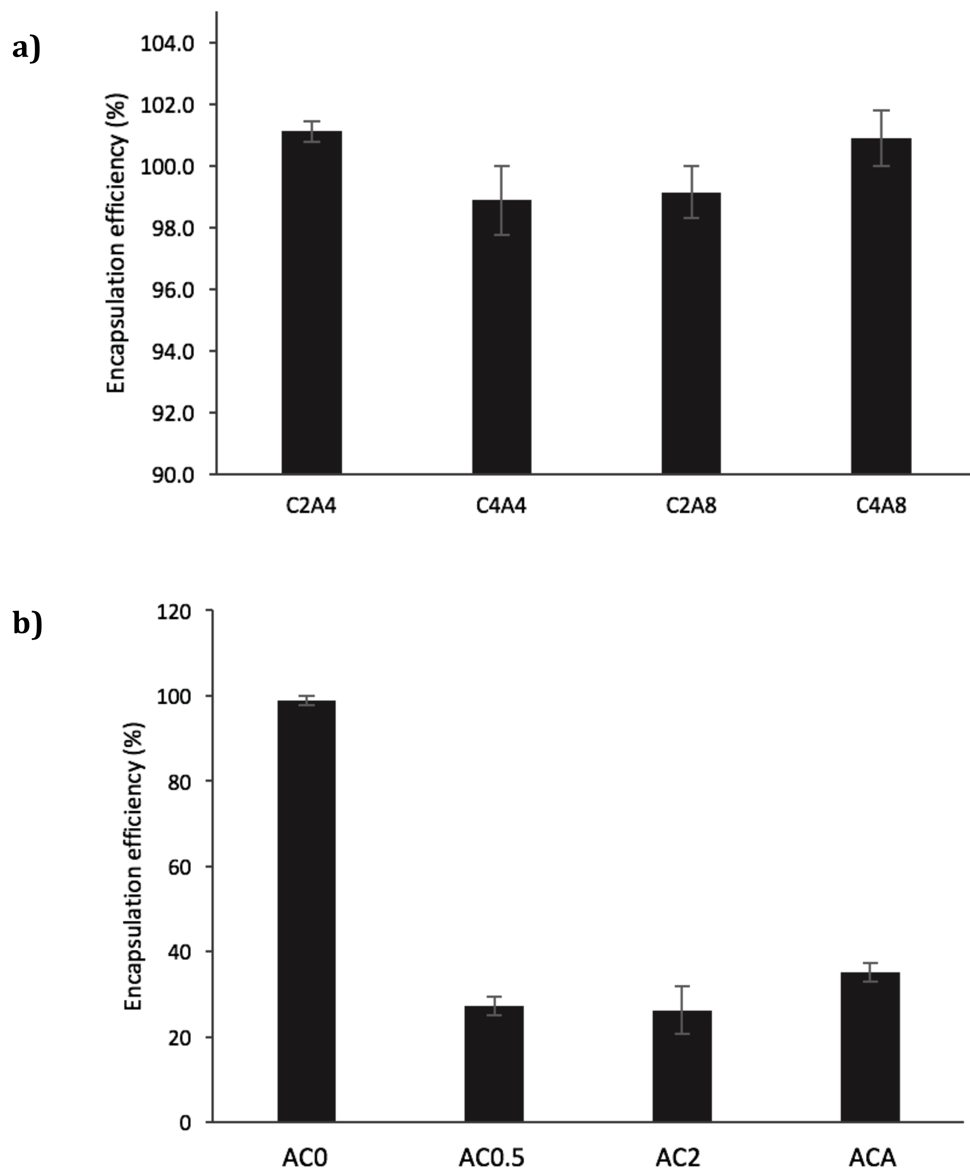


Figure 3.5 Encapsulation efficiency of the alginate beads. (a) Beads prepared with varying concentrations of alginate and $CaCl_2$. (b) Beads coated with multiple layers (alginate-chitosan-alginate) and single layer of chitosan of different concentrations.

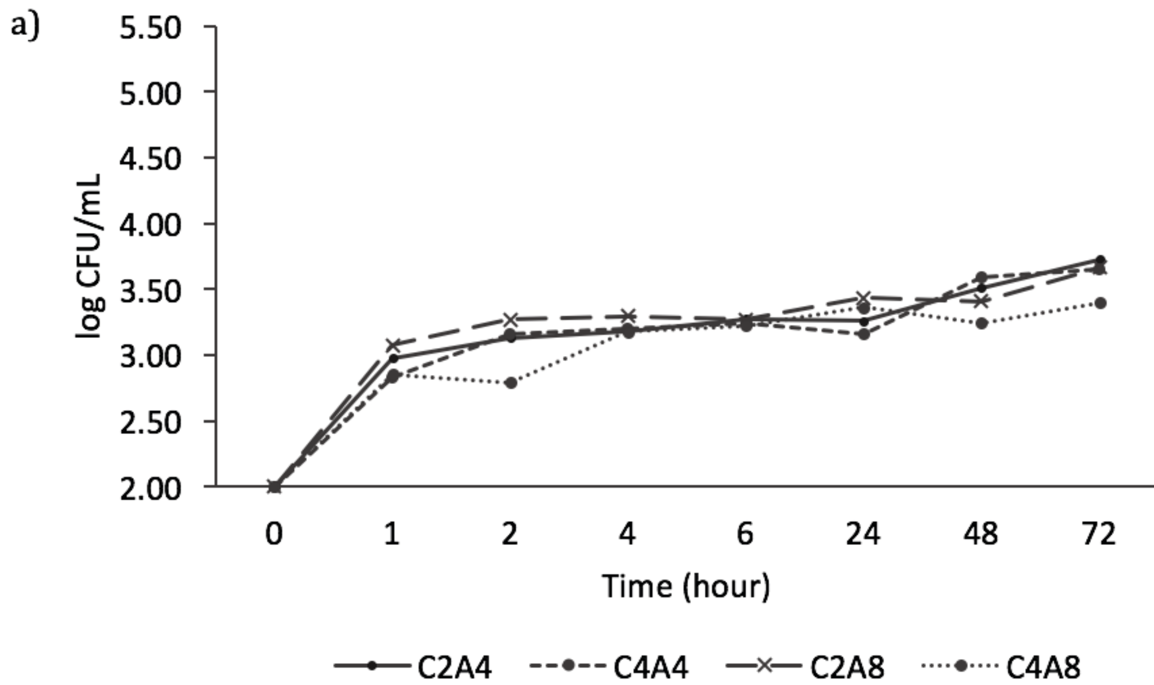
3.3.3 The effect of NaCl concentration on cell release

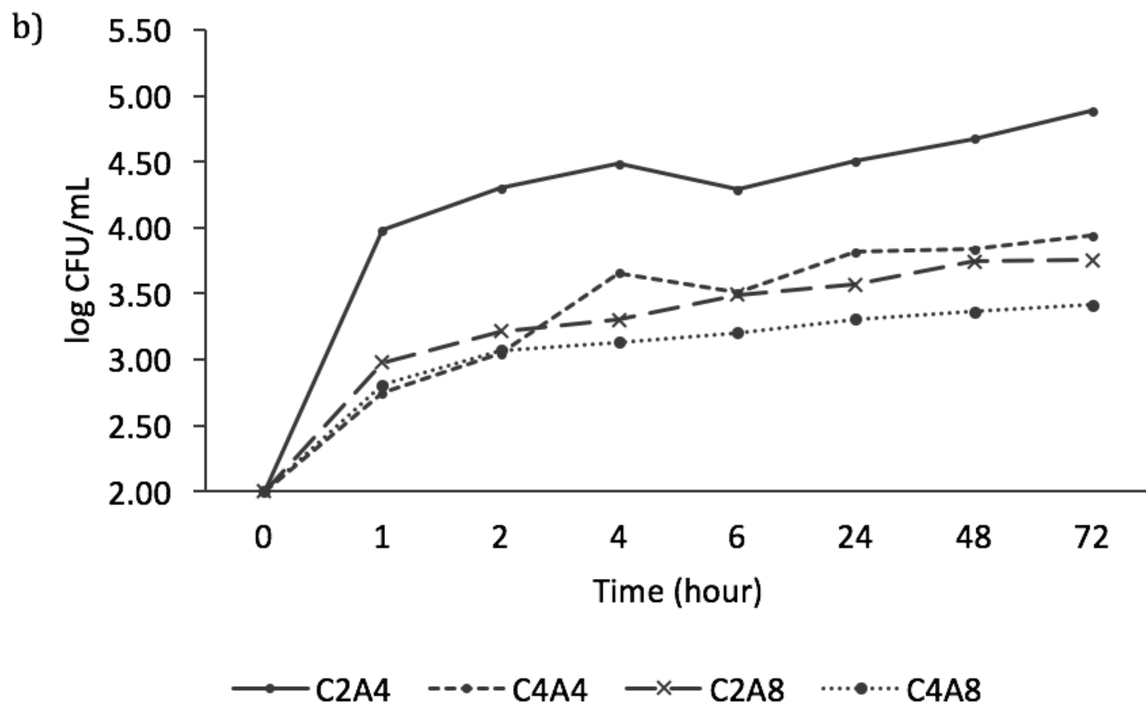
The release of yeast cells from the beads was found to be affected by NaCl in the medium, and it was proportional to the concentration of NaCl (Figure 3.6). The presence of NaCl caused the beads to disintegrate, resulting in the release of cells into the medium. Similar results were also observed in a study by Kebbouche-Gana et al. (2013), in which the alginate beads became increasingly less formed when NaCl concentration was increased (10 – 30%). However, when alginate concentration was increased (6 – 10%), the alginate beads could form perfect spherical shape. In the present study, increasing the concentration of alginate and $CaCl_2$ during hardening process was shown to reduce the amount of cells released due to beads disintegration (Figure 3.6).

The presence of NaCl in the medium can cause alginate beads to swell due to water uptake, which becomes higher with increasing amount of NaCl. Once reaching maximum degree of swelling, the beads start to show weight loss and finally dissolve completely. Ion-exchange phenomenon between Na^+ and Ca^{2+} ions has been reported to be responsible for the swelling process and beads breakage (Bajpai and Sharma, 2004). Ion exchange between Na^+ ions present in the medium and Ca^{2+} ions which bound with the COO^- groups mainly in the polymannuronate sequences, can generate electrostatic repulsion among negatively charged COO^- groups. This can cause chain relaxation and gel swelling along with water uptake. Furthermore, when ion exchange occurs between Na^+ and Ca^{2+} ions bound with polyguluronate, the egg-box structure becomes loose. As Ca^{2+} ions diffuse out into the medium, alginate beads start to disintegrate and dissolve.

As an alternative to alginate, van der Sluis et al. (2001a) used polyethylene oxide gel for encapsulating yeast during moromi fermentation in a stirred-tank bioreactor with high NaCl concentration (12.5 – 17%). The polyethylene oxide gel was found to be

more stable against abrasion compared to alginate. However, this only lasted for several days and the particle size increased during 14 days of fermentation, causing the particle to stick together on the sensor, thus interfering with process control. Further study is needed to enable such system to work in a long-term continuous process.





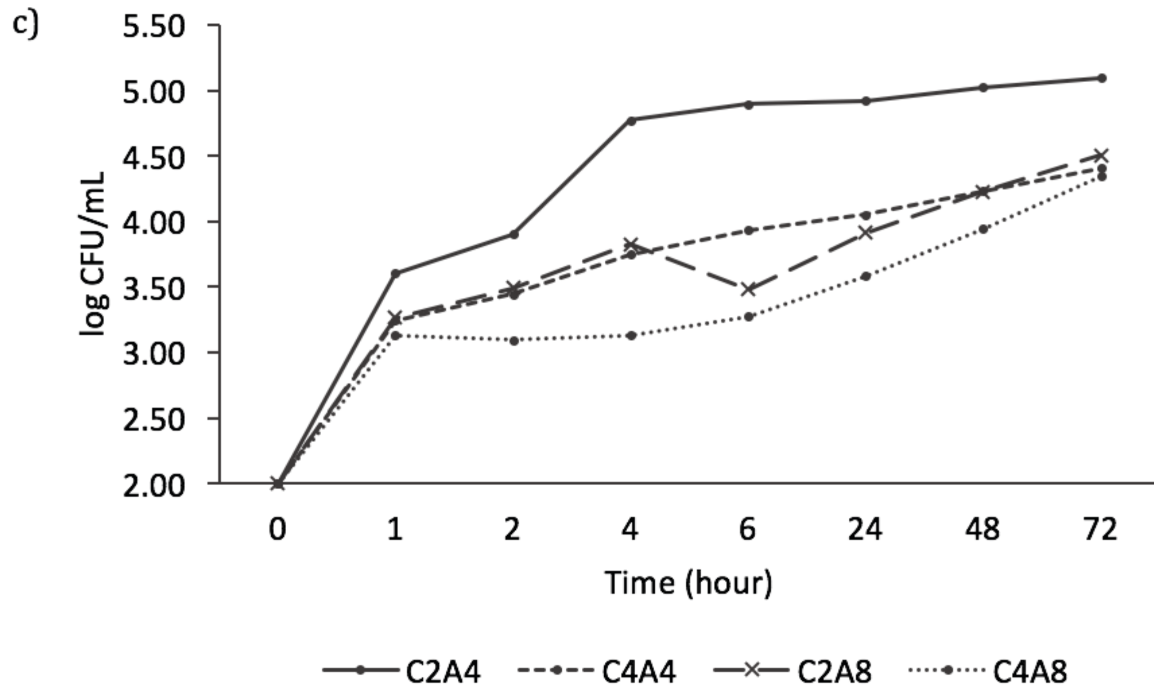


Figure 3.6 Release profile of *Z. rouxii* cells in solutions containing (a) 0% NaCl, (b) 5% NaCl, and (c) 10% NaCl.

3.4 Conclusion

The results in this study indicated that the alginate and chitosan-coated alginate were not suitable for *Z. rouxii* cells encapsulation in the presence of high NaCl concentration as such condition is required in soy sauce fermentation process. The improved stability resulted from increasing the alginate concentration was not sufficient as the beads started to disintegrate, accompanied by cell release within few days, which was not possible to be applied in soy sauce fermentation process which takes a longer duration. The addition of chitosan layer on the alginate bead surface was also not possible since it caused cell death. Due to all these reasons, alginate was not investigated further in this study for encapsulating *Z. rouxii* cells.

Chapter 4

Segregation of *Tetragenococcus halophilus* and *Zygosaccharomyces rouxii* using water-in-oil-in-water double emulsions for use in mixed culture fermentation

4.1 Background

In this chapter, DEs were investigated as an alternative encapsulation method for minimizing antagonism between *T. halophilus* and *Z. rouxii* during moromi fermentation. DEs are a type of emulsion that contains two aqueous compartments separated by an oil phase, creating a multi-compartmentalized structure that could be used for delivering multiple starter cultures during fermentation, when minimum interference between species is required. Segregation of multiple microbial species was previously studied by Nissen et al. (2003) and Kemsawasd et al. (2015) during mixed culture fermentation using dialysis tubing and double-compartment fermentation system separated by cellulose membranes, respectively. Such compartmentalization was shown to reduce antagonism caused by cell-to-cell contact and antimicrobial peptides secretion. Moreover, stable mixed culture of *Lactococcus lactis* and *Bifidobacterium longum* was obtained during continuous fermentation in two-reactor

system by separately immobilizing the two strains in κ-carrageenan/locust bean gum gel beads (Doleyres et al., 2004).

DEs were previously reported for its ability to protect probiotic bacteria against adverse environment in human gastrointestinal tract (Shima et al., 2006; Pimentel-González et al., 2009; Rodríguez-Huezo et al., 2014) and the controlled release of microbial cells based on osmotic pressure imbalance (El Kadri et al., 2015, 2016). However, the segregation of antagonistic cultures has not been studied using conditions relevant to fermentation. Furthermore, previous studies on microbial encapsulation in DEs are limited to bacteria. Therefore, for DEs to be used in soy sauce fermentation, it is important to understand its stability under relevant conditions and effect on microbiological and physicochemical changes.

The aim of this study was to investigate the feasibility of DEs as a delivery system of soy sauce starter cultures, including its stability and release, effect on cell viability, and species-to-species interaction under conditions relevant to moromi stage of soy sauce fermentation. Brine solution and soybean oil were used as water and oil phases, respectively, in order to create a formulation that reflects the moromi process. The effects of varying concentrations of *Z. rouxii* in the W_1 phase and glucose in the W_2 phase on DEs stability and release profile were investigated and the survival of the encapsulated *Z. rouxii* was monitored over storage. Also, the interaction between *T. halophilus* and *Z. rouxii* was investigated by monitoring the microbiological and physicochemical changes of the culture medium.

4.2 Material and Methods

4.2.1 Materials

Soybean oil (Alfa Aesar, United Kingdom) was used as the oil phase of the DEs. Polysorbate80 (Tween80, Sigma-Aldrich, United Kingdom) and polyglycerol polyricinoleate (PGPR, Danisco, Denmark) was used as water and oil soluble emulsifiers, respectively. Sodium chloride (NaCl, extra pure) and D(+)-glucose were purchased from Acros Organics (United Kingdom). Acridine orange (AO) stain was purchased from Sigma-Aldrich (United Kingdom).

Microorganisms used in this study have been described in section 2.2.1. In addition to microbial growth medium described in section 2.2.1, Tryptic Soy Agar (TSA, Oxoid Ltd., United Kingdom) and Tryptic Soy Broth (TSB, Oxoid Ltd., United Kingdom) media were also used.

4.2.2 Microbial cultures preparation

Microorganisms used in this study have been described in section 2.2.1. In addition to microbial growth medium described in section 2.2.1, Tryptic Soy Agar (TSA, Oxoid Ltd., United Kingdom) and Tryptic Soy Broth (TSB, Oxoid Ltd., United Kingdom) media were also used.

4.2.3 The growth ability of *Z. rouxii* in glucose solution

Prior to investigation of *Z. rouxii* cells release under various concentrations of glucose, a preliminary test was done to ensure that the quantified cells were solely due to release from the W_1 to W_2 phase, not the result of microbial growth. Three types of cells were used in this study: (i) live cells, (ii) heat-killed cells (90°C for 1 h), and (iii)

live cells with the addition of natamycin. *Z. rouxii* cell suspension was inoculated into 100 mL of 5% w/v NaCl solution containing 10% glucose with a final cell concentration of ~10^7 cells/mL, followed by incubation for 7 days at 30 °C with gentle shaking at 100 rpm. Cell number was counted at day 0, 3, and 7 using total plate count method and haemocytometer (Nageotte cell counting chamber).

4.2.4 Effect of PGPR and Tween80 on *Z. rouxii* growth

In order to ensure that *Z. rouxii* did not use PGPR and Tween80 as carbon sources to grow, *Z. rouxii* was incubated in 10% NaCl solution with and without PGPR and Tween80. *Z. rouxii* was inoculated into four different 10% NaCl solutions with a final cell concentration of ~10^7 cells/mL containing: (i) no PGPR or Tween80 (control), (ii) 0.4% PGPR, (iii) 2% PGPR, and (iv) 1% Tween80. The inoculated flasks were incubated for 7 days at 30 °C with gentle shaking at 100 rpm. Cell viability was monitored at day 0, 1, and 7 using total plate count method.

4.2.5 DEs preparation

The DEs were prepared using the 2-step emulsification method at ambient temperature by using a high shear mixer (Silverson L5M). In the first step, W_1/O primary emulsion was prepared by mixing sterile 10% (w/v) NaCl solution into the oil phase (soybean oil with 2% wt PGPR) at W_1 : oil phase ratio of 20 : 80 at 1700 rpm for 2 min. For yeast encapsulation, *Z. rouxii* suspensions in 10% (w/v) NaCl solution (10^8 cells/mL and 10^6 cells/mL) were used as W_1.

In the second stage, W_1/O was re-emulsified in the continuous phase (W_2; sterile 10% (w/v) NaCl in water with 1 % wt Tween80) at 2000 rpm for 1 min (W_1/O : W_2 ratio of 20 : 80). In order to study the effect of glucose on the stability of DEs and *Z.*

rouxii release profile, various concentrations of glucose (0%, 6%, 12%, and 30% w/v) were added to the W_2 in addition to 8.05% (w/v) NaCl (Table 4.1). The osmotic pressure gradient was calculated using Van't Hoff equation as follows:

$$\Delta\pi = (C_i - C_e)RT \qquad (1)$$

where C_i is the solute concentration in the internal W_1 phase, C_e is the solute concentration in the external W_2 phase, R is the ideal gas constant, and T is the absolute temperature.

DEs containing *T. halophilus* in the W_2 were prepared by directly adding 2 mL of *T. halophilus* (10^6 cells/mL) into the W_2 after mixing process. For the study that investigates the effects of DEs on *T. halophillus* and *Z. rouxii* interaction, both microorganisms in DEs or as free cells (as a single or mixed cultures) were transferred into double concentrated TSB supplemented with 10% w/v NaCl and 12% w/v glucose. *T. halophillus* and *Z. rouxii* were inoculated at final concentrations of 10^6 CFU/mL and 10^5 CFU/mL, respectively, followed by incubation in 30 °C static incubator for 30 days.

Table 4.1 Formulation of $W_1/O/W_2$ double emulsions (DEs) with varying glucose concentrations in the W_2 phase and directions of osmotic pressure gradients.

NaCl		Glucose		$\Delta\pi$ (atm)	Molar concentration of solute
W_1	W_2	W_1	W_2		
10%	8.05%	0%	0%	16.54	$W_1 > W_2$
			6%	8.28	$W_1 > W_2$
			12%	0	$W_1 = W_2$
			30%	-24.84	$W_1 < W_2$

4.2.6 DEs stability characterization

Oil globule size measurement: The volume mean diameter (D 4,3) and particle size distribution of the DEs were determined using Mastersizer 2000 (Malvern Instruments Ltd., Malvern, Worcestershire, UK) equipped with a He–Ne laser (λ = 633 nm). The analysis was done for the freshly prepared DEs and as a function of storage time. The dispersion unit stirring speed was maintained at 2000 rpm and the measurement range was 0.02–2000 μm. The refractive index for the soybean oil and water were set at 1.474 and 1.330, respectively. The measurement was run at concentrations corresponding to obscuration of 10-20%.

Creaming volume measurement: The cream volume of DEs after preparation and during storage were monitored as described by El Kadri et al. (2015). Briefly, after gentle mixing, 1 mL sample was collected using 1 mL syringe and left standing upright for 1 h until the cream layer is formed on the top. The creaming volume percentage was calculated as follows:

$$\textit{Creaming volume (\%)} = \textit{(Creaming layer volume/Total volume of DEs)} \times 100\% \qquad (2)$$

Microscopy observation: DEs microstructure was observed by placing the samples onto microscope slides followed by observation under a light microscope (Olympus BX50) with a 10x objective lens. Images were taken using Moticam 10 camera via Motic Images Plus video acquisition software at 17fps.

In order to track the entrapped cells during storage, *Z. rouxii* cells were stained with AO before the entrapment process. Samples were placed onto microscope slides

and gently covered with cover slips and imaged using Zeiss Axioplan fluorescent microscope equipped with objective lens 40x magnification at ambient temperature. Images were captured using digital color camera system Motic Moticam 10 using a 10 megapixel CMOC camera via Motic Images Plus video acquisition software.

4.2.7 Determination of the encapsulation efficiency and encapsulation stability of DEs

Encapsulation efficiency is defined as the percentage of *Z. rouxii* cells that are entrapped in the W_1 immediately after the emulsification process while encapsulation stability is described as the percentage of *Z. rouxii* cell that remains entrapped in the W_1 during storage.

The encapsulation efficiency and encapsulation stability were determined by counting the number of the non-encapsulated *Z. rouxii* cells in the serum phase (W_2). Five millilitre sample of DEs was collected and serum phase was removed using syringe. Cells were counted using Nageotte cell counting chamber under optical microscope (20x magnification). Cell concentration (cell/mL) was calculated using this following formula:

$$\textit{Cell concentration (cell/mL)} = (\textit{Total number of cells} \times 25 \times 10^4)/\textit{Number of squares} \quad (3)$$

Encapsulation efficiency (EE) and encapsulation stability (ES) were determined using the following equations:

$$\textit{Encapsulation efficiency (\%)} = ((N_0 - N_{w2})/N_0) \times 100\% \quad (4)$$

$$\textit{Encapsulation stability (\%)} = ((N_0 - N_{w2(t)})/N_0) \times 100\% \quad (5)$$

where N_0 is the number of free *Z. rouxii* cells initially added in the inner phase, while N_{w2} and $N_{w2(t)}$ are the number of non-encapsulated *Z. rouxii* cells measured immediately after DEs were formed and as a function of storage time, respectively.

4.2.8 *T. halophilus* and *Z. rouxii* cell enumeration

Viable cell counts were made by taking 0.1 mL of samples subjected to serial dilution in PBS (phosphate buffered saline) buffer solution followed by plating on BHI agar supplemented with 7% (w/v) NaCl and 21.6 mg/L natamycin for *T. halophilus* and YM agar with the addition of 5% (w/v) NaCl and 100 mg/L chloramphenicol for *Z. rouxii*. Bacteria and yeast colonies were counted after 2 days of incubation at 30 °C.

4.2.9 Physicochemical changes

Glucose concentration was measured using Accu-chek Aviva glucose monitor with Accu-chek Aviva glucose test strips (Roche Diagnostics, United Kingdom). Lactic acid was analyzed using enzymatic assay kit (Megazyme, International Ireland Ltd., Ireland) according to the manufacturer instructions. Acetic acid and ethanol were determined using gas chromatography (GC).

GC analysis was performed using GC-2010 (Shimadzu, Japan), equipped with a flame ionization detector (FID). Prior to analysis, samples were filtrated through 0.22 μm pore size filter (Millex GP, Millipore, United Kingdom) and 300 μL of samples were added with 200 μL hexylene glycol (Sigma Aldrich, United Kingdom) as an internal standard at final concentration of 742 mg/L. Samples (1 μL) were injected using auto sampler with split ratio of 100:1 at 260 °C. Compound separation was done by using ZB-WAX plus column (30 m, 0.25 mm I.D., 0.25 μm film thickness, Phenomenex, United

States) and helium as the carrier gas at a pressure of 104.99 kPa. The oven temperature was programmed at an initial temperature of 30 °C for 5 min, followed by an increase to 50 °C at 4 °C/min (held for 5 min), 150 °C at 20 °C/min (held for 5 min), 200 °C at 10 °C/min (held for 5 min), and finally increase to 220 °C at 4 °C/min. FID temperature was set to 300 °C.

4.2.10 Statistical analysis

Each measurement was conducted in triplicate (N = 3) in two independent experiments and the results were expressed as mean ± standard deviation. Significant differences among means were tested by one-way analysis of variances (ANOVA) using IBM SPSS Statistics Software version 21 at $p < 0.05$ and Tukey's test was applied for means comparison.

4.3 Results and discussion

4.3.1 Encapsulation efficiency and stability during storage

The amount of encapsulated *Z. rouxii* cells was monitored over storage (Figure 4.1). *Z. rouxii* was successfully encapsulated in the internal W_1 phase of DEs with high encapsulation efficiency (>99%; Figure 4.1a) regardless of low (10^5 CFU/mL) or high (10^7 CFU/mL) cell concentrations. Relatively high encapsulation stability of DEs was maintained up to 14 days of storage (>75%), and significantly ($p<0.05$) decreased at day 30 to 13.28% and 30.72%, for low and high cell concentration, respectively. This observation was associated with the fluorescence microscopy images (Figure 4.1b-d) in which non-encapsulated cells were observable in the external W_2 phase at day 30.

Furthermore, the stability of DEs decreased over time regardless of the presence and amount of encapsulated cells, as indicated by the loss in inner W_1 phase after 30 days (Figure 4.2a). Such time dependent loss of inner W_1 phase could occur due to coalescence between the W_1 droplets as well as coalescence between W_1 droplets and the oil globule's interface (Chávez-Páez et al., 2012). Ficheux et al. (1998) found that Tween80 migrates from the oil globule's interface through the oil phase to the W_1 droplet's interface and displaces the lipophilic surfactant (Span 80) molecules which causes an increase in coalescence events between the W_1 droplet and the oil globule's interface leading to DEs to become a single O/W emulsion. In this study, such coalescence events may have occurred resulting in the release of hydrophilic substances including *Z. rouxii* cells into the W_2 phase. Although the amount of W_1 phase decreased, size distribution (Figure 4.2b) and the average size (Figure 4.2c) of the oil globules were apparently preserved throughout storage and this might be attributed to coalescence occurring between the W_1 droplets and the oil globule's interface as well as between the oil globules.

Such coalescence events have shown to increase the size of the interfacial film of the oil globules despite loss in the W_1 phase maintaining the oil globule's size (Ficheux et al., 1998). These results indicate the possibility to use such inherent instability of DEs as a mechanism for the release of *Z. rouxii* cells during fermentation.

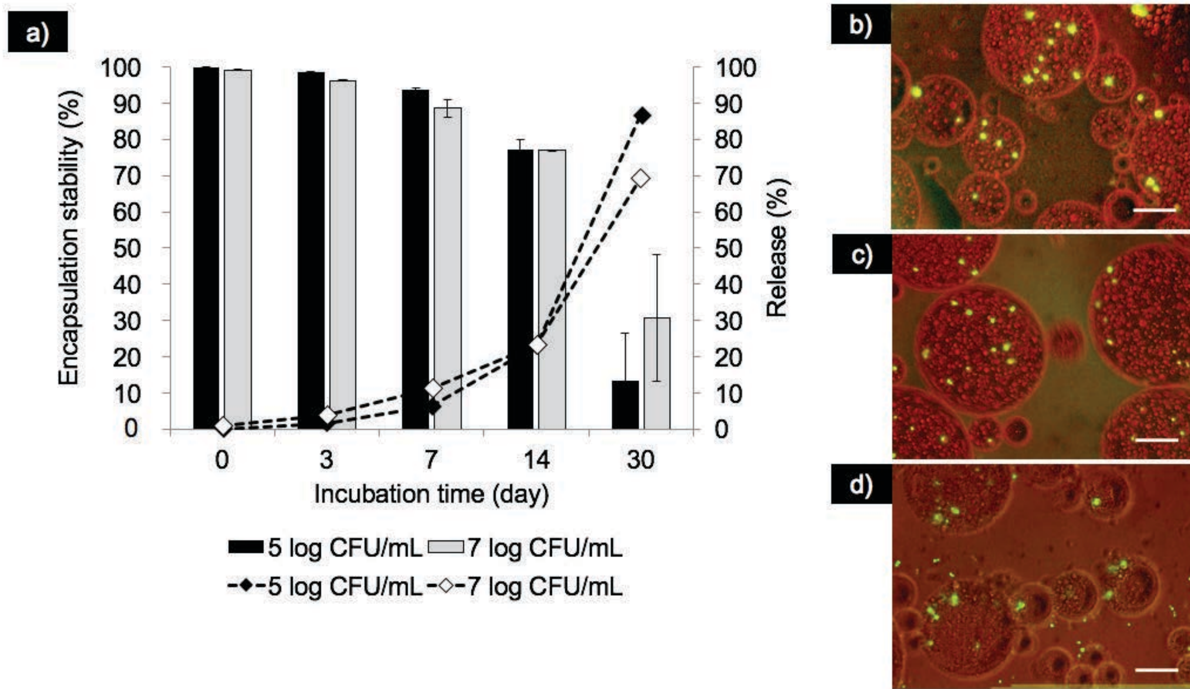


Figure 4.1 (a) Changes in the percentage of entrapped (bar chart) and released (line chart) *Z. rouxii* cells in DEs prepared under iso-osmotic conditions over 30 days of storage at 30 °C. (b) Fluorescence microscopy images of the entrapped *Z. rouxii* cells at day 0, (c) day 7, and (d) day 30. Scale bar: 100 μm.

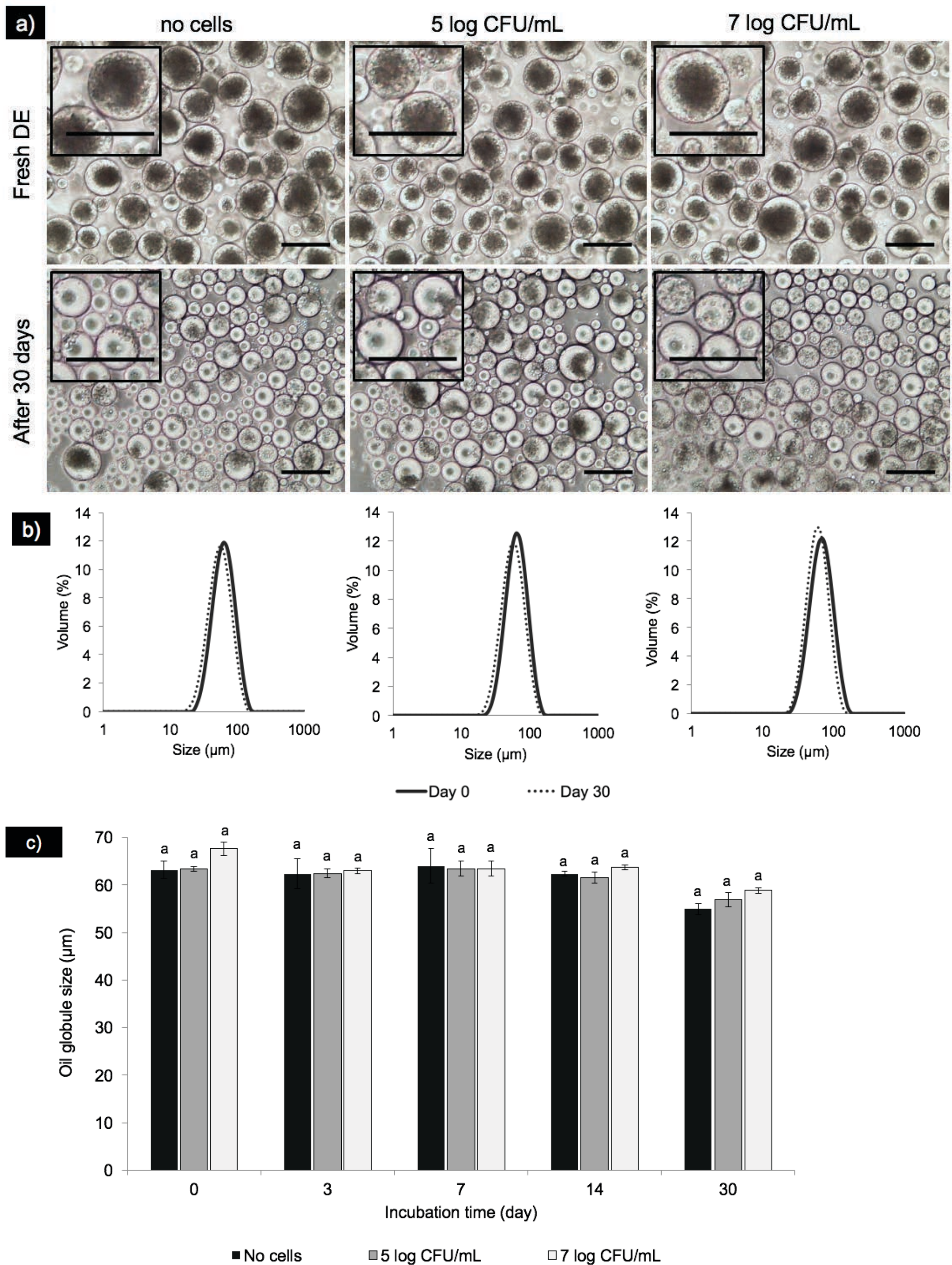


Figure 4.2 DEs with no cells, 5 log CFU/mL, and 7 log CFU/mL before and after 30 days of storage at 30 °C under iso-osmotic condition. (a) Optical micrographs; (b) Oil globule size distribution; (c) Average oil globule size. Scale bar: 100 μm. Mean values with same letters are not significantly different ($p<0.05$).

4.3.2 The effect of glucose concentration on cell release and DEs stability

During moromi fermentation of soy sauce, *Z. rouxii* converts glucose into biomass and ethanol. Changes in glucose concentration would alter the osmotic pressure balance between the two phases of DEs, therefore affecting its microstructure and encapsulation stability. For this reason, the microstructure of DEs (Figure 4.3a), *Z. rouxii* cells release profile (Figure 4.3b), and the oil globule size (Figure 4.4a-c), were monitored by varying glucose concentration (0%, 6%, 12%, and 30%) in the external W_2 phase, which created osmotic pressure gradient between W_1 and W_2 phase, except for 12% which was designed to have balanced osmotic pressure (Table 4.1). Prior to investigation, the ability of *Z. rouxii* to grow in glucose solution (10%) in the absence of other nutrients was tested and the viable cells decreased by 2.53 log CFU/mL after 7 days of incubation (Figure A1). This aimed to ensure that the quantified cells during the release study were solely due to release from the W_1 to W_2 phase and not the result of microbial growth.

The release profile was found to be influenced by the amount of glucose in the W_2 phase (Figure 4.3b) and it followed a similar pattern to the loss in the W_1 phase (Figure 4.3a), by which the complete loss in the W_1 phase was observed when maximum cell release occurred. However, the DEs instability and cell release rate were found to be driven by increasing amount of glucose, rather than the osmotic pressure difference between the two phases. In the presence of 30% glucose ($\Delta\pi$ = -24.84 atm), the DEs were transformed into O/W single emulsion due to complete loss of the inner W_1 phase within 3 days, accompanied with a sharp increase in the number of released cells which was followed by a plateau thereafter. Meanwhile, the release of *Z. rouxii* cells in 6% glucose was gradual throughout storage and took place in a manner comparable

to control (0% glucose). The destabilization of DE containing 0% and 6% glucose was reduced as the oil globules maintaining their inner W_1 phase were still noticeable by the end of storage. Although DEs with 12% glucose were designed to be osmotically balanced ($\Delta\pi$ = 0 atm), the DEs microstructure was found to be more unstable compared to DEs with 0% ($\Delta\pi$ = 16.54 atm) and 6% glucose ($\Delta\pi$ = 8.28 atm) as they were transformed into O/W single emulsion by the end of storage. This also resulted in higher amount of cell release compared to DEs with 0% and 6% glucose. These results suggest that the faster release of *Z. rouxii* was associated with increased destabilization of the DEs.

The excess amount of lipophilic emulsifier can increase the flux of water through reverse micelles formation (Dickinson, 2011). In the present study, glucose might also behave as lipophilic emulsifier facilitating water movement to the external W_2 phase through reverse micellar transport, which eventually led to release of *Z. rouxii* cells since yeast cells are hydrophilic and therefore would preferentially reside within the aqueous W_2 phase and not the oil phase. Furthermore, the release of *Z. rouxii* cells might also be driven by bursting mechanism. According to El Kadri et al. (2016, 2015), osmotic pressure balance alteration can lead to oil globule bursting which can be used to modulate the release of bacterial cells. However, the release of *Z. rouxii* cells from DEs might involve not one but various mechanism and further investigation is required for a better understanding on how *Z. rouxii* cells are being released.

DEs prepared with the highest concentration of glucose (30%) in the W_2 phase possessed the lowest initial oil globule size (37.28 ± 0.74 μm; Figure 5.4a), even though the mixing speed and conditions during the two-step homogenizing process was maintained for all the formulations. This is expected as the addition of glucose increases

the viscosity of the W_2 phase which leads to smaller oil globules to form (Khalid et al., 2013).

Furthermore, it has been reported that glucose can further reduce the interfacial tension which also contributes to the observed reduction in oil globule size (Pawlik et al., 2010). Decrease in size of oil globule during storage occurred in cases of 0%, 6%, and 12% glucose although these responses were not statistically significant ($p<0.05$) (Figure 5.4a). In contrast, DEs containing 30% glucose showed significant ($P<0.05$) increase in oil globule size at day 3 which then stabilized until the end of storage period, although the oil globules lost their inner W_1. This can be attributed to the increase in coalescence events between the oil globules as it becomes less stable in the presence of glucose. These results show that the stability of DEs and release of *Z. rouxii* are influenced by the glucose concentration regardless of the osmotic pressure gradient between the two phases. However, the responses do not follow the same direction or linearity in all cases.

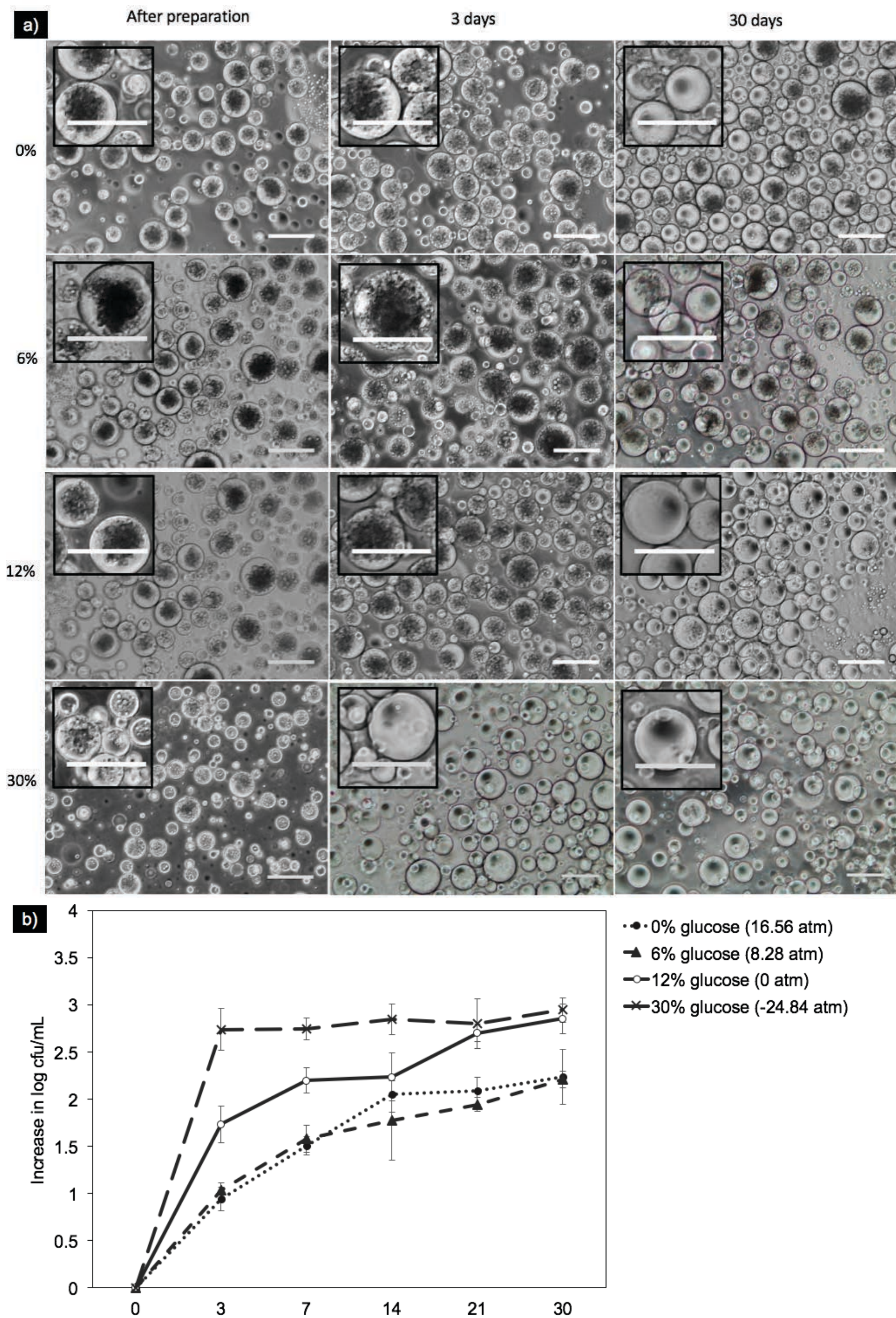


Figure 4.3 Double emulsion with 0%, 6%, 12%, and 30% glucose in the W_2 phase after preparation, 3, and 30 days of storage at 30 °C. (a) Optical micrographs of $W_1/O/W_2$. (b) *Z. rouxii* cell release profile into W_2 phase. Scale bar: 100 μm.

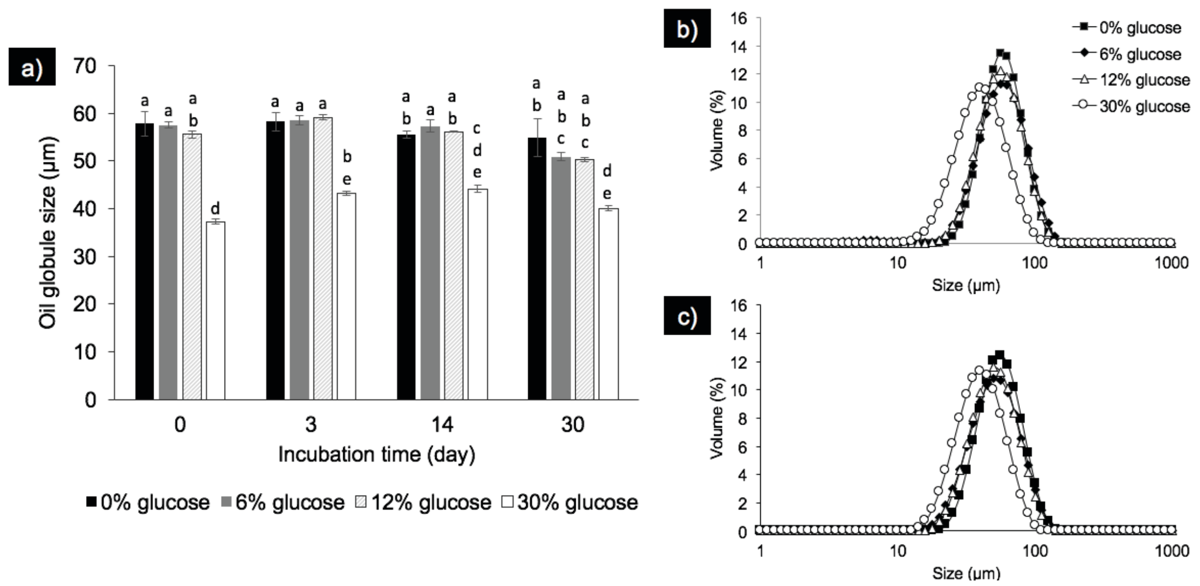


Figure 4.4 DEs before and after 30 days of storage at 30 °C with different glucose concentrations in the external W_2 phase. (a) Average oil globule size; (b) Oil globule size distribution of DEs before storage and (c) after storage. Mean values with different letters are significantly different ($p<0.05$).

4.3.3 *Z. rouxii* cell viability after emulsification and during storage

To investigate the effect of emulsification and encapsulation on survival of *Z. rouxii*, cell viability was assessed immediately after encapsulation and during storage. The relative viability of *Z. rouxii* cells soon after the emulsification process was ~100% (Figure 4.5), showing that the encapsulation technique as well as the surfactants used did not affect the yeast. This was reported in other studies for bacterial cells (El Kadri et al., 2015; Shima et al., 2006). Interestingly, the encapsulated cells viability remained high during 30 days of storage in the absence of nutrient (~2 log CFU/mL decrease), while no viable cells were detected in non-encapsulated cells by the end of incubation period (Figure 4.5).

The oil layer which functions as a barrier, might reduce mass transport and biological communication between the *Z. rouxii* cells and the environment and thus result in molecular gradient that could switch cells to the non-dividing resting state (G0) (Wang et al., 2008). Furthermore, the cells resistance towards environmental stress increases once it enters the resting state, including the ability to survive extended periods of starvation (Herman, 2002). It could be argued that *Z. rouxii* may have utilized the surfactants (PGPR and Tween80) as carbon sources (Luh, 1995), thus enabling the yeast to grow. However, no growth was observed when *Z. rouxii* was incubated with PGPR or Tween80 only (Figure A2). These results indicate that encapsulation in DEs is able to prolong life of *Z. rouxii* in the absence of nutrients.

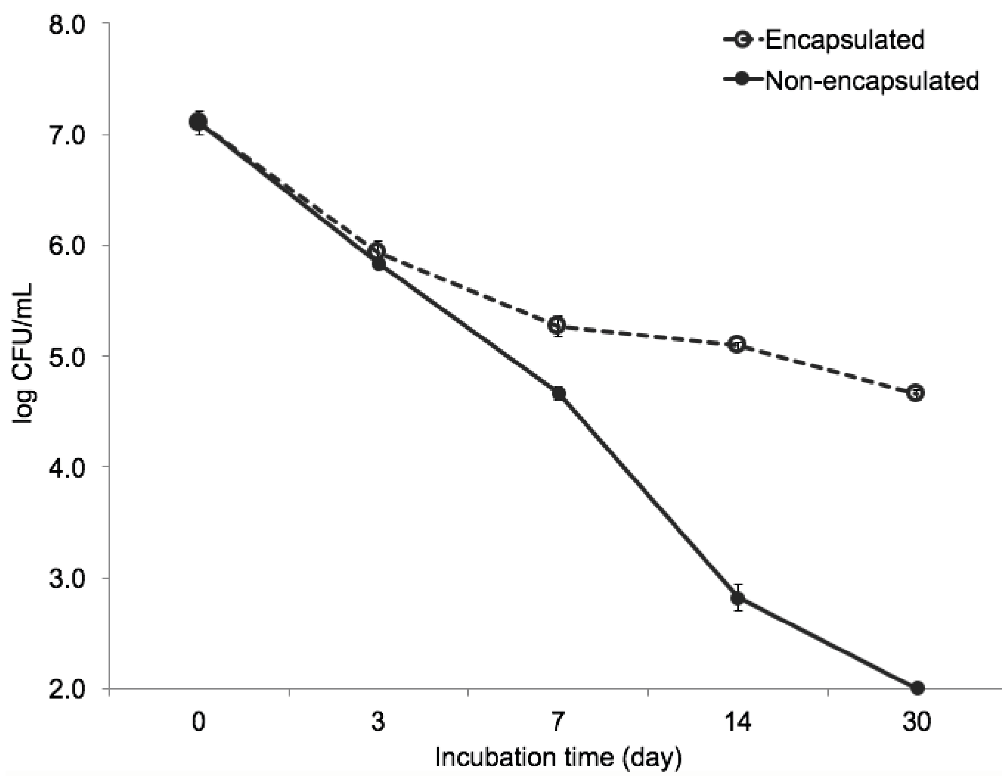


Figure 4.5 Changes in *Z. rouxii* cells viability over 30 days of storage at 30°C.

4.3.4 The effects of encapsulation on *T. halophilus* and *Z. rouxii* interactions.

Interaction between microbial species during fermentation would influence their growth which further affects the proportion of microbial population and their metabolic activity. In this study, the co-presence of *T. halophilus* and *Z. rouxii* resulted in antagonism as *T. halophilus* growth was inhibited, as indicated by a sharp decrease in *T. halophilus* cell count to undetectable level (< 2 log CFU/mL) at day 15 (Figure 4.6c). This observation was in contrast to the previous study described in Chapter 2 in which the growth inhibition was observed on *Z. rouxii* instead of *T. halophilus*, when both were co-present in a moromi model system. According to a study by Noda et al (1980), metabolite produced by *Pediococcus halophilus* (later reclassified as *T. halophilus*) during moromi fermentation can inhibit the growth of osmophilic *shoyu* yeasts such as *Saccharomyces rouxii* (later reclassified as *Z. rouxii*) and *Torulopsis versatilis*. However, a study by Inamori, Miyauchi, Uchida, & Yoshino (1984) showed that the growth inhibition in mixed cultures could occur to *P. halophilus* under aerobic conditions (without a covered liquid paraffin) or *S. rouxii* under anaerobic conditions (with a covered liquid paraffin) in static culture. Growth inhibition of *T. halophilus* in this study was possibly due to the aerobic conditions used during incubation. Also, inhibitory effect towards *Z. rouxii* which was previously described in Chapter 2 and a study by Noda et al. (1980), was observed in a digested liquid mixture of pre-cooked soybean and roasted wheat, while in this study interaction assay was performed in a synthetic broth medium. Furthermore, the presence of acetic acid in this study was unlikely to cause growth inhibition on *Z. rouxii* as previously reported (Noda et al., 1982; Kusumegi et al., 1998). It was suggested that acetic acid could interfere with proton expulsive activity of *Z. rouxii* for its halo-tolerance mechanisms, causing growth inhibition at NaCl

concentration above 10%. In this study, we did not observe any decrease in *Z. rouxii* cells population which was possibly due to relatively low NaCl concentration (10%) used in the medium.

The compartmentalization of *T. halophilus* and *Z. rouxii* in DEs affected the growth kinetics in both single and co-culture. The growth of *T. halophilus* (Figure 4.6a) and *Z. rouxii* (Figure 4.6b) as single culture was slightly enhanced and the antagonism between *T. halophilus* and *Z. rouxii* was no longer observed when *Z. rouxii* was encapsulated in DEs (Figure 4.6d). *T. halophilus* was able to propagate steadily throughout the incubation period, reaching a final count of 7.23 log CFU/mL (Figure 4.6d). The final cell counts of *Z. rouxii* in DEs (6.87 log CFU/mL) did not differ significantly ($p<0.05$) from non-DEs culture (6.72 log CFU/mL), although a different growth pattern was observed, and its growth was not affected by the presence of *T. halophilus* in the W_2 phase. The oil layer functions as a physical barrier separating *T. halophilus* from *Z. rouxii*, thus minimizing antagonistic interaction between them. Also, the oil layer could serve as a selective membrane, allowing chemicals or molecules to diffuse in or out based on their molecular weight (Zhang et al., 2013). In this study, deleterious metabolite compounds produced by *Z. rouxii*, might not be able to pass through the oil layer to the bulk medium (W_2 phase) due to its molecular weight and lipophilicity, thus minimizing its harmful effects toward *T. halophilus*. The ability of DEs to gradually release the *Z. rouxii* into the bulk medium might also prevent inhibitory effects toward *T. halophilus*. However, high *Z. rouxii* cell population was observed in the bulk medium due to their propagation after being released and yet the inhibitory effect towards *T. halophilus* was absent. *T. halophilus* might have exhibited physiological

changes in the presence of DEs, increasing its tolerance against inhibitory effect of *Z. rouxii*.

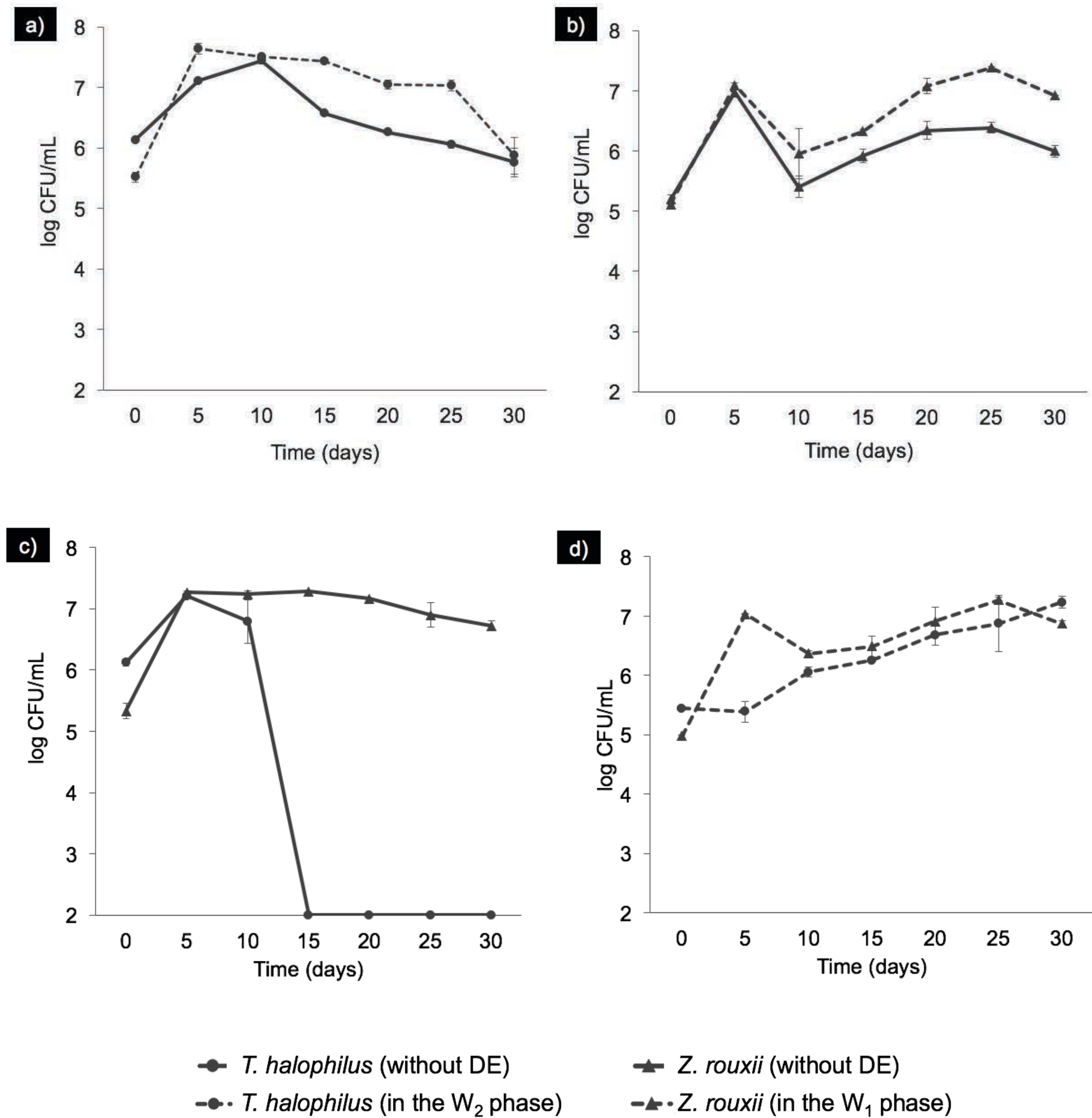


Figure 4.6 Changes in viable cell number of (a) *T. halophilus*, (b) *Z. rouxii*, and mixed culture (c) without and (d) with DE, during 30 days of incubation at 30°C.

4.3.5 Physicochemical changes in DEs during *T. halophilus* and *Z. rouxii* growth

To further understand how the presence of DEs with single or mixed cultures can affect the interaction between the two microorganisms the physicochemical changes during fermentation were monitored. As seen in Figures 5.7a-d, the presence of DEs caused alteration in the metabolic activity of both microorganisms as single or mixed cultures.

Glucose consumption (Figure 4.7a) was correlated with ethanol production (Figure 4.7b). Glucose was exclusively consumed by *Z. rouxii*, therefore, ethanol was only produced in its presence. Both glucose consumption and ethanol production were accelerated when *Z. rouxii* was encapsulated. With mixed cultures, glucose was consumed in a gradual manner in the absence of DEs which was accompanied by a slow production of ethanol, reaching maximum concentration of 12.39 g/L at day 30. In contrast, glucose was consumed faster in DEs as it was depleted at day 10, associated with maximum ethanol production (27.94 g/L) which was comparable to concentrations in good quality soy sauce (Luh, 1995). Similar level of ethanol was also obtained in rapid fermentation of soy sauce described by Muramatsu, Sano, Uzuka, & Company (1993). Once glucose was depleted, the ethanol production was terminated and its concentration continuously decreased throughout the incubation period. Encapsulation seemed to delay glucose consumption by *Z. rouxii* as only half amount of glucose was consumed during the first 5 days when *Z. rouxii* was encapsulated. However, this led to prolonged ethanol production for up to 10 days, producing higher maximum concentration of ethanol (23.56 g/L) compared to non-encapsulated cells (19.57 g/L) with single culture.

T. halophilus played a major role in both acetic acid (Figure 4.7c) and lactic acid (Figure 4.7d) formation. In mixed culture, acetic acid concentration gradually decreased when *Z. rouxii* was non-encapsulated, while the acetic acid concentration sharply decreased within the first 10 days to 1.86 g/L when *Z. rouxii* was encapsulated. This was comparable to the amount of acetic acid found in top-graded bottled soy sauces in China (Xu, 1990). However, the acetic acid production by *T. halophilus* as single culture markedly increased by 1.7 fold in the presence of DEs although *T. halophilus* was non-encapsulated. In contrast, lactic acid production was suppressed when DEs were present, as the amount of lactic acid remained stable from day 5 onwards, and the suppression was more obvious in mixed cultures. The yield of lactic acid in the presence of DEs was about half of the bottled soy sauces in China (Xu, 1990). In contrast, lactic acid increased exponentially in all non-DEs systems, reaching almost twice the amount of lactic acid produced in the presence of DEs. The presence of DEs might have caused a shift in metabolic pathway of *T. halophilus* cells from homofermentative to heterofermentative, thus decreasing the lactic acid yields (Krishnan et al., 2001).

These results suggest that the presence of DEs affects the physicochemical changes during *T. halophilus* and *Z. rouxii* growth in both single and mixed culture. Changes in microbial cells morphology and physiology due to immobilization have been reported in several studies reviewed by Lacroix & Yildirim (2007), including increase in the production of insoluble exopolysaccharides (Bergmaier et al., 2005), lactic acid (Lamboley et al., 1999), as well as a shift in metabolic pathway from homofermentative to heterofermentative, resulting in decreased lactic acid production (Krishnan et al., 2001). The altered metabolic activity may have contributed to the elimination of antagonism by reducing the production of inhibitory metabolites or enhancing the

production of metabolites essential for *T. halophilus* growth by *Z. rouxii*, as well as enhancing cell adaptation towards changing environmental conditions. However, further investigation is required to understand how the presence of DEs affects the cells both in the W_1 and W_2 phase at the metabolic level.

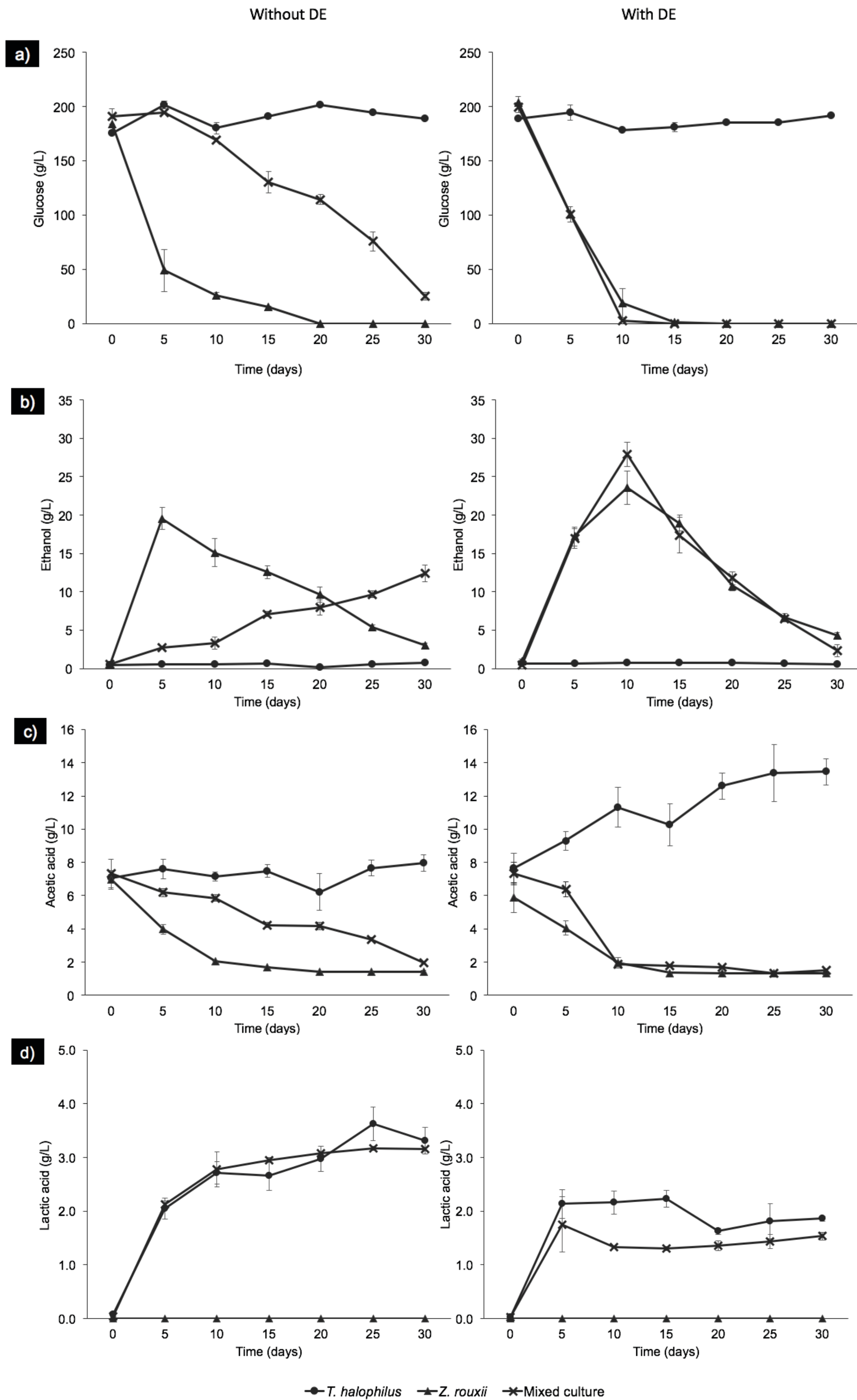


Figure 4.7 Changes in (a) glucose; (b) ethanol; (c) acetic acid; and (d) lactic acid; during fermentation with and without DE.

4.4 Conclusion

The results in this study suggest that DEs could be a suitable formulation for the delivery of mixed starter cultures in soy sauce fermentation. *Z. rouxii* was successfully encapsulated in DEs which enhanced survival during storage and eliminated antagonistic interaction with *T. halophilus*. The presence of DEs altered the metabolic activity of the two species, which could have contributed to the elimination of antagonism. Although the initial encapsulation efficiency was high, it decreased over time due to DEs instability and this could be utilized as a mechanism for gradual cell release depending on the glucose concentration in the W_2 phase. Such gradual release could be used to minimize the adverse effects of antagonistic interaction on cells. Furthermore, gradual release could also mimic the sequential inoculation described in chapter 2, which could result in more complex aroma formation during moromi fermentation. In conclusion, DEs could offer a valuable tool for standardizing the microbial activity and aroma development in soy sauce fermentation. However, further study is needed for these observations to be validated in real soy sauce fermentation.

Chapter 5

Water-in-oil-in-water double emulsions for the delivery of starter cultures in reduced-salt moromi fermentation of soy sauce

5.1 Background

As previously described in Chapter 2, the final aroma profile in moromi fermentation was affected due to antagonism between co-inoculated *T. halophilus* and *Z. rouxii.* However, inoculating them sequentially could improve the aroma complexity. Therefore, a formulation is needed to control the sequential delivery and activity of microbial cultures during the moromi stage. As discussed in Chapter 4, the inherent DEs instability could act as a mechanism for gradual release of *Z. rouxii* associated with changes in glucose concentration in the medium. It was demonstrated that DEs could minimize the antagonism between *T. halophilus* and *Z. rouxii* in TSB medium. Therefore, DEs could be a medium for encapsulating and controlling the release of *Z. rouxii* during moromi fermentation.

Low-salt soy sauce products have arisen in the market driven by the increasing consumer awareness of association of excessive sodium intake with hypertension, cardiovascular disease, and renal dysfunction. Furthermore, World Health Organization

(WHO) recommends a limitation of average daily intake of sodium to 2 g which is equivalent to 5 g of salts (WHO, 2012). On the other hand, the use of brine with high NaCl concentration in moromi fermentation is important to control the undesirable microorganisms and improve the flavor profile and texture of the final product (Song et al., 2015a). Therefore, the challenge is to produce low-salt soy sauce without compromising the quality characteristics and flavor production during fermentation.

Soy sauce production with reduced NaCl content have been investigated by following different approaches. Moromi fermentation in the absence of NaCl was possible by autolyzing koji under high temperature prior to fermentation (Muramatsu et al., 1993). However, the absence of salt during fermentation may not be sufficient to prevent the growth of spoilage microorganisms and the quality of the final product can differ from the original. Such problems could be counteracted by the addition of mixed cultures of indigenous yeast species (Song et al., 2015a) as well as combining LAB and yeasts (Singracha et al., 2017). However, this could result in antagonism as previously described in Chapter 2 and 4.

The application of sequential inoculation of mixed cultures to improve flavor quality of fermented foods and beverages has been reported in Chapter 2 as well as other studies. Modulation of the inoculation time was found to be the key in achieving the desired quality of apple cider (Ye et al., 2014). Furthermore, in whey fermentation, sequential inoculation of *Kluyveromyces lactis* B10 and *Torulaspora delbrueckii* B14 after 48 h was shown to improve aroma compounds production (e.g. alcohols and esters) (Andrade et al., 2017). Higher production of 3-sulfanylhexyl acetate (3SHA) and 3SH (3-sulfanylhexan-1-ol), which are the most important aromas in Sauvignon Blanc aroma, has been achieved with sequential culture of *T. delbrueckii* and *S. cerevisiae*.

In this chapter, the use of DEs for encapsulation and sequential delivery of *Z. rouxii* was tested in a moromi model system with reduced salt concentration. The stability of DE in moromi was examined by monitoring its microstructure, oil globules size, and distribution. Furthermore, microbial population and physicochemical changes as well as aroma compounds formation were monitored.

5.2 Material and Methods

5.2.1 Materials

All the materials that were used in this study were as described in section 2.2.1 and 4.2.1.

5.2.2 Microbial culture preparation

A. oryzae, Z. rouxii, and *T. halophilus* inoculums were prepared as previously described in section 2.2.2.

5.2.3 DEs preparation

The DEs were prepared using the 2-step emulsification method as previously described in section 4.2.3 with slight modification. In the first step, W_1/O primary emulsion was prepared by mixing sterile 6% (w/v) NaCl solution into the oil phase (soybean oil with 2% wt PGPR) at W_1 : oil phase ratio of 20 : 80 at 1700 rpm for 2 min. For yeast encapsulation, *Z. rouxii* suspension in 6% (w/v) NaCl solution (10^7 cells/mL) were used as W_1.

In the second stage, W_1/O was re-emulsified in the continuous phase (W_2; sterile 6% (w/v) NaCl in water with 1 % wt Tween80) at 2000 rpm for 1 min (W_1/O : W_2 ratio of 20 : 80). DEs containing *T. halophilus* in the W_2 were prepared by directly adding 2 mL of *T. halophilus* (10^6 cells/mL) into the W_2 after mixing process.

5.2.4 Soy sauce fermentation

Koji preparation: Koji was prepared according to method described in section 2.2.5.

Moromi preparation: Prior to moromi fermentation with DEs, the effect of moromi viscosity on DEs stability was tested. Moromi with different viscosity were prepared by varying the ratios of koji : brine. First, koji was mixed with 18% w/v NaCl solution with koji : brine ratio of 1 : 3, 1 : 5, and 1 : 7. DEs were then incorporated into the moromi, followed by incubation at 30 °C for 7 days in a static incubator.

Different concentrations of brine: 18% w/v NaCl; 6% w/v NaCl and 12% w/v KCl; 6% w/v NaCl, were added to the koji with ratio of 1 : 5 (koji : brine) to create moromi $A_{[18\%]}$, $B_{[6:12\%]}$, and $C_{[6\%]}$ respectively, followed by inoculation as shown in Figure 5.1. Moromi $A_{[18\%]}$ and $B_{[6:12\%]}$ were simultaneously inoculated with *T. halophilus* and *Z. rouxii*. Three different moromi $C_{[6\%]}$ were prepared according to the inoculation method of *Z. rouxii*. Moromi $C1_{[6\%]}$ was simultaneously inoculated with *T. halophilus and Z. rouxii*, while moromi $C2_{[6\%]}$ and $C3_{[6\%]}$ were inoculated with *Z. rouxii* after 1 week and 2 weeks, respectively. Moromi $C4_{[6\%]}$ was inoculated with *T. halophilus* and *Z. rouxii* which had been incorporated in the W_2 and W_1 phase, respectively, prior to inoculation. The inoculated moromi were then incubated at 30 °C for 4 weeks and samples were taken at

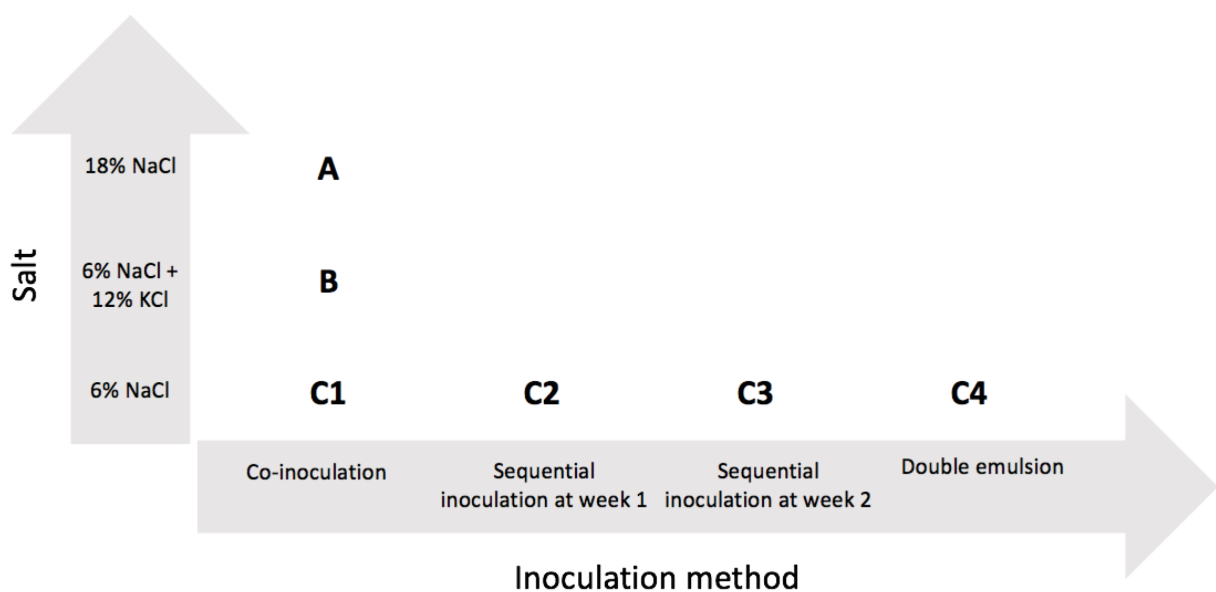


Figure 5.1 Set of moromi samples varying in salt composition and inoculation method.

week 0, 1, 2, 3, and 4. *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.

5.2.5 Rheological measurements

Rheological characterization of moromi was done by measuring the viscosity of koji mixed with varying concentrations of brine solution (18% NaCl w/v). The viscosity was measured for moromi containing koji : brine ratio of 1 : 3, 1 : 5, 1 : 7 and brine only at 30 °C using AR-G2 rheometer (TA instruments, New Castle, Delaware USA) on a parallel plate geometry (d: 40 mm). The apparent viscosity was measured over a shear rate range of 0.1–100 s^{-1}. Briefly, 1 mL of sample was placed between the cone and the plate, and measurement was started immediately. In total, 30 data points were recorded at 10 s intervals during the shearing. Shear stress was determined as a function of shear rate. Data was fitted to Power-law model (Barnes *et al*. 1989):

$$\eta = K \cdot \gamma^{n-1}$$

where; η refers to viscosity (Pa.s), K to consistency coefficient ($Pa.s^n$), γ to shear rate (s^{-1}), and n to flow behavior index (dimensionless).

5.2.6 Physicochemical analysis

Moromi samples were centrifuged at 15000 g for 15 min at ambient temperature. The supernatant regarded as raw soy sauce was transferred to microtubes and kept in -20 °C until analysis. Total reducing sugar (D-glucose and D-fructose), total lactic acid (L-lactic acid and D-lactic acid), ethanol, and L-glutamic acid were analyzed using enzymatic assay kit (Megazyme, International Ireland Ltd., Ireland) according to

the manufacturer instructions. Changes in pH were monitored using pH meter (SevenCompact S220, Mettler Toledo, Germany).

5.2.7 Aroma compound analysis (SPME GC-MS)

Aroma compounds extraction and analysis were conducted as previously described in section 2.2.8.

5.2.8 DE stability characterization

DE microstructure was observed as previously described in section 4.2.4. The oil droplet size distribution of DE was determined based on microscopy images using image analysis software (ImageJ) by measuring the diameter of at least 500 oil droplets from 3 different samples.

5.2.9 Statistical analysis

Microbial cell enumeration, physicochemical tests, and aroma compounds analysis were conducted in triplicate and repeated in two independent experiments. The results were presented as means ± standard deviation. Significant differences among means and multivariate analysis were done as previously described in section 2.2.9.

5.3 Results and discussion

5.3.1 The effect of viscosity on the stability of DEs in moromi

DEs were formulated using ingredients relevant to moromi constituents, including soybean oil, which was used as the oil phase. Since NaCl content in the moromi was reduced to 6% to reflect the exisiting low-salt soy sauce in the market, the internal W_1 and external W_2 phase of DE also contained 6% NaCl. This aimed to balance the osmotic pressure between the two phases, thus reducing instability of DEs due to water movement across the oil phase (Mezzenga et al., 2004). However, it is difficult to maintain the osmotic pressure balance during moromi fermentation, since moromi is a complex system that contains protein, glucose, and other molecules that also influence the osmotic pressure. Thus the destabilization of DEs could also be affected by other components in the moromi, including glucose as previously described in chapter 4.

Furthermore, the stability of DEs could also be affected by the viscosity of the W_2 phase (Oppermann et al., 2018). Increasing the viscosity of W_2 phase with thickener, such as pectin and xanthan, was found to promote the oil droplet breakup, which led to decrease in oil droplet size and yields. In order to describe relationship between the viscosity of moromi and DEs stability, moromi formulations with different viscosity were tested by varying the ratio of koji : brine (1 : 3, 1 : 5, and 1 : 7). The Power-Law model was used to describe the flow curves of the moromi. The rheological parameters of this model are presented in Table A3. All the moromi formulations exhibited non-Newtonian behavior at shear rates ranging between 0.1 and 100 s^{-1} at 30 °C (Figure 5.3a). Moreover, the plot of the viscosity against shear rate of the koji and brine mixtures yielded into a flow index n of less than 1 (shear thinning), indicating that their flow behavior had a non-Newtonian profile. Similar non-Newtonian behavior has been

reported for semi-solids of similar composition to koji which could be attributed to the presence of high molecular weight components, such as proteins or dextrin (Manohar et al., 1998).

DE maintained its microstructure after 4 weeks of fermentation (Figure 5.2a). However, the oil globule size significantly decreased from 27.88 μm to 11.40 μm (Figure 5.2b and 5.2c). This could be attributed to the high viscosity of the moromi system as previously reported in the study by (Oppermann et al., 2018). The viscosity increased when the amount of brine added was decreased (Figure 5.3a). After the incorporation into the moromi system, the DE stability was determined by observing its microstructure (i.e. inner W_1 phase) using microscopy and monitoring the oil globule size. The initial oil globule size (31.84 μm) decreased immediately after the incorporation into the moromi slurry and during storage (Figure 5.3b and 5.3c). However, the decrease in koji : brine 1 : 3 was more noticeable compared to those with higher fractions of brine. By the end of storage, the oil globule size of DE in koji : brine 1 : 3, 1 : 5, and 1 : 7 was 6.84 μm, 18.02 μm, and 15.29 μm, respectively. Moreover, all the oil globules in koji : brine 1 : 3 completely lost their inner phase, while in koji : brine 1 : 5 and 1 : 7, the DE structure was maintained (Figure 5.3d). This data indicates that DEs were destabilized in the moromi system. However, the destabilization was not proportional to the viscosity of the moromi. Apart from high viscosity, moromi with koji : brine 1 : 3 may also contain higher initial glucose concentration compared to the rest of samples. As previously discussed in chapter 4, increasing the concentration of glucose in the W_2 phase promotes destabilization of DEs.

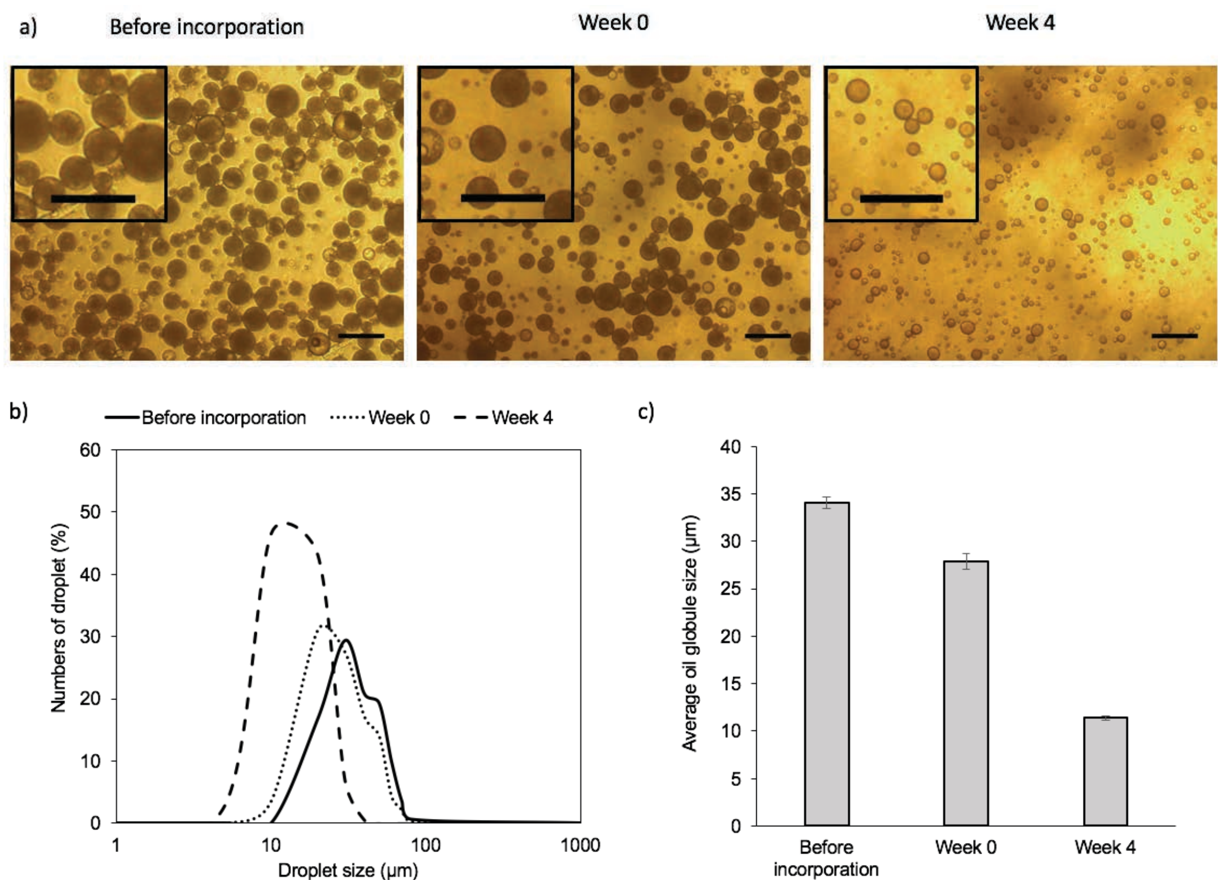
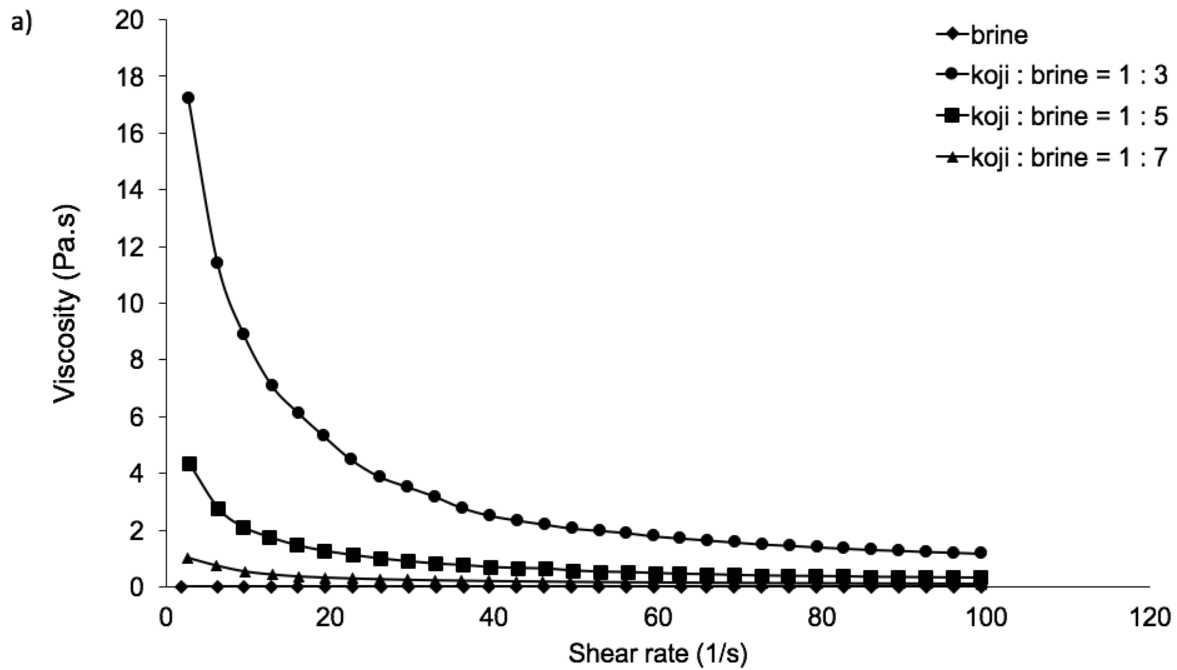
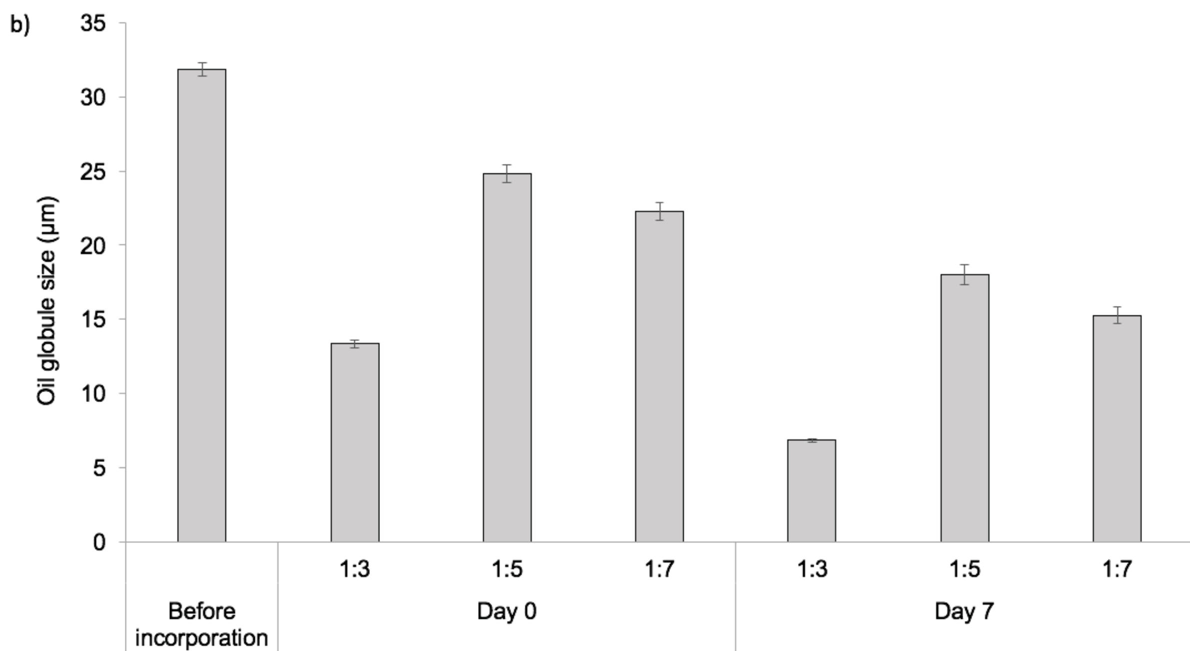


Figure 5.2 Optical micrograph of $W_1/O/W_2$ DE before and after incorporation into moromi, and after 4 weeks of fermentation. Scale bar: 100 µm. (b) Oil globule size distribution before and after fermentation. (c) Average oil globule size before and after fermentation.


a)
Viscosity (Pa.s)
Shear rate (1/s)
brine
koji : brine = 1 : 3
koji : brine = 1 : 5
koji : brine = 1 : 7
0
2
4
6
8
10
12
14
16
18
20
0
20
40
60
80
100
120


b)
Oil globule size (μm)
0
5
10
15
20
25
30
35
Before incorporation
1:3
1:5
1:7
Day 0
1:3
1:5
1:7
Day 7

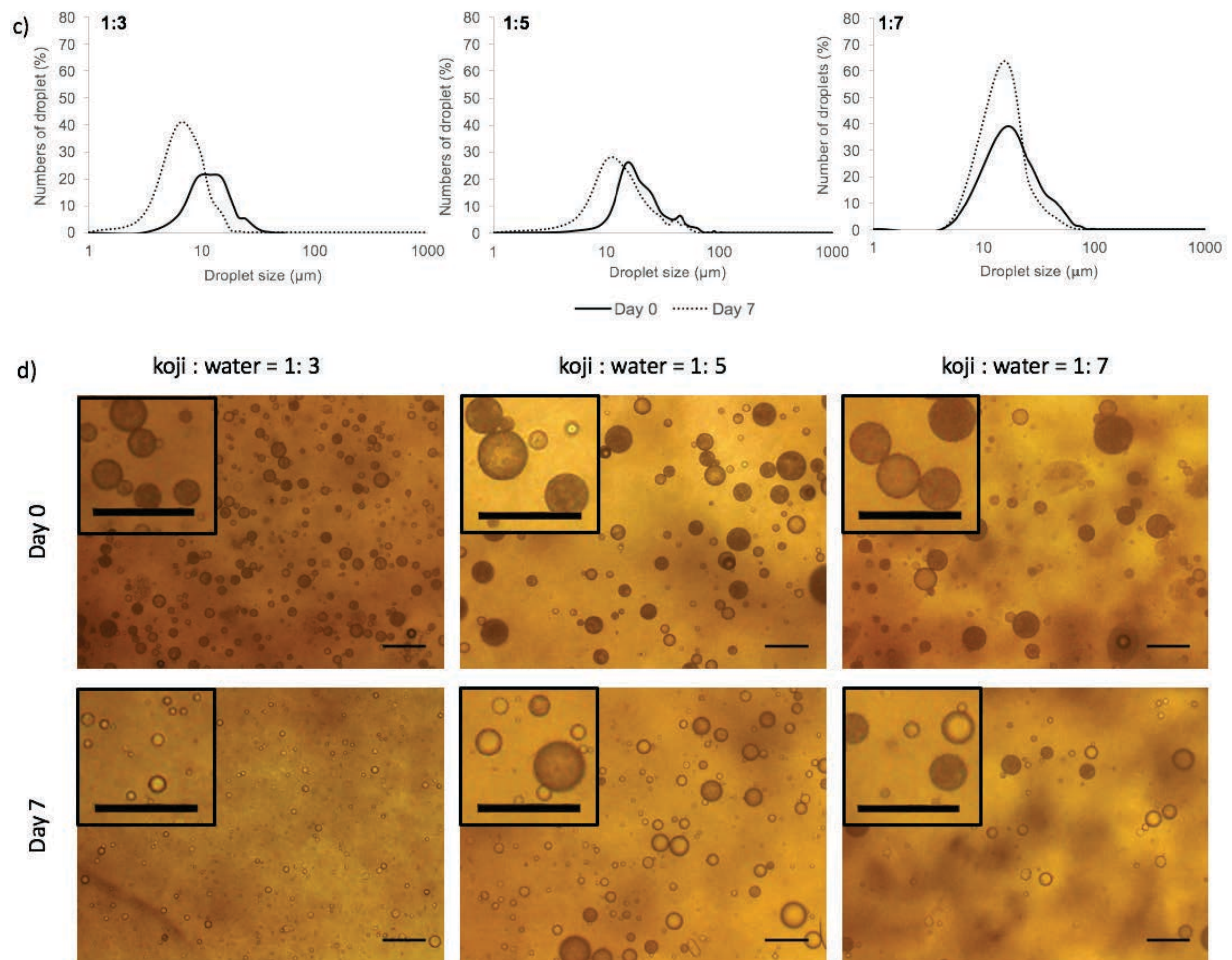


Figure 5.3 DE stability characteristics in moromi made with different ratios of koji and brine, before and after incorporation and during storage. (a) Viscosity of moromi. (b) Average oil globule size. (c) Droplet size distribution. (d) Optical micrographs. Scale bar : 100 μm.

5.3.2 The effect of salt reduction and inoculation sequence on the growth of *T. halophilus* and *Z. rouxii*

Salt concentration is a significant parameter that determines soy sauce fermentation process by affecting the microbial growth. High salt concentration is typically used in soy sauce fermentation in order to suppress the growth of undesirable microorganism as well as improving the organoleptic properties of the final product. *T. halophilus* growth was suppressed during the first 2 weeks of fermentation (from 6.30 log CFU/mL to 4.17 log CFU/mL) when 18% NaCl ($A_{[18\%]}$) was present in moromi (Figure 5.4a). Meanwhile, its growth was significantly enhanced when part of NaCl was replaced with KCl ($B_{[6:12\%]}$) and maintained high viability reaching 7.88 log CFU/mL. Interestingly, the growth of *T. halophilus* in $A_{[18\%]}$ recovered after 2 weeks and exceeded $B_{[6:12\%]}$ by the end of incubation period. In any case, the growth was higher at the lowest salt concentration ($C1_{[6\%]}$, $C2_{[6\%]}$, $C3_{[6\%]}$) throughout the fermentation, where the cell count sharply increased to 8.49 log CFU/mL within the first week and remained stable throughout the incubation period. Although *T. halophilus* is an osmophilic LAB that can tolerate up to 26% NaCl, it grows best at 5 to 10% w/v (Taniguchi et al., 1988). Therefore, raising the NaCl concentration can increase the osmotic stress, reducing the ability of *T. halophilus* to grow (Kobayashi et al., 2004). This indicated that *T. halophilus* could not grow immediately after inoculation in the presence of high NaCl concentration, as previously described by Taniguchi et al. (1988).

The growth of *T. halophilus* under reduced-salt environment was enhanced when it was simultaneously inoculated with *Z. rouxii* ($C1_{[6\%]}$) compared to sequential inoculation ($C2_{[6\%]}$, $C3_{[6\%]}$) and gradual release in DE ($C4_{[6\%]}$). The addition of *Z. rouxii* from the early stage of fermentation might have supplied a variety of metabolites such

as pyruvate, amino acids, and vitamins, which are essential for the early stage of bacterial growth (Sudun, Wulijideligen, Arakawa, Miyamoto, & Miyamoto, 2013).

Z. rouxii was not affected significantly by salt concentration during the first 3 weeks of fermentation. However, low salt moromi ($C1_{[6\%]}$) resulted in a decrease in its population at week 4, in contrast to the enhanced growth of *T. halophilus*. *Z. rouxii* is typically added to enhance flavor and aroma formation in soy sauce production through alcoholic fermentation (van der Sluis et al., 2001b; Wah et al., 2013). In a previous study by Singracha et al. (2017), the addition of *Z. rouxii* in combination with *T. halophilus* and *P. gulliermondii* was shown to increase the total population of lactic acid bacteria and yeast in reduced-salt moromi fermentation. Since *Z. rouxii* grows optimally at low pH, it would be better added at the later stage of fermentation, once moromi is acidified due to organic acids production by *T. halophilus*. In the present study, *Z. rouxii* sequential inoculation ($C2_{[6\%]}$and $C3_{[6\%]}$) and gradual release in DE ($C4_{[6\%]}$) did not have significant effect on growth, as this seemed to be depended primarily on the brine formulation and less on inoculation sequence (Figure 5.4b). This was in contrast to the finding discussed in chapter 2, where sequential inoculation of *Z. rouxii* resulted in the decrease of *Z. rouxii* population as the pH increase to >6.0. Such antagonism was not observed in the latter study, which most likely to be due to pH of moromi which remained low (~4.8). Differences in microbiological and physicochemical properties of moromi could be due to different fermentation conditions applied in both studies, such as NaCl concentration, koji : brine ratio, and *Z. rouxii* inoculation time.

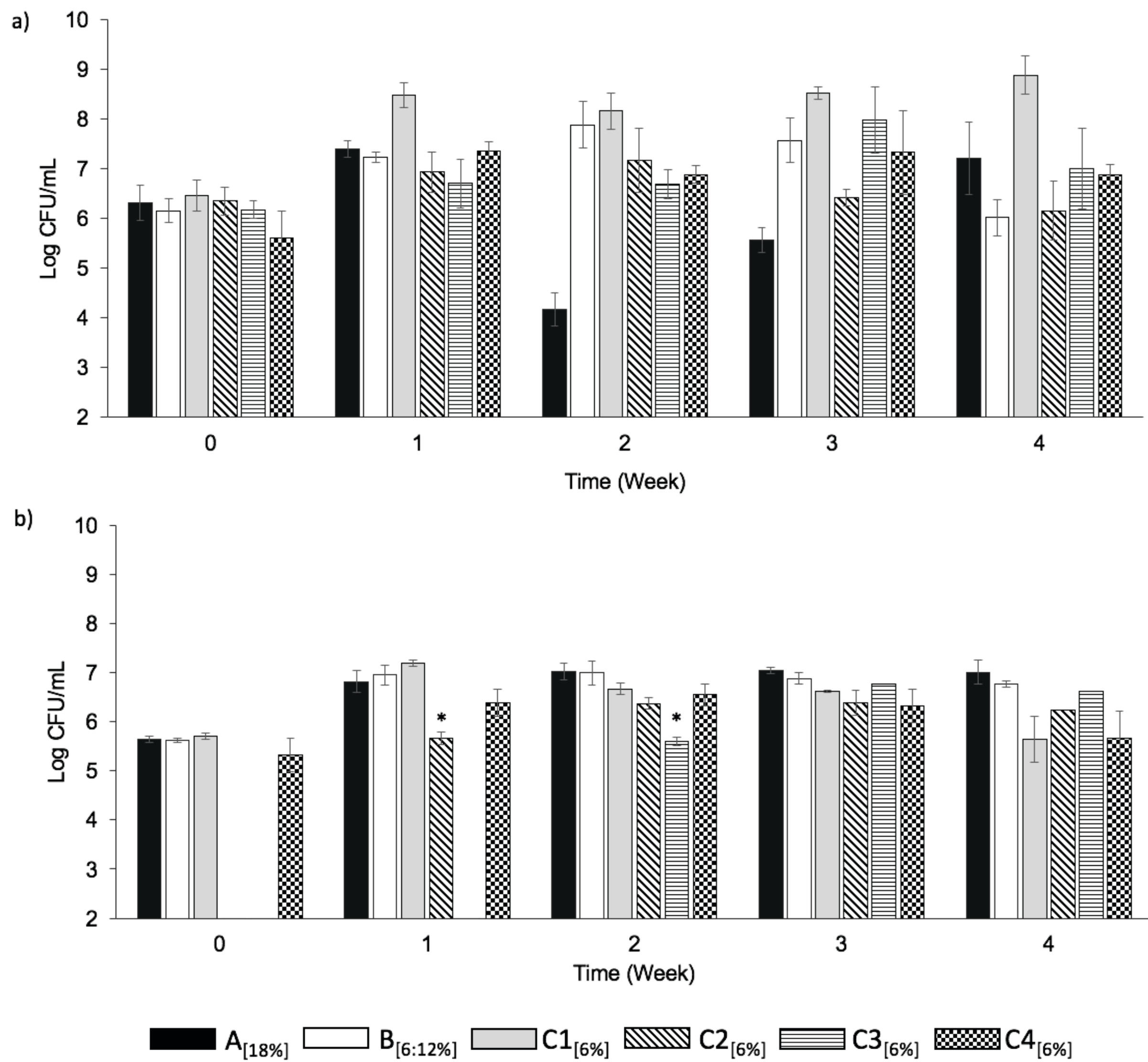


Figure 5.4 Changes in population of (a) *T. halophilus* and (b) *Z. rouxii* during fermentation of low and high salt moromi at 30 °C. The samples contained co-inoculated *T. halophilus* and *Z. rouxii* in 18% NaCl ($A_{[18\%]}$), 6% NaCl and 12% KCl ($B_{[6:12\%]}$), 6% NaCl ($C1_{[6\%]}$), and sequentially inoculated *T. halophilus* and *Z. rouxii* at week 1 ($C2_{[6\%]}$), week 2 ($C3_{[6\%]}$), or with DE ($C4_{[6\%]}$). The addition time of *Z. rouxii* cells for sequential inoculation is indicated by the asterisk mark (*).

5.3.3 Physicochemical changes during fermentation

The changes in pH, reducing sugar, lactic acid, ethanol, and glutamic acid were measured to monitor the fermentation progress, as they are associated with the growth of microorganisms (Figure 5.5). Besides increasing in population during soy sauce fermentation, LAB also utilize and convert carbohydrates into organic acids, which can bring the pH down. Reduction in pH can also occur due to the accumulation of free fatty acids, amino acids, and peptides containing carbonylic side chains, resulting from other microbial activities and raw materials hydrolysis (Hoang et al., 2016; Yanfang and Wenyi, 2009; van der Sluis et al., 2001b). As shown in Figure 5.5a, pH of all moromi samples decreased from ~5.3 to final pH of ~4.8, which was similar to values reported in previous study of traditional Korean (Song, Jeong, & Baik, 2015a) and reduced-salt soy sauce (Singracha et al., 2017). The pH decreased within two weeks and then remained constant throughout the fermentation period, except for $C1_{[6\%]}$ where pH increased to 5.49. The reduction in pH was associated with the increase in the lactic acid amount produced by *T. halophilus* (Figure 5.5c). Although lactic acid production was greatly suppressed by 18% NaCl, the reduction in pH was unaffected, which could be due to production of other organic acids. Although *T. halophilus* is known as homofermentative, some strains are regarded heterofermentative and they are able to produce acetic acid (Justé et al., 2012). Moreover, homofermentative strains of *T. halophilus* are reported to undergo mixed acid fermentation under certain growth conditions (Röling and van Verseveld, 1997).

Lactic acid is the most predominant organic acids produced during moromi fermentation. Chemical characteristic analysis of some soy sauce products in China showed that the lactic acid content was ranging from 0.0239 – 4.88 g/L (Xu, 1990),

while in South-east Asia, the lactic acid content was ranging from 0.0023 – 0.015 g/L (Syifaa et al., 2016). The production of lactic acid was significantly lower in the presence of high salt concentration (0.07 g/L), and high sodium content had a greater impact on the suppression (Figure 5.5c). In low salt concentration, microorganisms are able to perform faster metabolic activity, therefore producing higher amount of acids (Hoang et al., 2016). In the present study, lactic acid production in reduced-salt moromi was enhanced when the inoculation of *Z. rouxii* was modulated, sequentially at week 1 (0.58 g/L), at week 2 (0.74 g/L), or gradually by using DEs (0.67 g/L). In co-inoculation, *Z. rouxii* might have changed the physicochemical properties of the substrate which could suppress the fermentation of lactic acid (0.32 g/L) by *T. halophilus*, as reported in Chapter 2.

Reducing sugar is important during fermentation as it serves as carbon source for microbial growth as well as flavor and aroma formation. The initial content of total reducing sugar in all moromi samples were ranging from 2.68 – 3.49 g/L. Since the initial concentration of reducing sugar (glucose and fructose) was less than 6%, the destabilization of DEs was expected to be less, as discussed in chapter 4. Thus, *Z. rouxii* cells might have been released in a gradual manner. However, it is difficult to predict as moromi also contains other components that could affect the release.

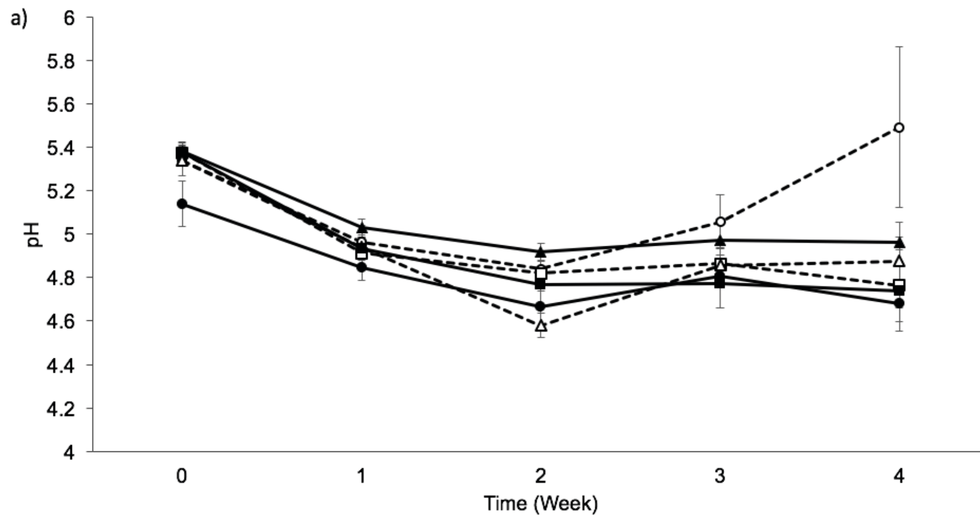
The reducing sugar content constantly decreased throughout the incubation period (Figure 5.5b), which was in agreement with the previous study by Zhang, Zhou, Cui, Huang, & Wu (2016). The reduction patterns were comparable regardless of salt concentration and sequence of inoculation. During fermentation, reducing sugar is consumed by microbes or possibly reacts with free amino acids during Maillard reactions (Kim and Lee, 2008). Since the fungal amylase, which breaks down the

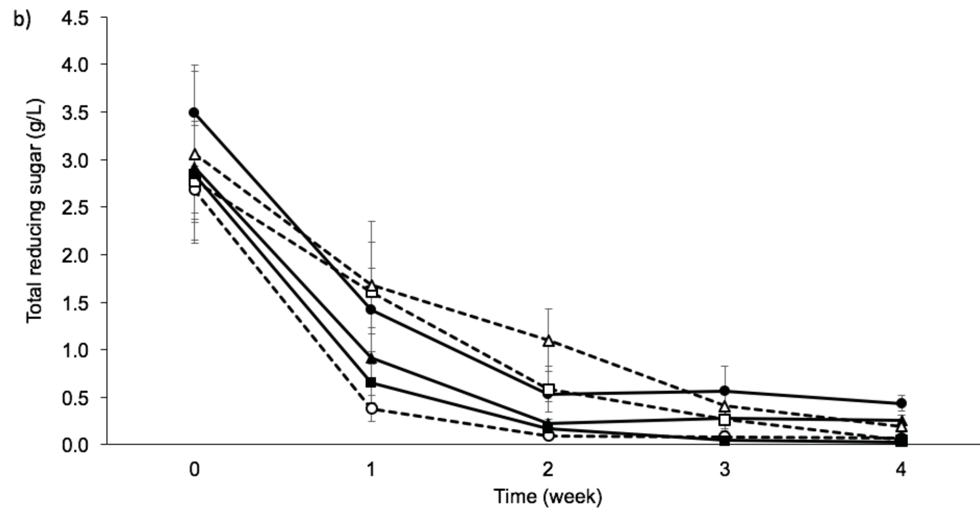
polysaccharide into simple sugars, was heat-inactivated prior to moromi stage, the amount of reducing sugar was expected to decrease over time. The reducing sugar content in moromi decreased faster when low salt concentration ($B_{[6:12\%]}$, $C1_{[6\%]}$, and $C4_{[6\%]}$) was used. This could be attributed to faster metabolic activity of the microbes, which was also corresponded to higher *T. halophilus* population and lactic acid production (Hoang et al., 2016). Furthermore, the reducing sugar content decreased in a slower rate when *Z. rouxii* was inoculated sequentially after 1 or 2 weeks of fermentation, but not when DE was used. This was expected since *Z. rouxii* is the main user of sugar for biomass and ethanol production. The activity of the released *Z. rouxii* cells might have caused faster sugar depletion in DE ($C4_{[6\%]}$).

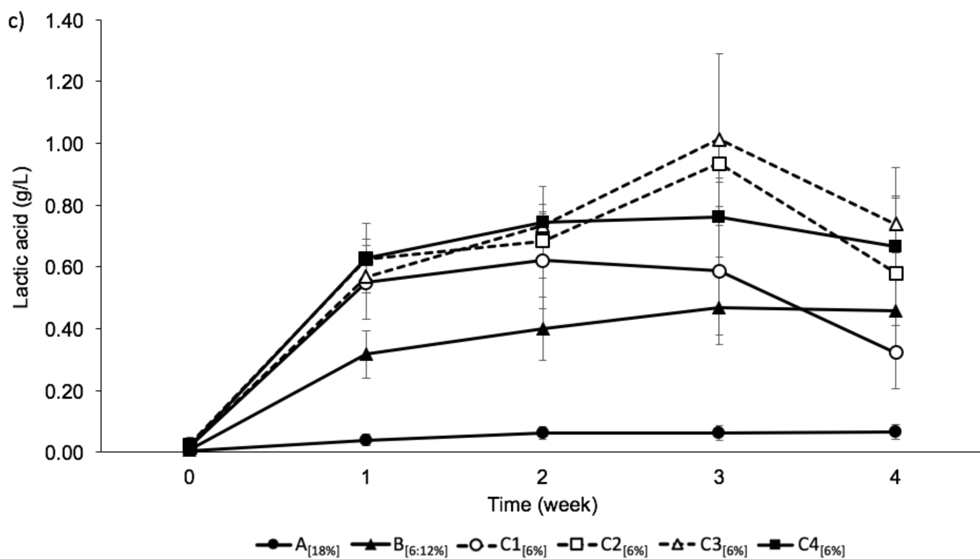
Ethanol production was highly affected by variation in salt concentration and sequence of inoculation (Figure 5.5d). In low salt moromi ($C1_{[6\%]}$), the amount of ethanol constantly decreased after 2 weeks of fermentation to 0.48 g/L compared to high concentration of salt ($A_{[18\%]}$ = 0.95 g/L and $B_{[6:12\%]}$ = 0.78 g/L). However, the concentration of ethanol in ($C1_{[6\%]}$) still exceeded its odor threshold (0.10 g/L), indicating that ethanol contributed in the overall aroma of moromi. The decrease in ethanol production was found to be compensated when *Z. rouxii* was added simultaneously ($C2_{[6\%]}$ and $C3_{[6\%]}$) or using DE ($C4_{[6\%]}$). Interestingly, ethanol production with similar pattern to $A_{[18\%]}$ and B and highest concentration was able to be achieved when *Z. rouxii* was encapsulated in DE.

Z. rouxii is known to produce extracellular glutaminase, which is a proteolytic enzyme that converts L-glutamine derived from soy protein to L-glutamic acid (Iyer and Singhal, 2008; Kashyap et al., 2002). Unlike the glutaminase produced by koji mold, *Z. rouxii* glutaminase is more tolerant against high salinity. L-glutamic acid is essential for

improving the flavor of the final product since it contributes to the "umami" taste of the soy sauce. Therefore, high activity of glutaminase is desirable in order to increase the production of L-glutamic acid. As shown in Figure 5.5e, the amount of glutamic acid increased after the fermentation process and the final concentration of glutamic acid between samples did not differ significantly ($p > 0.05$).


a)
pH
6
5.8
5.6
5.4
5.2
5
4.8
4.6
4.4
4.2
4
0
1
2
3
4
Time (Week)
A[18%] B[6:12%] C1[6%] C2[6%] C3[6%] C4[6%]


b)
Total reducing sugar (g/L)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
1
2
3
4
Time (week)
A[18%] B[6:12%] C1[6%] C2[6%] C3[6%] C4[6%]


c)
Lactic acid (g/L)
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0
1
2
3
4
Time (week)
A[18%] B[6:12%] C1[6%] C2[6%] C3[6%] C4[6%]

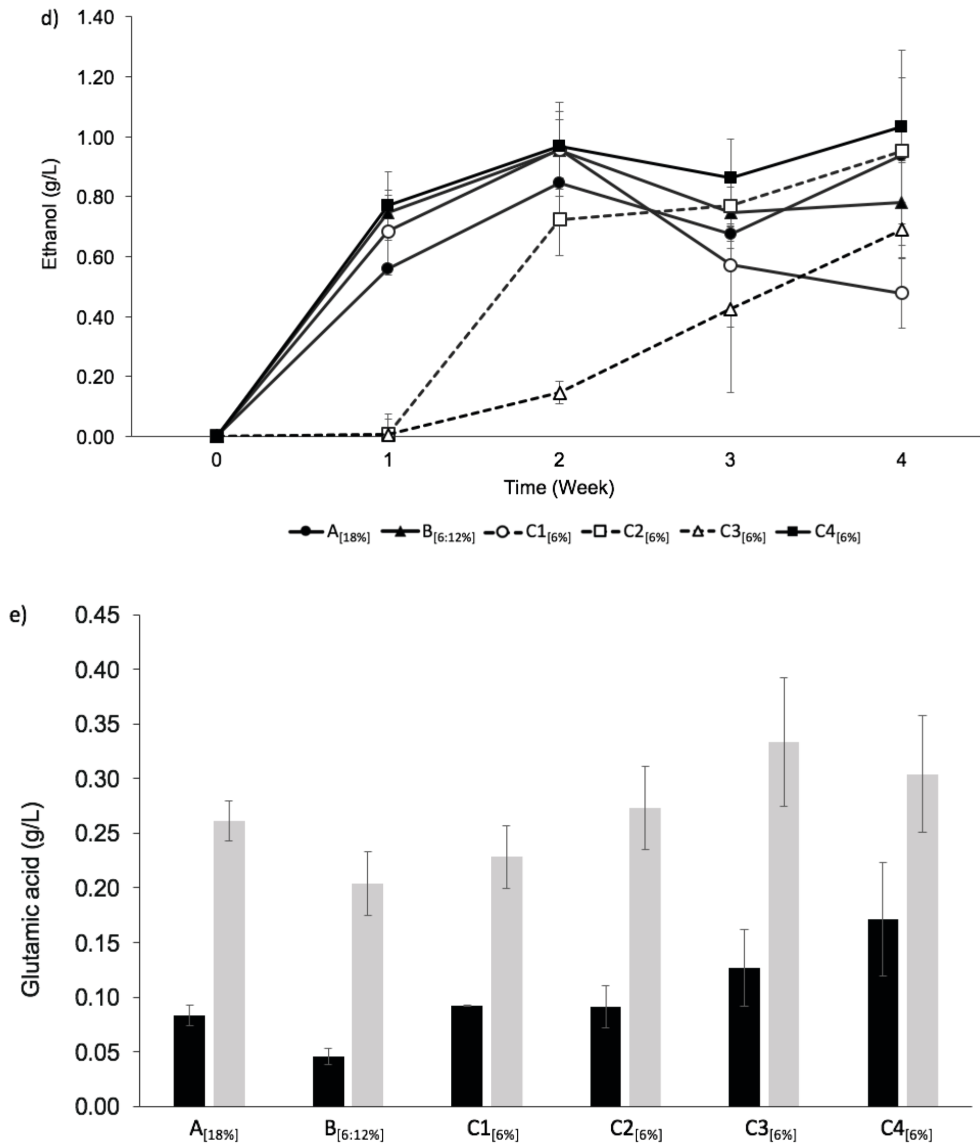


Figure 5.5 Changes in (a) pH, (b) total reducing sugar, (c) lactic acid, (d) ethanol, and (e) glutamic acid during fermentation of low and high salt moromi at 30°C.

5.3.4 Formation of aroma compounds

A total of 38 aroma compounds were detected in the moromi samples by using SPME-GC/MS, including 15 alcohols, 5 acids, 8 aldehydes, 4 esters, 1 furan, 1 phenol, 3 ketones, and 1 alkene (Table 5.1). Alcohol was found to be the most abundant compound in all samples, comprising more than 90% of the total aroma compounds, as previously found in high-salt liquid state fermentation, low-salt solid-state fermentation, and Koikuchi soy sauce (Feng et al., 2015).

Salt reduction ($C1_{[6\%]}$) was shown to have great influence on the aroma production in moromi, especially alcohols (Table 5.1). Yeasts contribute to the formation of alcohols through the reduction of related aldehydes (Sun et al., 2010; van der Sluis et al., 2001b). Lowering salt concentration to 6% w/v ($C1_{[6\%]}$) significantly ($p < 0.05$) enhanced the production of 2,4-dimethyl-3-pentanol, 2,6-dimethyl-4-heptanol, 3-methyl-1-butanol, 5-nonanol, and phenylethyl alcohol. On the other hand, the production of ethanol and propanol was reduced in low salt concentration ($C1_{[6\%]}$) which was in agreement with the previously studied reduced-salt Korean soy sauce (Song, Jeong, & Baik, 2015a). Partial salt substitution with KCl ($B_{[6:12\%]}$) did not affect the production of most volatile compounds, except for 2-furanmethanol, 2-methoxy-5-methyphenol, and 2-methyl-1-propanol which were significantly ($p < 0.05$) lower compared to sample $A_{[18\%]}$. In previous studies reported by Sasaki (1996) and Jansen, Veurink, Euverink, & Dijkhuizen (2003), the production of higher alcohols including phenylethyl alcohol, 3-methyl-1-butanol, propanol, and 2-methyl-1-propanol was found to decrease with an increase of NaCl concentration. However, the amount of propanol and 2-methyl-1-propanol decreased in the reduced NaCl condition ($B_{[6:12\%]}$). This might have arisen from decreasing uptake of the related amino acid by yeast, since these

compounds are mainly produced by *Z. rouxii* from their corresponding branched-chain amino acids via the Erlich pathway (van der Sluis et al., 2001b). The method of inoculation was found to affect the production of most alcohols in the reduced-salt moromi. Moromi with similar flavor pattern to those containing high salt concentration ($A_{[18\%]}$ and $B_{[6:12\%]}$) was achieved when *Z. rouxii* was added sequentially at week 1 ($C2_{[6\%]}$) or using DE ($C4_{[6\%]}$). The addition of *Z. rouxii* at week 2 resulted in significantly ($p < 0.05$) lower amount of 2-furanmethanol, 3-methylbutanol, ethanol, heptanol, hexanol, and propanol. This result corresponds to the ethanol measurement during fermentation by using enzymatic reaction (Figure 5.5d).

Salt reduction was also found to affect the production of several acids. The amount of 4-methyl-2-oxovaleric acid was enhanced in reduced-salt moromi, only when *Z. rouxii* was added simultaneously. Meanwhile, 2-methylpropanoic acid, that contributes to cheese/fatty odor, was found to be significantly lower in all reduced-salt moromi samples. However, noticeably higher amount of 2-methylpropanoic acid was detected when *Z. rouxii* was added at week 2. The production of some acids, including 3-methylbutanoic acid (cheese/sweet) and acetic acid (sour/vinegar-like odor) were found to be enhanced when *Z. rouxii* inoculation was delayed for 2 weeks. Acetic acid production was also similar when DE was used. These acids have been reported as the highest odor-active compounds in Chinese soy sauce (Feng et al., 2014). Among these acids, 2-methylpropanoic acid and 3-methylbutanoic acid are formed via branched-chain α-keto acid catabolism (Song et al., 2015a)

Aldehydes contribute to nutty and malty aroma in soy sauce (Feng et al., 2015). In the present study, most aldehyde compounds were not affected by salt reduction, except for 2-methylpropanal which was significantly enhanced in reduced-salt moromi

when mixed cultures were added simultaneously ($C1_{[6\%]}$). This branched-chain aldehyde is considered as important flavor compound, perceived as malty, chocolate-like, with low taste threshold (Smit et al., 2009). It is generated from branched-chain amino acid valine via Strecker degradation or microbial activity, which then can be converted to its corresponding alcohol (2-methylpropanol) and/or acid (2-methylpropanoic acid) (Ardö, 2006; Song et al., 2015a). The effect of modulating the inoculation time of *Z. rouxii* on aldehydes formation was hardly seen, except for benzaldehyde (burnt sugar/sweet) and furfural (bread/sweet), which were significantly enhanced in $C2_{[6\%]}$ and $C4_{[6\%]}$, respectively.

Replacing NaCl with KCl decreased the amount of 2-phenylethyl acetate, which contributes to honey, rosy odor. However, this could be compensated by adding *Z. rouxii* at week 1 of fermentation process. As described in Chapter 2, the production of 2-phenylethyl acetate could be enhanced by adding *Z. rouxii* sequentially rather than simultaneously. *Z. rouxii* enhances the production of ester through alcohols esterification with fatty acids (van der Sluis et al., 2001b). On the other hand, the production of isoamylacetate (banana aroma) was significantly enhanced in reduced-salt moromi. This was only observed in $C1_{[6\%]}$, while the amount of isoamylacetate in $C2_{[6\%]}$, $C3_{[6\%]}$, and $C4_{[6\%]}$ was similar to those found in high salt concentration ($A_{[18\%]}$and $B_{[6:12\%]}$).

The only furan and phenol compound detected in all moromi samples was 3-acetyl-2,5-dimethylfuran and 2-methoxy-5-methylphenol, respectively. These were produced in negligible amount when either salt or NaCl were reduced, except when *Z. rouxii* was added at week 1. Several ketones, such as 2-methyl-2-pentanone and acetoin were produced in significantly higher number in reduced-salt moromi. The amount of

these compounds were similar to moromi containing high salt ($A_{[18\%]}$ and $B_{[6:12\%]}$) when *Z. rouxii* was added sequentially, with or without DE.

Table 5.1 Aroma compounds found in moromi after 4 weeks of fermentation in low and high salt concentration. The values are relative to the peak area observed when the headspace above a 0.1 μg/mL 1-octen-3-ol solution was analyzed. Each value is based on three replicates.

Compound	LRI[1]	Day 30																	
		$A_{[18\%]}$			$B_{[6:12\%]}$			$C1_{[6\%]}$			$C2_{[6\%]}$			$C3_{[6\%]}$			$C4_{[6\%]}$		
		Mean (n=3)		SD	Mean (n=3)		SD	Mean (n=3)		SD	Mean (n=3)		SD	Mean (n=3)		SD	Mean (n=3)		SD
Alcohols																			
1-Octen-3-ol	1466	0.039	a	0.009	0.020	a	0.002	0.037	a	0.006	0.086	b	0.035	0.037	a	0.007	0.039	a	0.011
2-Ethylhexan-1-ol	1508	0.029	a	0.003	0.028	ab	0.008	0.014	abc	0.007	0.025	abc	0.011	0.012	bc	0.003	0.010	c	0.003
2-Furanmethanol	1690	0.084	a	0.006	0.046	b	0.003	0.039	b	0.002	0.089	a	0.011	0.051	b	0.004	0.085	a	0.016
2-Methyl-1-propanol	1115	0.385	a	0.055	0.186	bc	0.019	0.204	b	0.070	0.159	bc	0.030	0.055	c	0.012	0.185	bc	0.081
2,4-Dimethyl-3-pentanol	1395	0.013	a	0.001	0.019	a	0.001	0.298	b	0.203	0.021	a	0.004	0.025	a	0.003	0.009	a	0.001
2,6-Dimethyl-4-heptanol	1506	0.031	ab	0.010	0.005	a	0.001	0.090	b	0.025	0.032	ab	0.006	0.081	b	0.049	0.013	a	0.006
3-Methyl-3-buten-1-ol	1271	0.026	a	0.004	0.021	a	0.002	0.019	a	0.004	0.004	b	0.001	0.006	b	0.001	0.006	b	0.001
3-Methyl-1-butanol	1225	25.061	a	3.033	18.089	ac	1.609	40.297	b	8.876	19.532	ac	3.675	9.445	c	1.787	21.703	a	2.603
5-Nonanol	1473	0.002	a	0.000	0.001	a	0.000	0.006	b	0.003	0.005	ab	0.002	0.008	b	0.002	0.002	a	0.000
Ethanol	950	33.709	a	2.159	27.538	ab	1.129	14.398	b	1.106	39.284	a	3.380	16.496	b	1.616	36.572	a	12.076
Heptanol	1473	0.042	ab	0.006	0.016	a	0.002	0.016	a	0.002	0.015	a	0.006	0.007	a	0.001	0.066	b	0.033
Hexanol	1371	0.071	a	0.012	0.033	ab	0.002	0.032	ab	0.010	0.046	ab	0.018	0.014	b	0.002	0.072	a	0.032
Methanol	915	0.449	a	0.025	0.511	a	0.065	0.488	a	0.088	0.604	a	0.082	0.661	a	0.110	0.523	a	0.159
Phenylethyl alcohol	1957	2.425	a	0.371	2.059	ac	0.121	3.801	b	0.352	2.010	ac	0.193	1.408	c	0.535	1.658	ac	0.467
Propanol	1057	0.376	ac	0.030	0.281	ab	0.023	0.180	b	0.030	0.468	cd	0.057	0.204	b	0.038	0.547	d	0.088
Acids																			
4-Methyl-2-	1478	0.016	ac	0.005	0.006	a	0.000	0.040	b	0.006	0.012	ac	0.004	0.024	c	0.010	0.007	a	0.000

oxovaleric acid																			
2-Methylpropanoic acid	1596	0.113	a	0.046	0.057	ab	0.025	0.000	b	0.000	0.025	bc	0.007	0.086	ac	0.037	0.040	bc	0.004
3-Methylbutanoic acid	1699	0.400	ab	0.153	0.205	a	0.081	0.008	a	0.012	0.116	a	0.103	0.776	b	0.323	0.158	a	0.043
Acetic acid	1481	0.161	ab	0.098	0.041	ab	0.040	0.000	a	0.000	0.212	ab	0.055	0.300	b	0.133	0.312	b	0.178
Propionic acid	1565	0.010	a	0.010	0.018	a	0.023	0.015	a	0.025	0.061	a	0.090	0.045	a	0.058	0.108	a	0.107
Aldehydes																			
2-Methylbutanal	929	0.009	a	0.002	0.001	a	0.000	0.026	a	0.027	0.009	a	0.009	0.006	a	0.005	0.004	a	0.001
2-Methylpropanal	824	0.026	a	0.009	0.009	a	0.002	0.127	b	0.057	0.027	a	0.022	0.019	a	0.001	0.011	a	0.003
3-Methylbutanal	934	0.083	a	0.013	0.012	a	0.002	0.091	a	0.063	0.109	a	0.114	0.102	a	0.101	0.040	a	0.013
Benzaldehyde	1568	0.021	a	0.004	0.034	ab	0.001	0.013	a	0.001	0.060	b	0.030	0.023	a	0.003	0.022	a	0.001
Furfural	1500	0.014	ab	0.002	0.010	ab	0.001	0.009	a	0.000	0.015	b	0.003	0.010	ac	0.001	0.014	bc	0.003
Hexanal	1104	0.001	a	0.001	0.002	a	0.001	0.001	a	0.000	0.003	a	0.001	0.001	a	0.000	0.002	a	0.002
Pentanal	1001	0.014	a	0.007	0.015	a	0.007	0.015	a	0.004	0.029	a	0.010	0.023	a	0.007	0.024	a	0.006
Propanal	807	0.004	a	0.000	0.005	a	0.001	0.003	a	0.001	0.004	a	0.002	0.006	a	0.001	0.005	a	0.003
Esters																			
2-Phenylethyl acetate	1860	0.316	ac	0.054	0.136	b	0.006	0.094	b	0.024	0.378	c	0.071	0.163	b	0.061	0.191	ab	0.040
Ethyl acetate	906	0.196	a	0.046	0.079	a	0.026	0.073	a	0.023	0.100	a	0.077	0.019	a	0.009	0.245	a	0.189
Ethyl propionate	975	0.032	ab	0.008	0.006	a	0.000	0.010	ab	0.005	0.011	ab	0.009	0.003	a	0.002	0.038	b	0.024
Isoamylacetate	1141	0.256	a	0.074	0.029	a	0.006	0.878	b	0.280	0.115	a	0.119	0.044	a	0.015	0.243	a	0.121
Furan																			
3-Acetyl-2,5-dimethylfuran	1450	0.186	a	0.061	0.001	bc	0.000	0.001	b	0.000	0.069	c	0.014	0.009	bc	0.001	0.000	b	0.000
Phenol																			
2-Methoxy-5-methylphenol	1614	0.258	a	0.081	0.001	b	0.000	0.001	b	0.000	0.169	a	0.035	0.008	b	0.001	0.001	b	0.001
Ketone																			

3-Methyl-2-pentanone	1037	0.004	a	0.001	0.009	ac	0.001	0.032	b	0.011	0.000	a	0.000	0.018	bc	0.006	0.004	a	0.003
Acetoin	1318	0.656	a	0.039	0.451	a	0.043	1.624	b	0.572	0.066	a	0.013	0.227	a	0.146	0.175	a	0.103
Acetone	829	0.671	a	0.088	0.334	a	0.082	0.507	a	0.084	0.405	a	0.107	0.614	a	0.179	0.453	a	0.303
Others																			
D-Limonene	1217	0.006	a	0.003	0.001	a	0.001	0.004	a	0.004	0.002	a	0.001	0.183	a	0.179	0.008	a	0.006

5.3.5 Principal component analysis

PCA analysis was conducted in order to gain more understanding on the relationship between the fermentation conditions and profiles of aroma compounds production. The first (PC1) and second principal component (PC2) accounted for 30.60% and 21.43% of the total variance, respectively (Figure 5.6a-b). The PCA score plot demonstrates distinct separation of some moromi samples (Figure 5.6a). In the case of co-inoculated samples, low salt moromi sample ($C1_{[6\%]}$) was differentiated from high salt moromi sample ($A_{[18\%]}$) while reduced NaCl sample (substituted with 12% KCl; B) was positioned in the middle of PC1. This indicates that salt reduction affected the aroma profile of moromi. Replacing part of NaCl with KCl ($B_{[6:12\%]}$) was associated with lower content of 2-furanmethanol, 2-methyl-1-propanol, 2-phenylethylacetate, 3-acetyl-2,5-dimethylfuran, and 2-methoxy-5-methylphenol. $C1_{[6\%]}$ was associated with high amount of 3-methyl-1-butanol, phenylethyl alcohol, 2,4-dimethyl-3-pentanol, 2,6-dimethyl-4-heptanol, isoamyl acetate, 2-methylpropanal, 4-methyl-2-oxovaleric acid, 3-methyl-2-pentanone, and acetoin (Figure 5.6b).

The method of inoculation was found to affect the aroma profiles, and adding *Z. rouxii* encapsulated in DE or sequentially after 1 week matched the aroma profile obtained in high salt concentration. This was not the case when *Z. rouxii* was added sequentially after 2 weeks of fermentation. Clustering of samples $A_{[18\%]}$, $C2_{[6\%]}$, and $C4_{[6\%]}$was influenced by compounds such as 2-furanmethanol, 2,4-dimethyl-3-pentanol, 2,6-dimethyl-4-heptanol, 3-methyl-1-butanol, 5-nonanol, ethanol, hexanol, methanol, phenylethyl alcohol, 4-methyl-2-oxovaleric acid, 3-methylbutanoic acid, acetic acid, propionic acid, 2-methylbutanal, 2-methylpropanal, 3-methylbutanal, furfural, hexanal, pentanal, propanal, ethyl acetate, ethyl propionate, isoamylacetate, 3-methyl-2-pentanone, acetoin, acetone, and D-limonene.

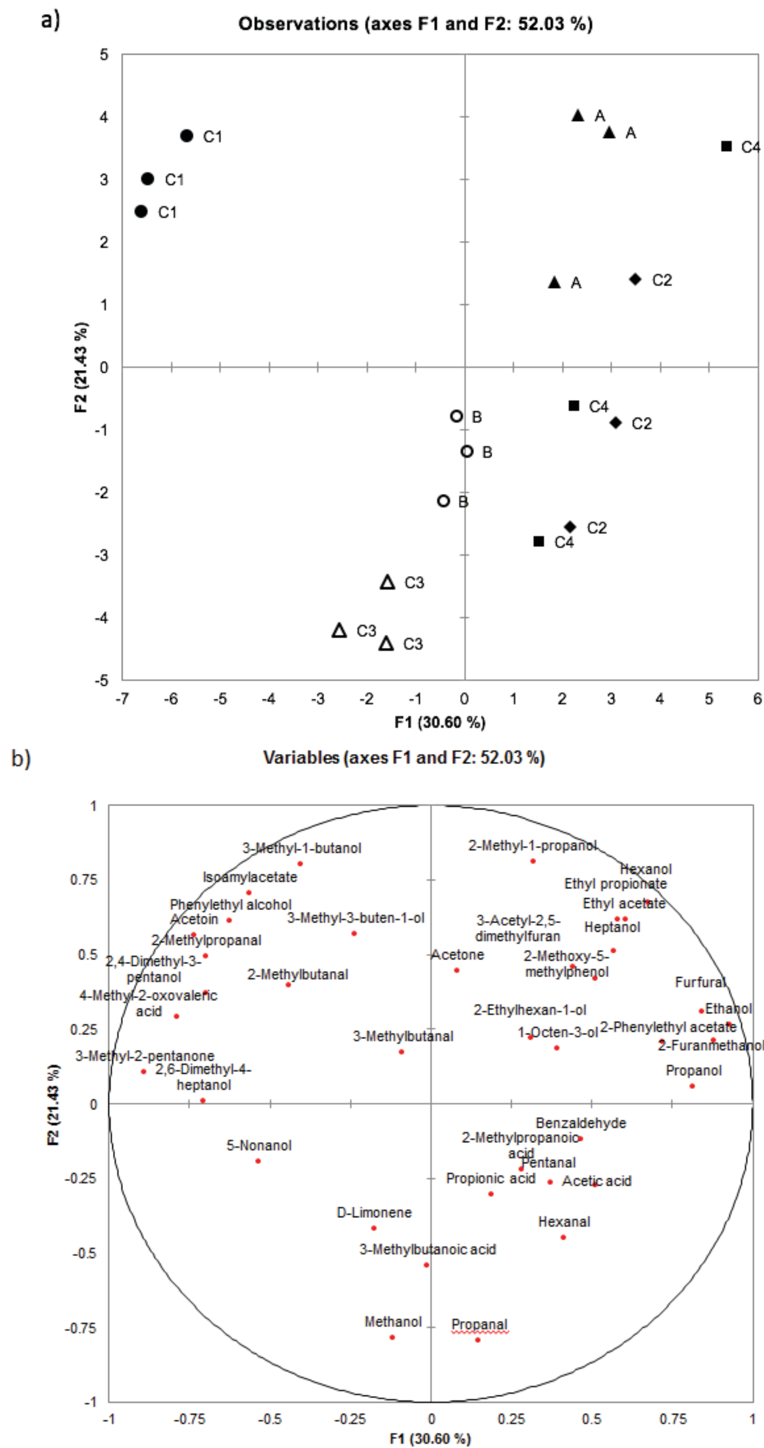


Figure 5.6 (a) PCA score plot of six moromi samples after 4-week fermentation. The scores are based on three replicates of each sample. The identical symbols represent triplicate measurements. (b) PCA loading plot of the aroma compounds detected in moromi after 4-week fermentation.

5.4 Conclusions

Salt reduction could affect the microbial growth and physicochemical changes during moromi fermentation. The application of low salt concentration could promote *T. halophilus* growth and enhance lactic acid production. However, the final overall aroma balance differed from the original soy sauce fermented with high salt concentration, indicated by lower content of some alcohols, acids, esters, furan, and phenol. The use of DE for delivering the mixed cultures of *T. halophilus* and *Z. rouxii* in reduced-salt moromi could compensate such changes by promoting the formation of some essential aroma compounds, including alcohols (e.g. 2-furanmethanol and ethanol) and esters (e.g. 2-phenylethyl acetate). This indicates the possibility of producing soy sauce under low salt environment with aroma profile pattern identical to the original high-salt soy sauce. The results obtained in this study provide soy sauce industry a new technique for standardizing the microbial activity and aroma development, which also offering health benefit to the consumers due to low salt content in the final product. However, since modulating the release has a great impact on the aroma formation, further study is needed in order to tailor the physicochemical properties of DE, therefore enabling the cell release in a more controlled manner.

Chapter 6

Conclusions and future work

***T. halophilus* and *Z. rouxii* exhibit antagonistic interaction, regardless of the inoculation sequence.** The ultimate aim of this thesis was to investigate the possibility of improving the formation of aroma compounds during moromi fermentation by controlling the activity of *T. halophilus* and *Z. rouxii*. Therefore, this study firstly looked at how mixed starter cultures of *T. halophilus* and *Z. rouxii* interact when they were inoculated simultaneously or sequentially. *T. halophilus* and *Z. rouxii* have been reported as the most important microorganisms in soy sauce production due to their ability to produce a wide range of key aroma compounds during moromi fermentation process. However, the results obtained in this study showed antagonistic interaction between *T. halophilus* and *Z. rouxii*, which was in agreement with the previous studies. This was indicated by the declining of *Z. rouxii* growth, regardless of sequence of inoculation. Although antagonism occurred in both cases, the inoculation sequence affected the activity of both microbial species, and physicochemical changes were observed. Furthermore, such changes seemed to favor the aroma profiles in the case of sequential inoculation.

Since the aroma profile is strongly influenced by the microbial growth and activity, it would be important to obtain a better understanding of species-to-species interactions by looking at the exchange of metabolites and growth factors or inhibiting compounds. Microbial encapsulation could also be an approach to minimize antagonism by

providing a selective barrier that can minimize interferences between species, followed by controlled release of cells into the medium, as described in Chapter 5.

Alginate is not suitable for encapsulation in the high salt environment and the use of chitosan as coating agent is not appropriate due to its antimicrobial activity. Since *Z. rouxii* growth was inhibited by *T. halophilus*, an attempt was made to protect *Z. rouxii* by encapsulating it in alginate beads. Despite a number of advantages offered, alginate has been reported to be sensitive to high NaCl concentration present in moromi. Therefore, in this study, the physical stability of alginate beads was assessed in the presence of NaCl, by varying the concentration of alginate. The results showed that the stability of alginate beads in the presence of NaCl improved when alginate concentration was increased. However, the stability obtained was not sufficient to be used in moromi fermentation as the beads started to disintegrate, accompanied by cell release within few days. The addition of chitosan layer on the beads surface to improve its physical stability failed. The alginate beads were shrinking during coating process and *Z. rouxii* cells were negatively affected due to the antimicrobial properties of chitosan.

In the future, for the preparation of alginate beads, factors such as composition, sequential structure, and molecular weight of alginate should be taken into account, since they affect the physical properties of the beads. Furthermore, crosslinking agent other than $CaCl_2$ could be used such as $BaCl_2$ and $AlCl_3$ to improve the physical stability of alginate beads. Finally, other cationic polymers such as poly-aminoacids (e.g poly-L-lysine and poly-ornithine) or synthetic polymers (e.g poly(allylamine) and poly(vinylamine) which have no or minimum antimicrobial activity could be used to coat the alginate beads.

DE could be a suitable formulation for the delivery of mixed starter cultures in soy sauce fermentation. Microbial encapsulation in DE has shown a great potential in food and biotechnological industries, from protecting probiotics against human gastrointestinal conditions, serving as bioreactor for biofilm formation, as well as controlling the release of bacteria to the external W_2 phase. Therefore, DE could be a suitable method to minimize the antagonistic interaction between *T. halophilus* and *Z. rouxii* by segregating the two species, followed by sustained release of *Z. rouxii* into the medium. Since the existing studies have only focused on encapsulation of bacteria, this study investigated the feasibility of DE to encapsulate *Z. rouxii* under conditions relevant to moromi fermentation. The results showed that *Z. rouxii* was successfully encapsulated in DE with very high encapsulation efficiency (>99%). This encapsulation method was not only able to enhance the survival of *Z. rouxii* during storage, but also eliminate its antagonistic interaction with *T. halophilus*. This was most likely due to the alteration of metabolic activities of both species in the presence of DE, as different patterns of physicochemical changes were observed in DE and non-DE culture/s. The inherent instability of DE could also be used to gradually release *Z. rouxii* cells into the medium, and this could be controlled by adjusting glucose concentration in the external W_2 phase.

Further study needs to be conducted to elucidate the mechanism of cell release. The effect of oil layer on mass transfer phenomena could also be further investigated as this may affect the alteration of metabolic activity of *T. halophilus* and *Z. rouxii*. In order to gain more insight into the interaction between the two species in the presence of DE, such interaction could be further studied at metabolic level.

Low-salt soy sauce with aroma profile similar to high-salt soy sauce can be produced by modulating the inoculation of *Z. rouxii* with DE. After gaining fundamental knowledge on DE with the presence of *Z. rouxii* in the internal W_1 phase and *T. halophilus* in the external W_2 phase, and also its behavior under the conditions relevant to moromi fermentation, the next step was to apply such system in a real moromi fermentation. Salt reduction was found to stimulate the growth of *T. halophilus* accompanied with enhanced lactic acid production, but the final aroma profile was altered. The application of DE in low-salt moromi was able to compensate such changes by promoting the formation of some essential aroma compounds, including alcohols (e.g. 2-furanmethanol and ethanol) and esters (e.g. 2-phenylethyl acetate).

In the future, a method to quantify cell release in moromi could be developed. Furthermore, mathematical models could also be developed for predicting the release of cells during moromi fermentation. Understanding the mechanism of release could also help to release the cells in a more controlled manner.

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Appendix 1. Ability of *Z. rouxii* to grow in glucose solution

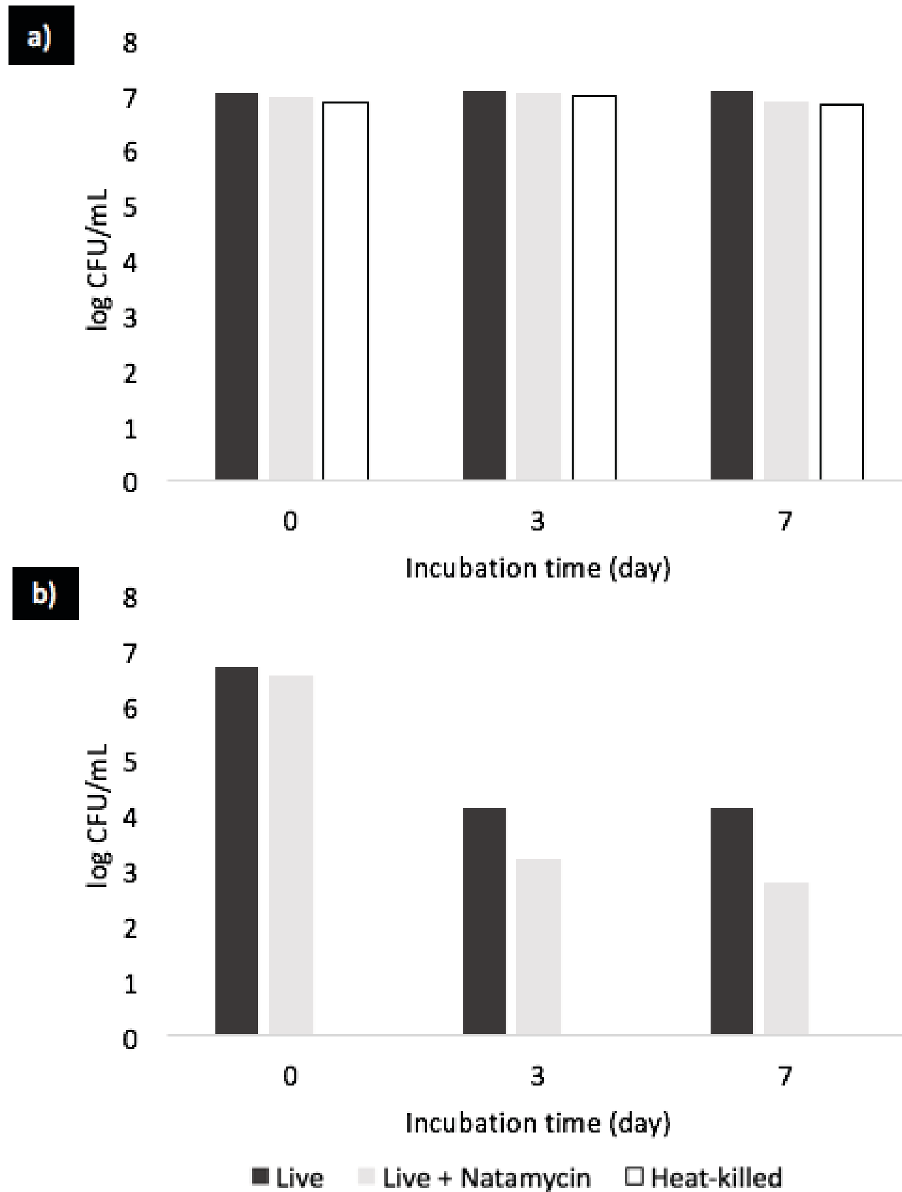

Figure A1. The population of *Z. rouxii* incubated in 10% glucose solution counted with (a) haemocytometer and (b) total plate count method.

Appendix 2. Effect of PGPR and Tween 80 on *Z. rouxii* growth

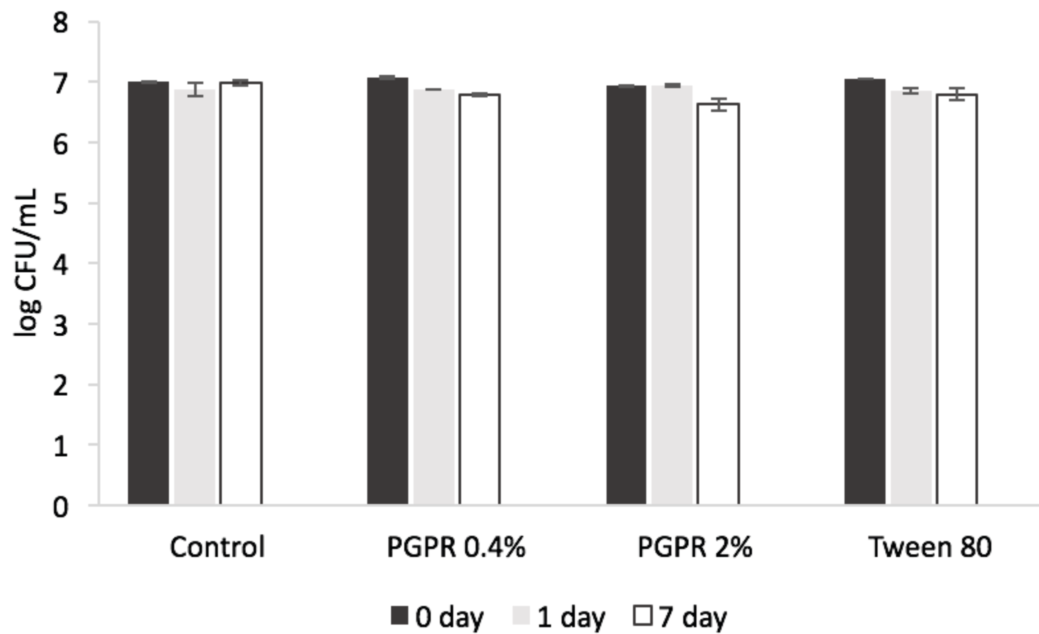

Figure A2. The population of *Z. rouxii* incubated in water containing PGPR or Tween 80

Appendix 3. Viscosity of moromi in relation to proportion of koji and brine

Table A3. Parameters of the Power-Law model of koji and brine mixtures (1:3, 1:5, and 1:7) at 30 °C

Koji : Brine	***Viscosity (Pa.s)***	***Consistency coefficient K ($Pa.s^n$)***	***Flow behaviour index n***	***R^2***
1 : 3	1.276±0.096[a]	52.401	0.178	0.988
1 : 4	0.331±0.085[b]	12.319	0.203	0.992
1 : 7	0.113±0.025[c]	2.228	0.341	0.995

The data was analysed using one-way ANOVA with with *post-hoc Tukey* HSD

[a, b, c] means ± standard error with different letters are significantly different

Publications

Research articles

- Devanthi, P. V. P., Linforth, R., Onyeaka, H., & Gkatzionis, K. (2018). Effects of co-inoculation and sequential inoculation of *Tetragenococcus halophilus* and *Zygosaccharomyces rouxii* on soy sauce fermentation. *Food Chemistry*, *240*, 1–8.
- Devanthi, P. V. P., El Kadri, H., Bowden, A., Spyropoulos, F., & Gkatzionis, K. (2018). Segregation of *Tetragenococcus halophilus* and Zygosaccharomyces rouxii using $W_1/O/W_2$ double emulsion for use in mixed culture fermentation. *Food Research International*, 105, 333–343.
- Devanthi, P. V. P., Linforth, R., El Kadri, H., & Gkatzionis, K. (2018). Water-in-oil-in-water double emulsion for the delivery of starter cultures in reduced-salt moromi fermentation of soy sauce. *Food Chemistry*, 257, 243-251.

Oral presentations (speaker underlined)

- Devanthi, P. V. P., El Kadri, H., Manoharan, A., Linforth, R., & Gkatzionis, K., *Tetragenococcus halophilus* and *Zygosaccharomyces rouxii* interactions in soy sauce fermentation and formulation in water-oil-water double emulsions, SfAM Summer Conference, Edinburgh, UK, 2016
- Devanthi, P. V. P., El Kadri, H., & Gkatzionis, K., Effects of *Zygosaccharomyces rouxii* entrapment in water-oil-water double emulsion on its interaction with *Tetragenococcus halophilus*, SfAM Summer Conference, Newcastle, UK, 2017

- Devanthi, P. V. P., El Kadri, H., Linforth, R., & Gkatzionis, K, Use of double emulsion for delivery of *T. halophilus* and *Z. rouxii* in soy sauce fermentation, 31st EFFoST International Conference, Sitges, Spain, 2017

Poster presentations

- Devanthi, P. V. P., El Kadri, H., Manoharan, A., Linforth, R., & Gkatzionis, K., *Tetragenococcus halophilus* and *Zygosaccharomyces rouxii* interactions in soy sauce fermentation and formulation in water-oil-water double emulsions, SfAM Summer Conference, Edinburgh, UK, 2016
- Devanthi, P. V. P., El Kadri, H., Manoharan, A., Onyeaka, H., & Gkatzionis, K, Studies on *Tetragenococcus halophilus* and *Zygosaccharomyces rouxii* interactions in soy sauce fermentation and formulation in water-oil-water double emulsions, Quantitative Tools for Sustainable Food and Energy in the food chain (Q-Safe), Malta, March 23rd – April 5th 2015
- Devanthi, P. V. P., El Kadri, H., Manoharan, A., Onyeaka, H., & Gkatzionis, K, *Tetragenococcus halophilus* and *Zygosaccharomyces rouxii* interactions in soy sauce fermentation and formulation in water-oil-water double emulsions, SfAM Summer Conference, Dublin, Ireland, 2015

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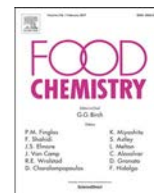

Effects of co-inoculation and sequential inoculation of *Tetragenococcus halophilus* and *Zygosaccharomyces rouxii* on soy sauce fermentation

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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

with magnetic cap (20 mm diameter, 5 mm centre, PTFE/silicone liner). Samples were allowed to equilibrate at 22 °C for 30 min before analysis. Three replicates were prepared for all samples.

The volatiles extraction was performed using a 1-cm Stableflex fibre coated with 50/30 µm divinylbenzene-Carboxen on polydimethylsiloxane bonded to a flexible fused silica core (Supelco, Bellefonte, PA). It was conditioned for 90 min at 300 °C in the injection port. The fibre was pushed out of the housing and inserted into the vials through the centre of the vial cap. The penetration depth was fixed at 22 mm. The extraction was carried out by exposing the fibre to the headspace for 10 min at 40 °C. For all analyses, desorption time was set to 10 min at 230 °C.

Chromatography was carried out using a Trace GC Ultra gas chromatograph (Thermo Electron Corporation, Hemel Hempstead, UK) equipped with a polar column ZB-Wax (30 m × 0.25 mm I.D.; film thickness: 1 µm) from Phenomenex (Torrance, CA). Mass spectrometry (MS) was performed with a DSQ mass spectrometer (Thermo Electron Corporation, Hemel Hempstead, UK). The GC–MS was set according to a previous study (Gkatzionis, Linforth, & Dodd, 2009): The temperature of the injection port was 230 °C. Helium was employed as the carrier gas, at a constant pressure of 17 psi. The oven temperature program was as follows: an initial temperature of 40 °C was maintained for 2 min, increasing at a rate of 8 °C /min to a final temperature of 220 °C. The transfer line from the gas chromatograph to the mass spectrometer was held at 250 °C. The mass spectrometer was operated in positive ionization electron impact mode (EI+) at 70 eV. The detector was operated in scan mode (2 scans/s) scanning from m/z 20 to 250. Source temperature was 200 °C.

Compounds were identified by comparing their retention times and mass spectra with those of standards, or their retention indices (RI) with those published in databases and their mass spectra with the National Institute of Standards and Technology (NIST) mass spectral library using XCalibur Software (Thermo Electron Corporation). The signal intensity for each compound was expressed relative to the signal observed when the headspace above a 0.1 µg/mL 1-octen-3-ol solution was sampled.

2.7. Statistical analysis

Microbial cell enumeration, physicochemical tests, and volatile compounds analysis were conducted in triplicate and repeated in two independent experiments. The results were presented as means ± standard deviation. Significant differences among means were tested by one-way analysis of variance (ANOVA) using IBM SPSS Statistics Software version 21 at $p < 0.05$ and Tukey's test was applied for comparison of means. Principal component analysis (PCA) was performed using XLSTAT™ version 2015.6.01.24027 (Addinsoft, Paris, France) to reduce the dimensionality of the dataset and show the differences in volatile compounds among the soy sauce samples. Observations/variables was chosen as data format and Pearson's correlation matrix was used as PCA type.

3. Results and discussion

3.1. The effect of T. Halophilus and Z. Rouxii interaction on growth during moromi fermentation

An antagonistic relationship between *T. halophilus* and *Z. rouxii* was evident in mixed culture fermentation regardless of the inoculation sequence. *Z. rouxii* viable cell counts markedly decreased to less than 2 log CFU/mL by the end of fermentation period while counts in single culture remained > 6 log CFU/mL (Fig. 1C and D). On the other hand, *T. halophilus* growth was stimulated in co-inoculation and sequential inoculation, to final counts of 8.62 log CFU/mL (Fig. 1C) and 8.47 log CFU/mL (Fig. 1D) respectively, compared to growth in single culture, which was constant (6.67 log CFU/mL; Fig. 1A).

A different pattern was observed for *T. halophilus* growth between co-inoculation and sequential inoculation. *T. halophilus* in co-inoculation reached its stationary phase (~9 log CFU/mL) at Day 15, while in sequential inoculation its stationary phase was reached at Day 25 (Fig. 1C and D). These results indicate that delaying *Z. rouxii* inoculation could slow down the growth of *T. halophilus*.

Marked increase in *T. halophilus* growth in mixed culture could possibly be due to metabolites production by *Z. rouxii*, such as pyruvate, amino acids, and vitamins, which are essential for bacterial growth (Sudun, Arakawa, Miyamoto, & Miyamoto, 2013). Moreover, the dynamics of *T. halophilus* and *Z. rouxii* viable cells were apparently influenced by soy mash pH values during moromi fermentation. Initially, the pH values of all moromi samples were similar (~5.5). When the pH was constantly low (~5.0) throughout the fermentation period, the viable cell counts of *T. halophilus* and *Z. rouxii* in single culture remained constant (Fig. 1A and B). The final pH values of moromi inoculated with pure culture of *T. halophilus* and *Z. rouxii* were 4.83 and 5.38, respectively. In contrast, as the pH of moromi increased (> 6.0), *T. halophilus* growth was enhanced in parallel with *Z. rouxii* growth declining (Fig. 1C and D). The increase in pH could stimulate *T. halophilus* growth since the optimal pH for its growth is around 7.0 (Justé et al., 2008; Röling & van Verseveld, 1997). On the other hand, since the optimal pH range for *Z. rouxii* is 3.5–5.0, the pH increase in moromi could inhibit *Z. rouxii* growth (Membré, Kubaczka, & Chéné, 1999). Moreover, *Z. rouxii* cannot maintain its salt tolerance when the extracellular pH goes above 5.5, due to loss of the proton gradient across the plasma membrane (Watanabe & Tamai, 1992). In addition, the growth retardation of *Z. rouxii* might also be influenced by the inoculum size and ratio in the mixed culture. According to the finding of Kedia, Wang, Patel, and Pandiella (2007), in a mixed culture with *Lactobacillus reuteri* (LAB), yeast growth was inhibited at LAB:yeast ratio of 1:1 and 2:1, but enhanced at the ratio of 1:2.

3.2. Physicochemical changes in moromi fermentation

The reducing sugar content in all models containing *Z. rouxii* decreased to undetectable levels 5 days after inoculation (Fig. 2A). This reducing sugar depletion was due to sugar utilisation by yeast for propagation as well as ethanol conversion. Since *A. oryzae* in the moromi model was heat-inactivated before fermentation started, the mould could no longer perform starch hydrolysis resulting in lower availability of reducing sugar for *Z. rouxii*.

In contrast, the amount of reducing sugar in control and single culture of *T. halophilus* remained high throughout the incubation period. By the end of fermentation, 10.39 g/L and 10.42 g/L of reducing sugar were found in control and single culture of *T. halophilus*, respectively, which were relatively higher than the initial amount. Such increases could result from the hydrolysing activity of amylase produced by *A. oryzae*, which remains active during moromi fermentation (Chou & Ling, 1998; Cui et al., 2014). Higher amount of reducing sugar in sample treated with *T. halophilus* also indicates limited ability of *T. halophilus* in utilizing D-glucose and D-fructose.

The changing reducing sugar content was associated with the ethanol production during moromi fermentation (Fig. 2B). Ethanol was monitored since it is one of the main alcohols produced by *Z. rouxii* during brine fermentation *via* sugars conversion (Van Der Sluis et al., 2001). Reducing sugar depletion in all *Z. rouxii*-containing samples occurred in parallel with maximum ethanol production 5 days after *Z. rouxii* was inoculated. As expected, ethanol was not detected in the sample treated with single culture of *T. halophilus* and control (Fig. 2b), since the reducing sugar content remained stable. Once sugar in all *Z. rouxii*-containing samples was exhausted, ethanol content dropped to below detection level.

As expected, *T. halophilus* was found to play a major role in both lactic acid and acetic acid production (Fig. 2C and D). Surprisingly, even though *T. halophilus* growth was enhanced, production of both

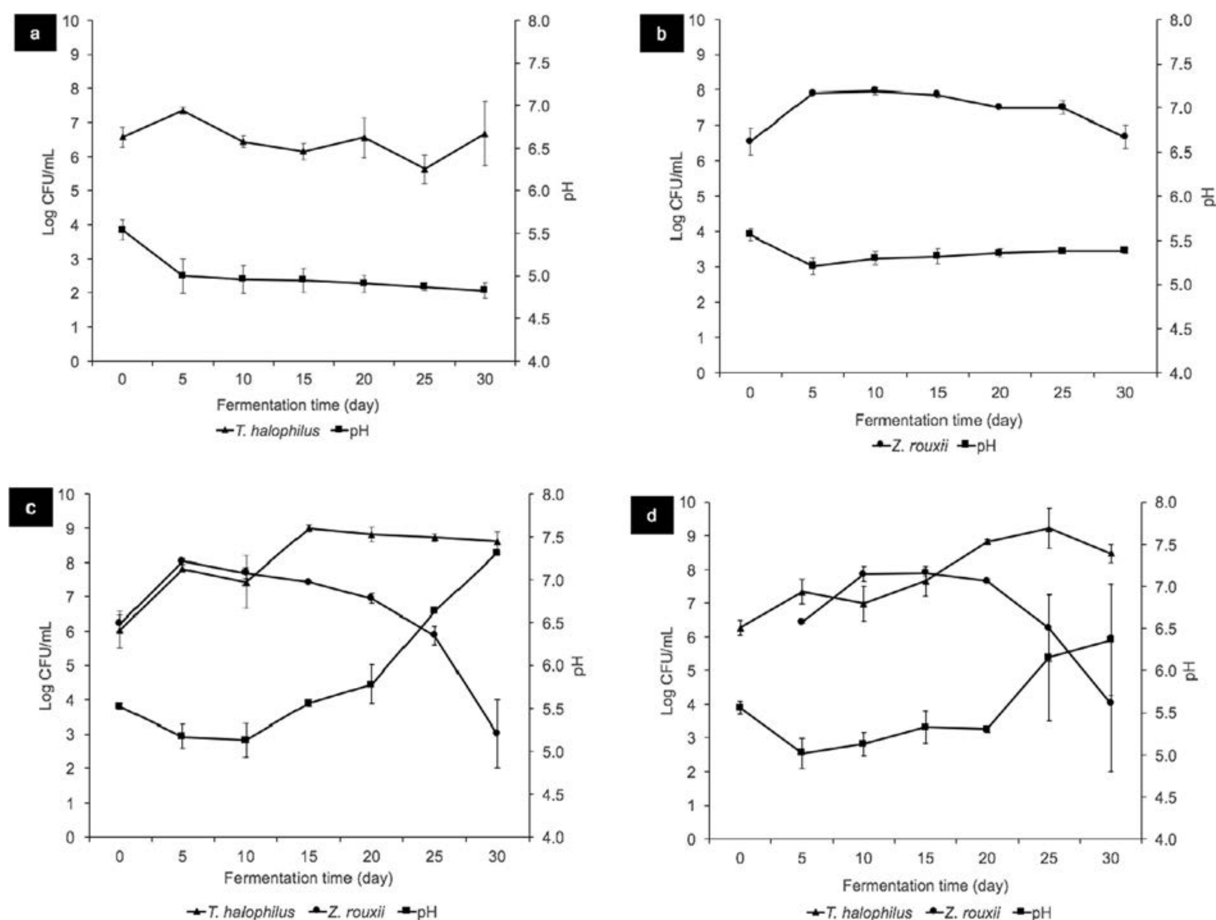

Fig. 1. Changes in pH and growth (cfu) of *T. halophilus* and *Z. rouxii* growth during moromi fermentation with pure culture of *T. halophilus* (a), pure culture of *Z. rouxii* (b), co-inoculation (c), and sequential inoculation (d).

acids was suppressed when *Z. rouxii* was present. In single culture, *T. halophilus* was able to produce lactic acid and acetic acid to a maximum level of 0.58 g/L and 0.26 g/L, respectively, which were the highest among all samples.

The amino nitrogen contents in all soy sauce samples were generally constant with some fluctuation throughout the fermentation process. Amino nitrogen changes might be due to the metabolic activity of *T. halophilus* or Maillard reaction (Cui et al., 2014), which is responsible for deep brown colour formation in soy sauce. This reaction involves condensation of carbonyls, such as reducing sugars and aldehydes, with compounds possessing a free amino group (Lertsiri, Maungma, Assavanig, & Bhumiratana, 2001; Martins, Jongen, & Van Boekel, 2000).

3.3. The effect of co-inoculation and sequential inoculation of T. halophilus and Z. rouxii on formation of volatile compounds

A total of 23 major volatile compounds was identified in the moromi samples, including eight alcohols, three acids, five aldehydes, two esters, two pyrazines, one furan, and two ketones (Table 1). Alcohols were found to be the most abundant compounds detected in all soy sauce samples which is in agreement with previous studies conducted under high (Feng et al., 2015) and low salt concentration (Singracha et al., 2017).

Higher amount of total alcoholic compounds was detected in the moromi treated with *Z. rouxii* either as single or mixed cultures, suggesting that *Z. rouxii* was mainly responsible for the alcohols formation. Fusel alcohols such as phenylethyl alcohol (floral, sweet), 3-methyl-1-butanol (malty, rancid, and pungent), and 2-methyl-1-propanol (bitter) were found to be the major alcohol components of the final moromi. Song et al. (2015a) also reported high production of the above fusel alcohols in reduced-salt moromi by adding indigenous yeast isolated from different stages during traditional moromi fermentation. These fusel alcohols could have been generated from their corresponding amino acids by *Z. rouxii* through the Ehrlich pathway (Jansen et al., 2003). Extracellular amino acids present during moromi fermentation can undergo deamination or transamination through the Ehrlich pathway, producing α-keto acids. The α-keto acids serve as the main key intermediates in the formation of fusel alcohols. Fusel alcohols are formed through decarboxylation and subsequent reduction of the corresponding α-keto acids (Van Der Sluis et al., 2001).

Combination of *Z. rouxii* and *T. halophilus* significantly increased 3-octanol (mushroom-like) production compared with the single cultures. Ethanol was obtained in significantly lower amount ($p < 0.05$) in single culture and co-culture compared to those treated with sequential inoculation. Ethanol is generally produced by *Z. rouxii* from glucose through the glycolytic pathway and anaerobic fermentation (Benitez & Codon, 2004).

Other alcohols such as 2-furanmethanol (sugar burnt) and methanol were detected in all samples before fermentation started. All samples contained 1-octen-3-ol (mushroom-like), but its amount decreased due to microbial activity during fermentation. Although most alcohols in soy sauce are originated from sugars and amino acids metabolism, small amounts of alcohols are also produced by yeast through metabolism of related aldehyde compounds (Sun, Jiang, & Zhao, 2010).

Aldehydes (furfural, benzaldehyde, 2-methylpropanal, 3-methylpropanal, 3-methylbutanal, and 2-methylbutanal) were the second main group of aroma compounds found in the soy mash. They were present in soy mash from the beginning of fermentation. However, their amount decreased significantly due to the activity of *Z. rouxii*,

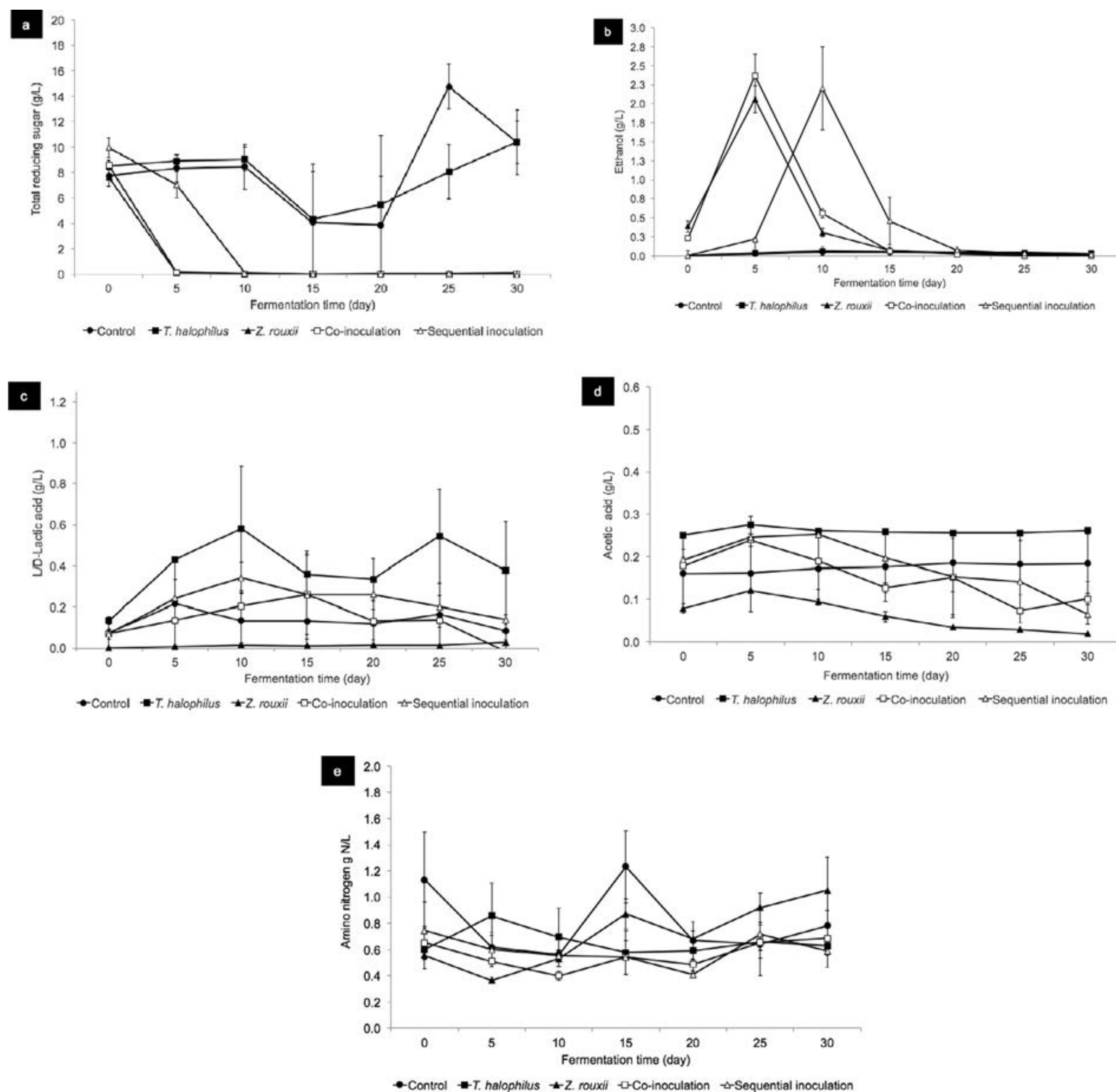

Fig. 2. The changes of total reducing sugar (a), ethanol (b), L/D-lactic acid (c), acetic acid (d), and amino nitrogen (e) during 30-day period of moromi fermentation at 30 °C with different combinations of microorganisms.

especially in mixed culture. The decrease might be due to aldehyde conversion to its corresponding alcohol (Song et al., 2015a). This hypothesis is supported by the fact that the amount of alcohols obtained in the samples treated with *Z. rouxii* is higher than control and single culture of *T. halophilus*. These aldehydes are responsible for soy sauce malty and nutty aroma (Feng et al., 2015).

Acetic acid was found to be the major volatile in single culture of *T. halophilus*. Its amount was significantly ($p < 0.05$) higher compared to other samples, since *T. halophilus* is known to produce acetic acid (Justé et al., 2012; Tanasupawat, Thongsanit, Okada, & Komagata, 2002). Acetic acid can give sour odour to the final product. Production of 3-methylbutanoic acid was favoured in single cultures of *T. halophilus* and *Z. rouxii*. It has been reported to be the major aroma compound in Chinese soy sauce and it is responsible for strong pungent, sweaty, and cheese-like odour notes (Sun et al., 2010). This branched-chain acid might be produced by *Z. rouxii* from branched-chain amino acids contained in soybean *via* the Ehrlich pathway (Lee et al., 2013). The presence of mixed culture seemed to suppress the production of 3-methylbutanoic acid.

Acetate esters comprising isoamyl acetate (banana aroma) and 2-phenylethyl acetate (honey, rosy) were detected in soy sauce samples fermented with *Z. rouxii*. Mixed cultures significantly enhanced the production of isoamyl acetate ($p < 0.05$). The production of 2-phenylethyl acetate was higher in samples treated with *Z. rouxii* either alone or in mixed cultures. Interestingly, sequential inoculation produces significantly higher amounts of 2-phenylethyl acetate than co-inoculation. Higher esters production in the presence of yeast could happen, since yeast is capable of esterifying alcohols with fatty acids (Van Der Sluis et al., 2001). Fatty acids can be produced through fungal lipase activity on the lipid contained in the soybean (Chung, 1999).

Pyrazines were detected in all samples and they might be produced through Maillard reaction between saccharide and amino residues (Sun et al., 2010). Trimethylpyrazine (burnt) and 2,5-dimethyl pyrazine (roasted nuts) were found in all samples, although 2,5-dimethylpyrazine was significantly higher in the mixed culture samples. The main furan compound detected was 2-pentylfuran and its levels were similar among samples. It is known as a singlet oxidation compound produced from linoleic acid (Lee et al., 2013).

Acetone seemed to be the major ketone found in all samples, which is in agreement with the findings of Lee et al. (2013), and

Table 1
Aroma compounds found in moromi before and after 30 days of fermentation with different combinations of microorganisms. The values are relative to the peak area observed when the headspace above a 0.1 µg/mL 1-octen-3-ol solution was analysed. Each value is based on three replicates.

Compound	LRI[1]	Day 0		Day 30									
				Control		*T. halophilus*		*Z. rouxii*		Co-inoculation		Sequential inoculation	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Alcohols													
Methanol	915	0.67a	0.12	0.82a	0.14	0.78a	0.08	0.75a	0.07	1.01a	0.07	0.55a	0.06
Ethanol	952	0.48a	0.10	4.86b	0.95	4.70b	0.40	1.51ac	0.19	0.29a	0.03	3.51bc	0.37
2-Methyl-1-propanol	1117	0.00a	0.00	0.01a	0.00	0.01a	0.00	0.06b	0.01	0.08b	0.00	0.07b	0.02
3-Methyl-1-butanol	1227	0.03a	0.00	0.87a	0.17	0.88a	0.06	14.93b	1.03	13.35b	0.49	11.68b	2.05
3-Octanol	1408	0.01a	0.00	0.00a	0.00	0.00a	0.00	0.07b	0.02	0.04c	0.01	0.04c	0.00
1-Octen-3-ol	1468	0.65a	0.06	0.28b	0.07	0.16b	0.02	0.18b	0.03	0.23b	0.04	0.21b	0.02
2-Phenylethanol	1961	0.06a	0.05	0.06a	0.04	0.07a	0.06	19.50b	1.96	16.45b	1.85	10.38c	0.59
2-Furanmethanol	1693	0.26a	0.03	0.23a	0.03	0.25a	0.03	0.01b	0.00	0.04b	0.00	0.02b	0.00
Acids													
Acetic acid	1486	0.12a	0.09	0.02a	0.04	14.34b	0.64	0.39a	0.31	0.09a	0.09	0.10a	0.12
2-methylpropanoic acid	1599	0.01a	0.01	0.00a	0.00	0.17a	0.01	0.42b	0.01	0.04a	0.02	0.16a	0.05
3-methylbutanoic acid	1700	0.05a	0.06	0.04a	0.04	2.03b	0.11	1.71b	0.14	0.14a	0.06	1.18ab	0.34
Aldehydes													
2-Methylpropanal	825	0.31a	0.06	0.06bc	0.03	0.09b	0.01	0.11b	0.01	0.00c	0.00	0.00c	0.00
2-Methylbutanal	930	0.18a	0.05	0.02b	0.01	0.03b	0.00	0.05b	0.01	0.00b	0.00	0.01b	0.00
3-Methylbutanal	934	0.14a	0.02	0.06b	0.03	0.07b	0.01	0.05b	0.00	0.00c	0.00	0.02c	0.01
Furfural	1502	1.11a	0.18	0.23b	0.04	0.14b	0.01	0.01b	0.00	0.01b	0.00	0.01b	0.00
Benzaldehyde	1570	0.31ab	0.05	0.44a	0.09	0.17bc	0.03	0.14c	0.02	0.03c	0.01	0.02c	0.00
Esters													
Isoamyl acetate	1140	0.00a	0.00	0.01a	0.00	0.03a	0.00	0.05a	0.01	0.27b	0.03	0.45b	0.16
2-Phenylethyl acetate	1860	0.01a	0.00	0.00a	0.00	0.00a	0.00	0.17b	0.03	0.15b	0.04	0.43c	0.02
Pyrazines													
2,5-Dimethylpyrazine	1353	0.03a	0.00	0.06a	0.01	0.17a	0.02	0.05a	0.01	2.00b	0.38	0.10ac	0.00
Trimethylpyrazine	1434	0.05a	0.01	0.05a	0.01	0.05a	0.01	0.05a	0.01	0.15a	0.02	0.05a	0.00
Furan													
2-Pentylfuran	1251	0.07a	0.02	0.05a	0.04	0.08a	0.01	0.08a	0.01	0.07a	0.02	0.08a	0.03
Ketones													
Acetoin	1321	0.15a	0.02	0.16a	0.02	1.23a	0.09	0.14a	0.01	0.00a	0.00	0.01a	0.00
Acetone	830	1.47a	0.23	0.62b	0.16	0.63b	0.09	0.59 b	0.01	0.15c	0.02	0.63b	0.04

Means within the same row with different letters (a, b, c) are significantly different ($p < 0.05$).
[1] LRI: linear retention indices of the compounds relative to an alkane series.

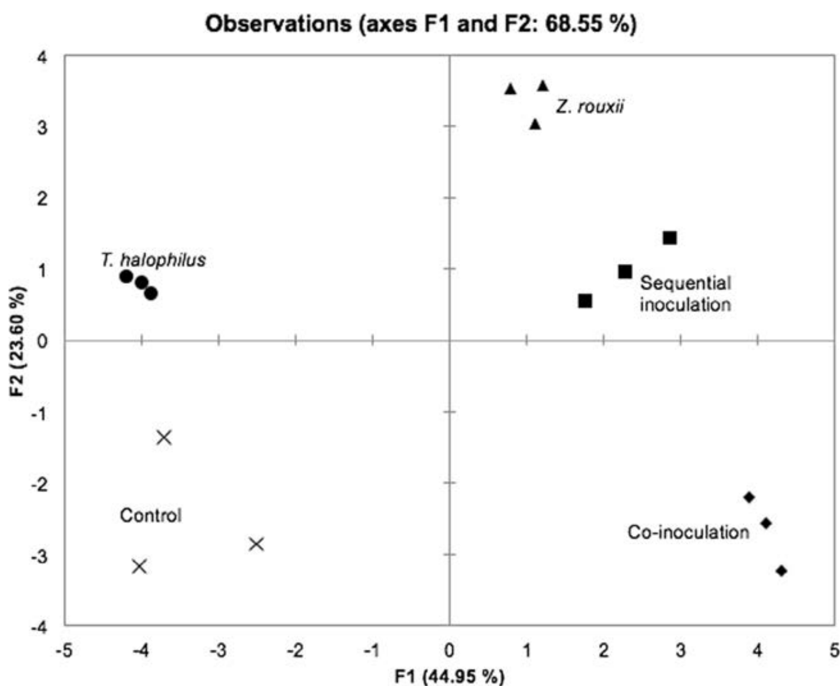

Fig. 3. PCA score plot of five moromi samples after 30-day fermentation. The scores are based on three replicates of each sample. The identical symbols represent triplicate measurements.

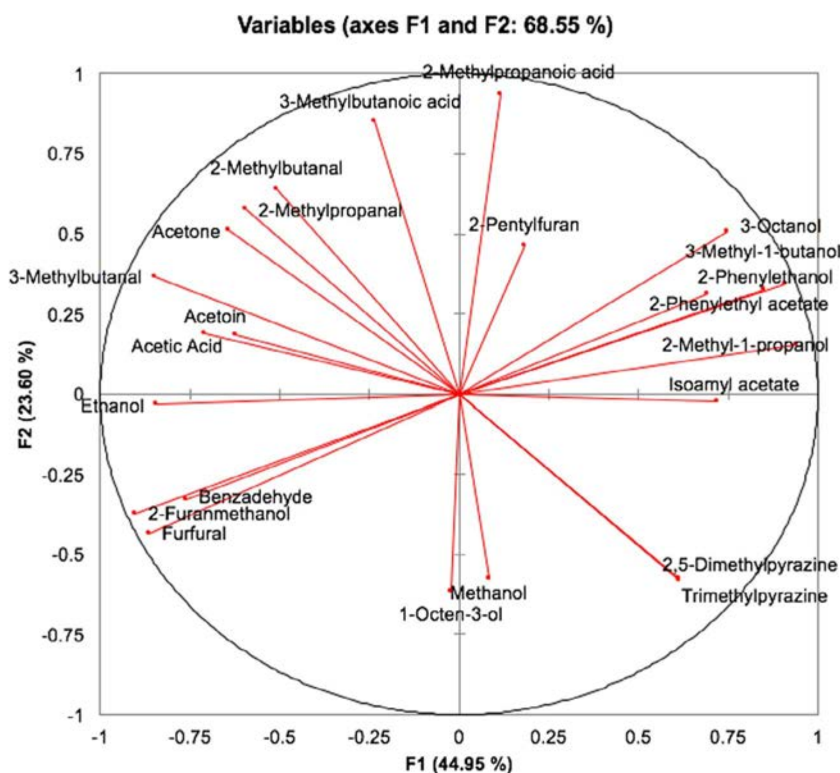

Fig. 4. PCA loading plot of the aroma compounds detected in moromi after 30-day fermentation with either single culture of *T. halophilus* and *Z. rouxii*, co-inoculation, and sequential inoculation.

Wanakhachornkrai and Lertsiri (2003). The formation of acetone is related to metabolism of microorganisms, especially yeast, *via* lipid oxidation (Lee et al., 2013; Song et al., 2015b). However, its amount was significantly higher at Day 0, suggesting that the acetone might have originated from koji. According to Feng et al. (2013), acetone found in koji could be derived from raw materials used in the koji making. Co-inoculation was found to significantly reduce the amount of acetone after 30 days' fermentation. Acetoin contributing to buttery odour was detected in all samples and its amount did not significantly vary. Therefore, acetoin might be derived from koji fermentation (Lee et al., 2013).

3.4. Principal component analysis

PCA was performed in order to understand the relationship between formation of volatile compounds, microbial species, and their order of inoculation. According to the PCA score plot (Fig. 3), all moromi samples were clearly differentiated, indicating that the microbial species and their order of inoculation contributed to different aroma profiles in each sample. Single culture of *Z. rouxii*, co-inoculation, and sequential inoculation were positioned in positive PC1 region. On the other hand, control and single culture of *T. halophilus* were located in the negative PC1, suggesting that *T. halophilus* alone is not sufficient to develop complex aroma profiles. The PCA loading plot (Fig. 4) shows this separation to be driven by a group of compounds dominated by alcohols (3-octanol, 3-methyl-1-butanol, 2-phenylethanol, 2-methyl-1-propanol, 2-phenylethyl acetate, isoamyl acetate, trimethylpyrazine and 2,5-dimethylpyrazine) while aldehydes are positioned on the left side of the plot (PC1; 44.95%). This is in agreement with the observed physicochemical changes discussed in Section 3.2, where higher amount of alcohols were detected in all moromi containing *Z. rouxii*.

Interestingly, sequential inoculation was distinguished from co-inoculation on PC2 (23.60%). Co-inoculation was distinguished by pyrazines and random groups of compounds. However, sequential inoculation accounted for significantly higher numbers of volatile compounds (13 out of 21) indicating production of more complex flavour profiles. PCA results demonstrated that simultaneous or subsequent inoculation of *Z. rouxii* matters to the development of flavour characteristics in moromi fermentation.

4. Conclusion

The inoculation sequence demonstrated the antagonistic relationship between *T. halophilus* and *Z. rouxii*, which also affected the physicochemical and volatile profile changes during reduced-salt moromi fermentation. Although the antagonistic interaction occurred in both co-inoculation and sequential inoculation, it resulted in different physicochemical changes, which seemed to favour the aroma profiles in the case of sequential inoculation. Since the microbial proliferation and activity is very important to the development of moromi, understanding microbial interactions can assist strategies for controlling microbial release and activity (e.g., encapsulation) or modulation of the inoculation time. Furthermore, varying the inoculum size and ratio between *T. halophilus* and *Z. rouxii* should also be investigated, in order to fully explore the interaction between the two microbes.

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Segregation of *Tetragenococcus halophilus* and *Zygosaccharomyces rouxii* using $W_1/O/W_2$ double emulsion for use in mixed culture fermentation

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ABSTRACT

Antagonism in mixed culture fermentation can result in undesirable metabolic activity and negatively affect the fermentation process. Water-oil-water ($W_1/O/W_2$) double emulsions (DE) could be utilized in fermentation for segregating multiple species and controlling their release and activity. *Zygosaccharomyces rouxii* and *Tetragenococcus halophilus*, two predominant microbial species in soy sauce fermentation, were incorporated in the internal W_1 and external W_2 phase of a $W_1/O/W_2$, respectively. The suitability of DE for controlling *T. halophilus* and *Z. rouxii* in soy sauce fermentation was studied in relation to emulsion stability and microbial release profile. The effects of varying concentrations of *Z. rouxii* cells (5 and 7 log CFU/mL) and glucose (0%, 6%, 12%, 30% w/v) in the W_2 phase were investigated. DE stability was determined by monitoring encapsulation stability (%), oil globule size, and microstructure with fluorescence and optical microscopy. Furthermore, the effect of DE on the interaction between *T. halophilus* and *Z. rouxii* was studied in Tryptic Soy Broth containing 10% w/v NaCl and 12% w/v glucose and physicochemical changes (glucose, ethanol, lactic acid, and acetic acid) were monitored. DE destabilization resulted in cell release which was proportional to the glucose concentration in W_2. Encapsulated *Z. rouxii* presented higher survival during storage (~3 log). The application of DE affected microbial cells growth and physiology, which led to the elimination of antagonism. These results demonstrate the potential use of DE as a delivery system of mixed starter cultures in food fermentation, where multiple species are required to act sequentially in a controlled manner.

1. Introduction

The utilization of mixed cultures in food fermentation is preferred over single culture since it offers benefits such as improved flavor production and aroma complexity (Narvhus & Gadaga, 2003; Smit, Smit, & Engels, 2005), food safety (Freire, Ramos, & Schwan, 2015), and health benefits (Chen et al., 2017). Each species of the mixed microbial community contributes to the fermentation process individually. However, antagonistic interactions have been observed due to production of growth-inhibitory compounds (Kemsawasd et al., 2015; Li & Liu, 2016), changes in physicochemical properties of the substrate (e.g. pH) (Devanthi, Linforth, Onyeaka, & Gkatzionis, 2018), competition for nutrients (Medina, Boido, Dellacassa, & Carrau, 2012), and cell-to-cell contact-mediated inhibition (Nissen, Nielsen, & Arneborg, 2003). Such interactions negatively affect the equilibrium in the mixed microbial community and performance of each species. Therefore, a formulation is needed for controlling the delivery and activity of these species, thus minimizing the effects of antagonistic interaction and maximizing flavor development. Compared to free cells system in fermentation, microbial encapsulation offers the benefits of higher cell density for faster fermentation and enhanced tolerance against adverse conditions.

Tetragenococcus halophilus and *Zygosaccharomyces rouxii* are predominant lactic acid bacteria (LAB) and yeast, respectively, during the second stage of soy sauce fermentation (or brine fermentation) called *moromi* (van der Sluis, Tramper, & Wijffels, 2001). The activity of these species is crucial since they produce key volatile compounds, taste active amino acids, peptides, and sugars that contribute to the final flavor of soy sauce (Harada et al., 2016; Zhao, Schieber, & Gänzle, 2016; Zhu & Tramper, 2013). However, our previous study has shown antagonistic interactions between the two microbes that result in compromising flavor development due to the rapid growth of *T. halophilus* producing lactic acid and acetic acid, suppressing the alcoholic fermentation by yeast (Devanthi et al., 2018). On the other hand, excessive alcohol

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production by *Z. rouxii* at the beginning of fermentation inhibits lactic acid fermentation (Kusumegi, Yoshida, & Tomiyama, 1998).

Encapsulation in alginate gel beads has been investigated on *Z. rouxii* and *Candida versatilis* during moromi stage of soy sauce process, in order to shorten the fermentation time (Hamada, Sugishita, Fukushima, Tetsuro, & Motai, 1991). However, this system was found to be unstable over time due to alginate's sensitivity to high salt concentration present in the brine added during moromi fermentation. As an alternative, encapsulation of *Z. rouxii* in polyethylene-oxide gel was found more stable in high salt concentration compared to alginate gel (van der Sluis et al., 2000). However, the production of these polymers is time consuming, costly, and not compatible with the composition of soy sauce.

Water-oil-water ($W_1/O/W_2$) double emulsion (DE) is a type of emulsion that contains two aqueous compartments separated by an oil phase. Its multi-compartmentalized structure could be used for delivering multiple starter cultures during fermentation, when minimum interference between species is required. Segregation of multiple microbial species was previously studied by Nissen et al. (2003) and Kemsawasd et al. (2015) during mixed culture fermentation using dialysis tubing and double-compartment fermentation system separated by cellulose membranes, respectively. Such compartmentalization was shown to reduce antagonism caused by cell-to-cell contact and antimicrobial peptides secretion. Moreover, stable mixed culture of *Lactococcus lactis* and *Bifidobacterium longum* was obtained during continuous fermentation in two-reactor system by separately immobilizing the two strains in κ-carrageenan/locust bean gum gel beads (Doleyres, Fliss, & Lacroix, 2004).

DE was previously reported for its ability to protect probiotic bacteria against adverse environment in human gastrointestinal tract (Pimentel-González, Campos-Montiel, Lobato-Calleros, Pedroza-Islas, & Vernon-Carter, 2009; Rodríguez-Huezo et al., 2014; Shima, Morita, Yamashita, & Adachi, 2006) and the controlled release of microbial cells based on osmotic pressure imbalance (El Kadri, Gun, Overton, Bakalis, & Gkatzionis, 2016; El Kadri, Overton, Bakalis, & Gkatzionis, 2015). However, the segregation of antagonistic cultures has not been studied using conditions relevant to fermentation. Furthermore, previous studies on microbial encapsulation in DE are limited to bacteria. Therefore, for DE to be used in soy sauce fermentation, it is important to understand its stability under relevant conditions and effect on microbiological and physicochemical changes.

The aim of this study was to investigate the feasibility of DE as a delivery system of soy sauce starter cultures, including its stability and release, effect on cell viability, and species-to-species interaction under conditions relevant to moromi stage of soy sauce fermentation. Brine solution and soybean oil were used as water and oil phases, respectively, in order to create a formulation that reflects the moromi process. The effects of varying concentrations of *Z. rouxii* in the W_1 phase and glucose in the W_2 phase on DE stability and release profile were investigated and the survival of the encapsulated *Z. rouxii* was monitored over storage. Also, the interaction between *T. halophilus* and *Z. rouxii* was investigated by monitoring the microbiological and physicochemical changes of the culture medium.

2. Material and methods

2.1. Materials

Soybean oil (Alfa Aesar, United Kingdom) was used as the oil phase of the DE. Polysorbate80 (Tween80, Sigma-Aldrich, United Kingdom) and polyglycerol polyricinoleate (PGPR, Danisco, Denmark) was used as water and oil soluble emulsifiers, respectively. Sodium chloride (NaCl, extra pure) and D(+)-glucose were purchased from Acros Organics (United Kingdom). The stain acridine orange (AO) was purchased from Sigma-Aldrich (United Kingdom).

Tetragenococcus halophilus 9477 and *Zygosaccharomyces rouxii* 1682 were purchased from National Collection of Industrial Food and Marine Bacteria Ltd. (NCIMB, United Kingdom) and National Collection of Yeast Cultures (NCYC, United Kingdom), respectively. For microbial growth, Brain Heart Infusion agar (BHI agar, Oxoid Ltd., United Kingdom), de Man, Rogosa, and Sharpe broth (MRS broth, Oxoid Ltd., United Kingdom), Yeast Malt agar (YM agar, Sigma-Aldrich, UK), Yeast Malt broth (YM broth, Sigma-Aldrich, UK), Tryptic Soy Agar (TSA, Oxoid Ltd., United Kingdom) and Tryptic Soy Broth (TSB, Oxoid Ltd., United Kingdom) media were used. Natamycin (Sigma-Aldrich, United Kingdom) and chloramphenicol (Oxoid Ltd., United Kingdom) were used for selective microbial growth.

2.2. Cultures preparation

T. halophilus was maintained on BHI agar supplemented with 10% (w/v) NaCl at 37 °C. Cells were transferred into MRS broth containing 7% (w/v) NaCl followed by incubation for 36 h in 37 °C static incubator. Final cell concentration was adjusted to 10^6 cells/mL. *Z. rouxii* was maintained on YM agar with 5% (w/v) NaCl and incubated at 30 °C. *Z. rouxii* cells were transferred into YM broth containing 5% (w/v) NaCl and incubated at 30 °C for 24 h with agitation (150 rpm). Cells (10^7 cells/mL) were harvested and washed by centrifuging at 10,000*g* for 15 min. In order to test the effect of initial cell concentration on emulsion stability, cell concentrations were adjusted to 10^8 cells/mL and 10^6 cells/mL.

2.3. DE preparation

The DEs were prepared using the 2-step emulsification method at ambient temperature by using a high shear mixer (Silverson L5M). In the first step, W_1/O primary emulsion was prepared by mixing sterile 10% (w/v) NaCl solution into the oil phase (soybean oil with 2% wt PGPR) at W_1:oil phase ratio of 20:80 at 1700 rpm for 2 min. For yeast encapsulation, *Z. rouxii* suspensions in 10% (w/v) NaCl solution (10^8 cells/mL and 10^6 cells/mL) were used as W_1.

In the second stage, W_1/O was re-emulsified in the continuous phase (W_2; sterile 10% (w/v) NaCl in water with 1% wt Tween80) at 2000 rpm for 1 min ($W_1/O:W_2$ ratio of 20:80). In order to study the effect of glucose on the stability of DE and *Z. rouxii* release profile, various concentrations of glucose (0%, 6%, 12%, and 30% w/v) were added to the W_2 in addition to 8.05% (w/v) NaCl (Table 1). The osmotic pressure gradient was calculated using Van't Hoff equation as follows:

$$\Delta\pi = (C_i - C_e)RT \tag{1}$$

where C_i is the solute concentration in the internal W_1 phase, C_e is the solute concentration in the external W_2 phase, R is the ideal gas constant, and T is the absolute temperature.

DEs containing *T. halophilus* in the W_2 were prepared by directly adding 2 mL of *T. halophilus* (10^6 cells/mL) into the W_2 after mixing process. For the study that investigates the effects of DE on *T. halophilus* and *Z. rouxii* interaction, both microorganisms in DE or as free cells (as a single or mixed cultures) were transferred into double concentrated TSB supplemented with 10% w/v NaCl and 12% w/v glucose. *T.*

Table 1
Formulation of $W_1/O/W_2$ double emulsions (DE) with varying glucose concentrations in the W_2 phase and directions of osmotic pressure gradients.

NaCl		Glucose		Δπ (atm)	Molar concentration of solute
W_1	W_2	W_1	W_2		
10%	8.05%	0%	0%	16.54	$W_1 > W_2$
			6%	8.28	$W_1 > W_2$
			12%	0	$W_1 = W_2$
			30%	− 24.84	$W_1 < W_2$

halophilus and *Z. rouxii* were inoculated at final concentrations of 10^6 CFU/mL and 10^5 CFU/mL, respectively, followed by incubation in 30 °C static incubator for 30 days.

2.4. DE stability characterization

2.4.1. Oil globule size measurement

The volume mean diameter (D 4,3) and particle size distribution of the DE were determined using Mastersizer 2000 (Malvern Instruments Ltd., Malvern, Worcestershire, UK) equipped with a He–Ne laser ($\lambda = 633$ nm). The analysis was done for the freshly prepared DE and as a function of storage time. The dispersion unit stirring speed was maintained at 2000 rpm and the measurement range was 0.02–2000 μm. The refractive index for the soybean oil and water were set at 1.474 and 1.330, respectively. The measurement was run at concentrations corresponding to obscuration of 10–20%.

2.4.2. Creaming volume measurement

The cream volume of DEs after preparation and during storage was monitored as described by El Kadri et al. (2015). Briefly, after gentle mixing, 1 mL sample was collected using 1 mL syringe and left standing upright for 1 h until the cream layer is formed on the top. The creaming volume percentage was calculated as follows:

$$\text{Creaming volume (\%)} = (\text{Creaming layer volume}/\text{Total volume of DE}) \times 100\% \quad (2)$$

2.4.3. Microscopy observation

DEs microstructure was observed by placing the samples onto microscope slides followed by observation under a light microscope (Olympus BX50) with a 10 × objective lens. Images were taken using Moticam 10 camera via Motic Images Plus video acquisition software at 17 fps.

In order to track the entrapped cells during storage, *Z. rouxii* cells were stained with AO before the entrapment process. Samples were placed onto microscope slides and gently covered with cover slips and imaged using Zeiss Axioplan fluorescent microscope equipped with objective lens 40 × magnification at ambient temperature. Images were captured using digital colour camera system Motic Moticam 10 using a 10 megapixel CMOC camera via Motic Images Plus video acquisition software.

2.5. Determination of the encapsulation efficiency and encapsulation stability of DEs

The encapsulation characteristics of DEs in this study are described as encapsulation efficiency and encapsulation stability. Encapsulation efficiency is defined as the percentage of *Z. rouxii* cells that are entrapped in the W_1 immediately after the emulsification process while encapsulation stability is described as the percentage of *Z. rouxii* cell that remains entrapped in the W_1 during storage.

The encapsulation efficiency and encapsulation stability were determined by counting the number of the non-encapsulated *Z. rouxii* cells in the serum phase (W_2). Five millilitre sample of DEs was collected and serum phase was removed using syringe. Cells were counted using Nageotte cell counting chamber under optical microscope (20 × magnification). Cell concentration (cell/mL) was calculated using this following formula:

$$\text{Cell concentration (cell/mL)} = (\text{Total number of cells} \times 25 \times 10^4)/\text{Number of squares} \quad (3)$$

Encapsulation efficiency (EE) and encapsulation stability (ES) were determined using the following equations:

$$\text{Encapsulation efficiency (\%)} = ((N_0 - N_{w2})/N_0) \times 100\% \quad (4)$$

$$\text{Encapsulation stability (\%)} = ((N_0 - N_{w2(t)})/N_0) \times 100\% \quad (5)$$

where N_0 is the number of free *Z. rouxii* cells initially added in the inner phase, while N_{w2} and $N_{w2(t)}$ are the number of non-encapsulated *Z. rouxii* cells measured immediately after DEs were formed and as a function of storage time, respectively.

2.6. *T. halophilus* and *Z. rouxii* cell enumeration

Viable cell counts were made by taking 0.1 mL of samples subjected to serial dilution in PBS (phosphate buffered saline) buffer solution followed by plating on BHI agar supplemented with 7% (w/v) NaCl and 21.6 mg/L natamycin for *T. halophilus* and YM agar with the addition of 5% (w/v) NaCl and 100 mg/L chloramphenicol for *Z. rouxii*. Bacteria and yeast colonies were counted after 2 days of incubation at 30 °C.

2.7. Physicochemical changes

Glucose concentration was measured using Accu-chek Aviva glucose monitor with Accu-chek Aviva glucose test strips (Roche Diagnostics, United Kingdom). Lactic acid was analyzed using enzymatic assay kit (Megazyme, International Ireland Ltd., Ireland) according to the manufacturer instructions. Acetic acid and ethanol were determined using gas chromatography (GC).

GC analysis was performed using GC-2010 (Shimadzu, Japan), equipped with a flame ionization detector (FID). Prior to analysis, samples were filtrated through 0.22 μm pore size filter (Millex GP, Millipore, United Kingdom) and 300 μL of samples were added with 200 μL hexylene glycol (Sigma Aldrich, United Kingdom) as an internal standard at final concentration of 742 mg/L. Samples (1 μL) were injected using auto sampler with split ratio of 100:1 at 260 °C. Compound separation was done by using ZB-WAX plus column (30 m, 0.25 mm I.D., 0.25 μm film thickness, Phenomenex, United States) and helium as the carrier gas at a pressure of 104.99 kPa. The oven temperature was programmed at an initial temperature of 30 °C for 5 min, followed by an increase to 50 °C at 4 °C/min (held for 5 min), 150 °C at 20 °C/min (held for 5 min), 200 °C at 10 °C/min (held for 5 min), and finally increase to 220 °C at 4 °C/min. FID temperature was set to 300 °C.

2.8. Statistics

Each experiment was conducted in triplicate ($N = 3$) and the results are expressed as mean ± standard deviation. Significant differences among means were tested by one-way analysis of variances (ANOVA) using IBM SPSS Statistics Software version 21 at $p < 0.05$ and Tukey's test was applied for means comparison.

3. Results and discussion

3.1. Encapsulation efficiency and stability during storage

The amount of encapsulated *Z. rouxii* cells was monitored over storage (Fig. 1). *Z. rouxii* was successfully encapsulated in the internal W_1 phase of DEs with high encapsulation efficiency (> 99%; Fig. 1a) regardless of low (10^5 CFU/mL) or high (10^7 CFU/mL) cell concentrations. Relatively high encapsulation stability of DE was maintained up to 14 days of storage (> 75%), and significantly ($p < 0.05$) decreased at day 30 to 13.28% and 30.72%, for low and high cell concentration, respectively. This observation was associated with the fluorescence microscopy images (Fig. 1b–d) in which non-encapsulated cells were observable in the external W_2 phase at day 30. Furthermore, the stability of DE decreased over time regardless of the presence and amount of encapsulated cells, as indicated by the loss in inner W_1 phase after 30 days (Fig. 2a). Such time dependent loss of inner W_1 phase could occur due to coalescence between the W_1 droplets as well as coalescence between W_1 droplets and the oil globule's interface (Chávez-Páez,

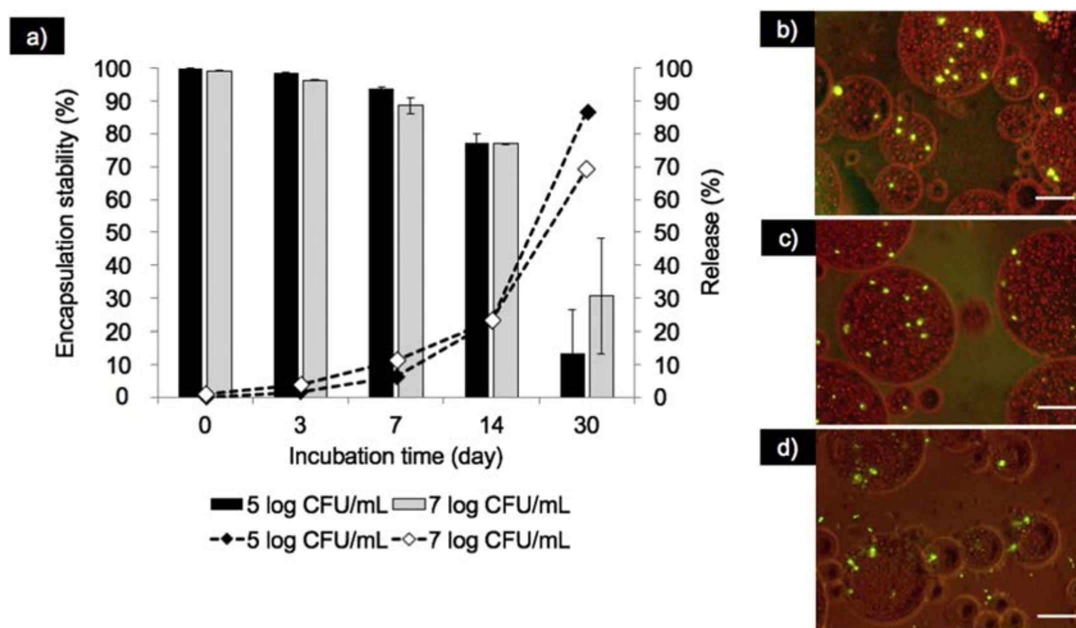

Fig. 1. (a) Changes in the percentage of entrapped (bar chart) and released (line chart) *Z. rouxii* cells in DEs prepared under iso-osmotic conditions over 30 days of storage at 30 °C. (b) Fluorescence microscopy images of the entrapped *Z. rouxii* cells at day 0, (c) day 7, and (d) day 30. Scale bar: 100 μm.

Quezada, Ibarra-Bracamontes, González-Ochoa, & Arauz-Lara, 2012). Ficheux, Bonakdar, and Bibette (1998) found that Tween80 migrates from the oil globule's interface through the oil phase to the W_1 droplet's interface and displaces the lipophilic surfactant (Span 80) molecules which causes an increase in coalescence events between the W_1 droplet and the oil globule's interface leading to DE to become a single O/W emulsion. In this study, such coalescence events may have occurred resulting in the release of hydrophilic substances including *Z. rouxii* cells into the W_2 phase. Although the amount of W_1 phase decreased, the average size (Fig. 2b) and size distribution of the oil globules (Fig. 2c–e) were apparently preserved throughout storage and this might be attributed to coalescence occurring between the W_1 droplets and the oil globule's interface as well as between the oil globules. Such coalescence events have shown to increase the size of the interfacial film of the oil globules despite loss in the W_1 phase maintaining the oil globule's size (Ficheux et al., 1998). These results indicate the possibility to use such inherent instability of DE as a mechanism for the release of *Z. rouxii* cells during fermentation.

3.2. The effect of glucose concentration on cell release and DE stability

During moromi fermentation of soy sauce, *Z. rouxii* converts glucose into biomass and ethanol. Changes in glucose concentration would alter the osmotic pressure balance between the two phases of DE, therefore affecting its microstructure and encapsulation stability. For this reason, the microstructure of DE (Fig. 3a), *Z. rouxii* cells release profile (Fig. 3b), and the oil globule size (Fig. 4a–c), were monitored by varying glucose concentration (0%, 6%, 12%, and 30%) in the external W_2 phase, which created osmotic pressure gradient between W_1 and W_2 phase, except for 12% which was designed to have balanced osmotic pressure (Table 1). Prior to investigation, the ability of *Z. rouxii* to grow in glucose solution (5%) in the absence of other nutrients was tested and the viable cells decreased by 2.53 log CFU/mL after 7 days of incubation (data not shown). This aimed to ensure that the quantified cells during the release study were solely due to release from the W_1 to W_2 phase and not the result of microbial growth.

The release profile was found to be influenced by the amount of glucose in the W_2 phase (Fig. 3b) and it followed a similar pattern to the loss in the W_1 phase (Fig. 3a), by which the complete loss in the W_1 phase was observed when maximum cell release occurred. However, the DE instability and cell release rate were found to be driven by increasing amount of glucose, rather than the osmotic pressure difference between the two phases. In the presence of 30% glucose ($\Delta\pi = -24.84$ atm), the DE was transformed into O/W single emulsion due to complete loss of the inner W_1 phase within 3 days, accompanied with a sharp increase in the number of released cells which was followed by a plateau thereafter. Meanwhile, the release of *Z. rouxii* cells in 6% glucose was gradual throughout storage and took place in a manner comparable to control (0% glucose). The destabilization of DE containing 0% and 6% glucose was reduced as the oil globules maintaining their inner W_1 phase were still noticeable by the end of storage. Although DE with 12% glucose was designed to be osmotically balanced ($\Delta\pi = 0$ atm), the DE microstructure was found to be more unstable compared to DEs with 0% ($\Delta\pi = 16.54$ atm) and 6% glucose ($\Delta\pi = 8.28$ atm) as it was transformed into O/W single emulsion by the end of storage. This also resulted in higher amount of cell release compared to DEs with 0% and 6% glucose. These results suggest that the faster release of *Z. rouxii* was associated with increased destabilization of the DE.

The phenomena observed in this study are in contrast to the previous studies reporting the effect of glucose in W_2 on osmotic pressure alteration of DE. The presence of glucose causes an osmotic pressure imbalance which forces water to migrate from the W_1 to W_2 phase and vice versa depending on the direction of the osmotic pressure gradient. This can destabilize the DE resulting in morphological changes as well as the release of entrapped materials (Frasch-Melnik, Spyropoulos, & Norton, 2010; Mezzenga, Folmer, & Hughes, 2004). However, increased salt release proportional to the glucose concentration was also observed by Pawlik, Cox, and Norton (2010). The authors suggested that PGPR was able to increase glucose lipophilicity, therefore it became surface active. According to Garti (1997), the increasing amount of lipophilic surfactant can increase the transport rate of water, surfactant, and water soluble molecules even when there is no osmotic pressure gradient. The excess amount of lipophilic emulsifier can increase the flux of water through reverse micelles formation. In the present study, glucose might also behave as lipophilic emulsifier facilitating water movement to the external W_2 phase through reverse micellar transport, which eventually led to release of *Z. rouxii* cells since yeast cells are

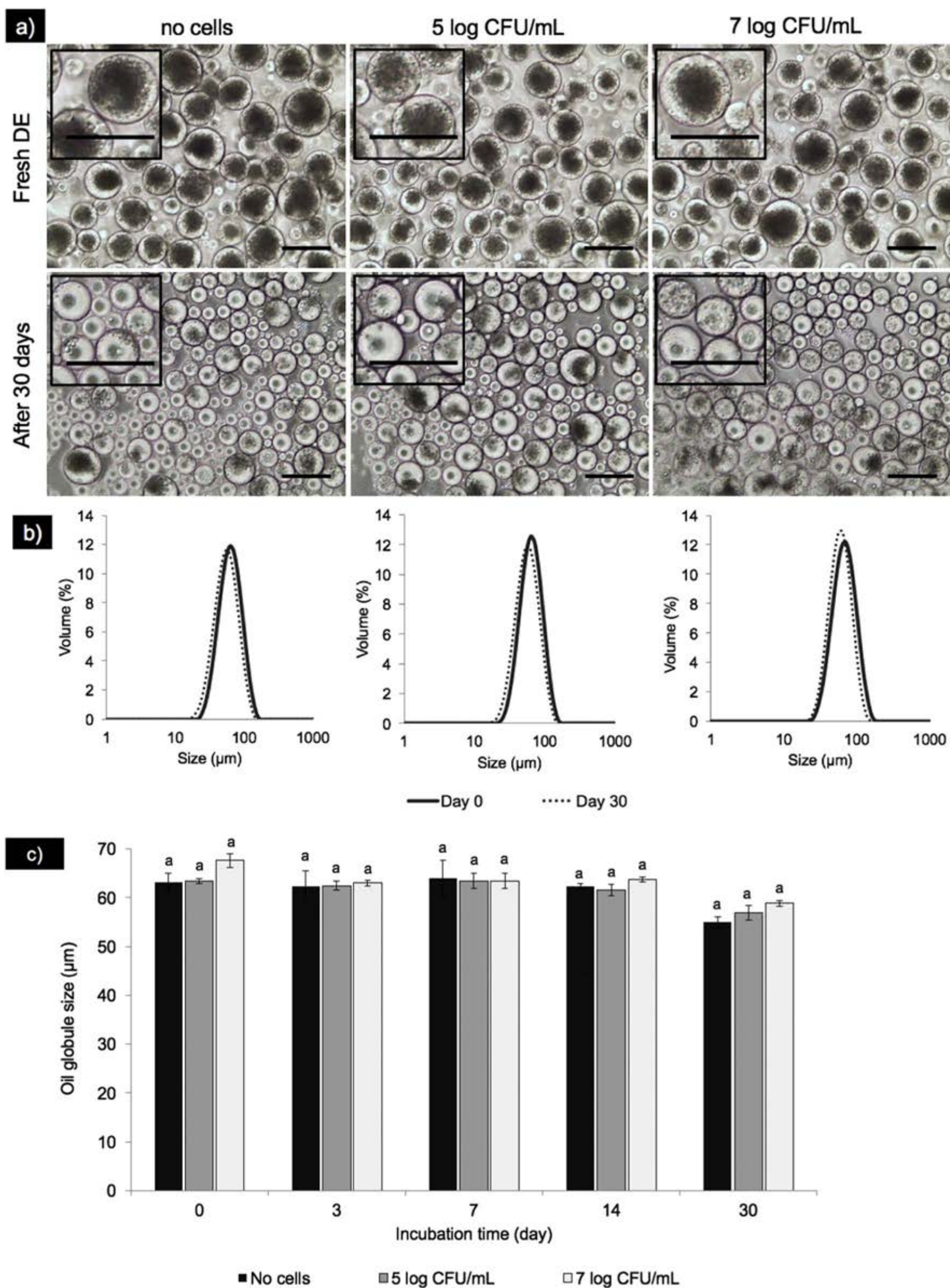

Fig. 2. DEs with no cells, 5 log CFU/mL, and 7 log CFU/mL before and after 30 days of storage at 30 °C under iso-osmotic condition. (a) Optical micrographs; (b) Oil globule size distribution; (c) Average oil globule size. Scale bar: 100 μm. Mean values with different letters are significantly different ($p < 0.05$).

hydrophilic and therefore would preferentially reside within the aqueous W_2 phase and not the oil phase. Furthermore, the release of *Z. rouxii* cells might also be driven by bursting mechanism. According to El Kadri et al. (2016, 2015), osmotic pressure balance alteration can lead to oil globule bursting which can be used to modulate the release of bacterial cells. However, the release of *Z. rouxii* cells from DE might involve not one but various mechanism and further investigation is required for a better understanding on how *Z. rouxii* cells are being released.

DE prepared with the highest concentration of glucose (30%) in its W_2 phase possessed the lowest initial oil globule size (37.28 ± 0.74 μm; Fig. 4a), even though the mixing speed and conditions during the two-step homogenizing process was maintained for all the formulations. This is expected as the addition of glucose

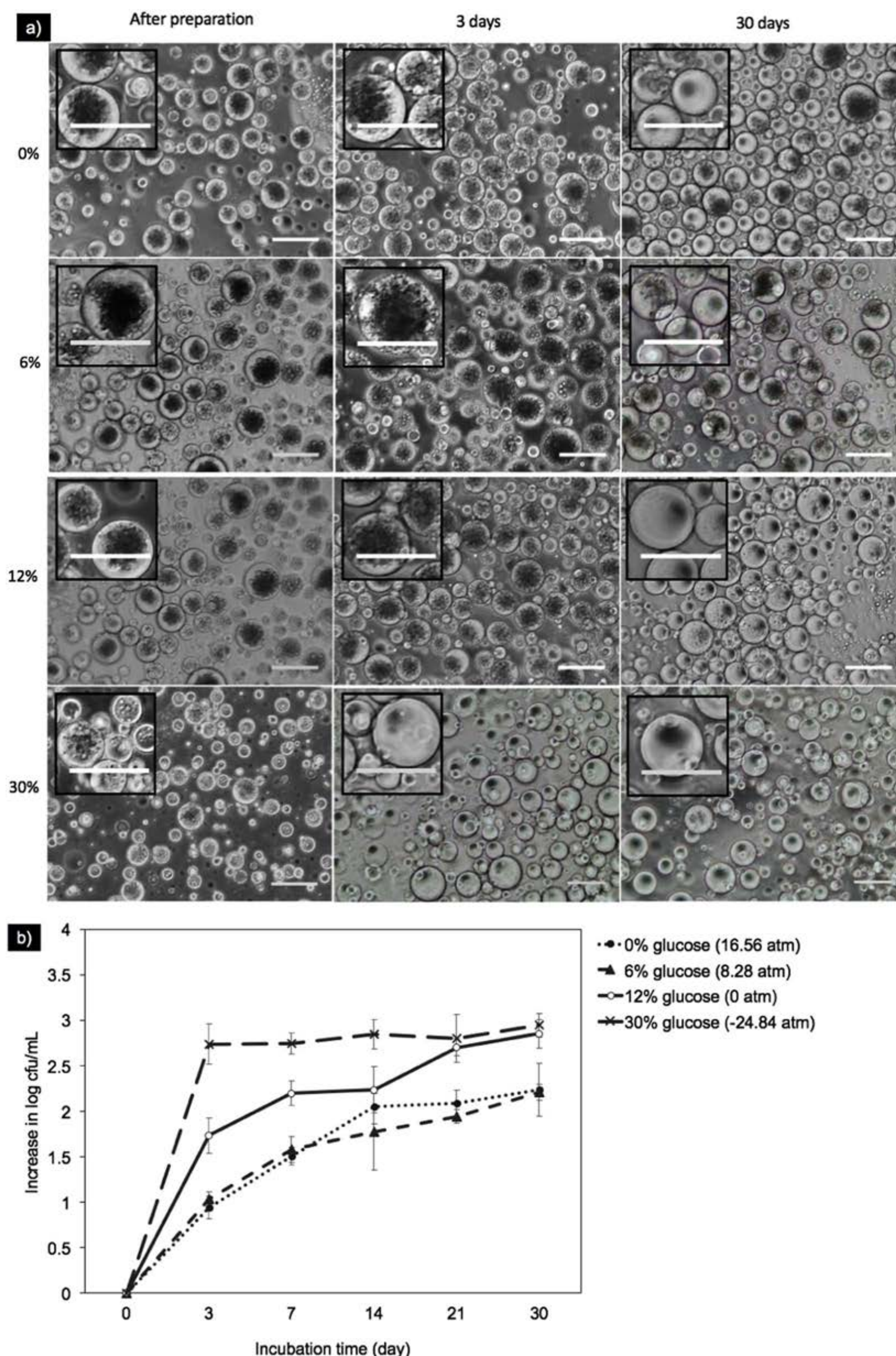

Fig. 3. Double emulsion with 0%, 6%, 12%, and 30% glucose in the W_2 phase after preparation, 3, and 30 days of storage at 30 °C. (a) Optical micrographs of $W_1/O/W_2$. (b) *Z. rouxii* cell release profile into W_2 phase. Scale bar: 100 μm.

increases the viscosity of the W_2 phase which leads to smaller oil globules to form (Khalid, Kobayashi, Neves, Uemura, & Nakajima, 2013). Furthermore, it has been reported that glucose can further reduce the interfacial tension which also contributes to the observed reduction in oil globule size (Pawlik et al., 2010). Decrease in size of oil globule during storage occurred in cases of 0%, 6%, and 12% glucose although these responses were not statistically significant ($p < 0.05$) (Fig. 4a). In contrast, DE containing 30% glucose showed significant ($p < 0.05$) increase in oil globule size at day 3 which then stabilised until the end of storage period, although the oil globules lost their inner W_1. This can be attributed to the increase in coalescence events between the oil globules as it becomes less stable in the presence of glucose. These

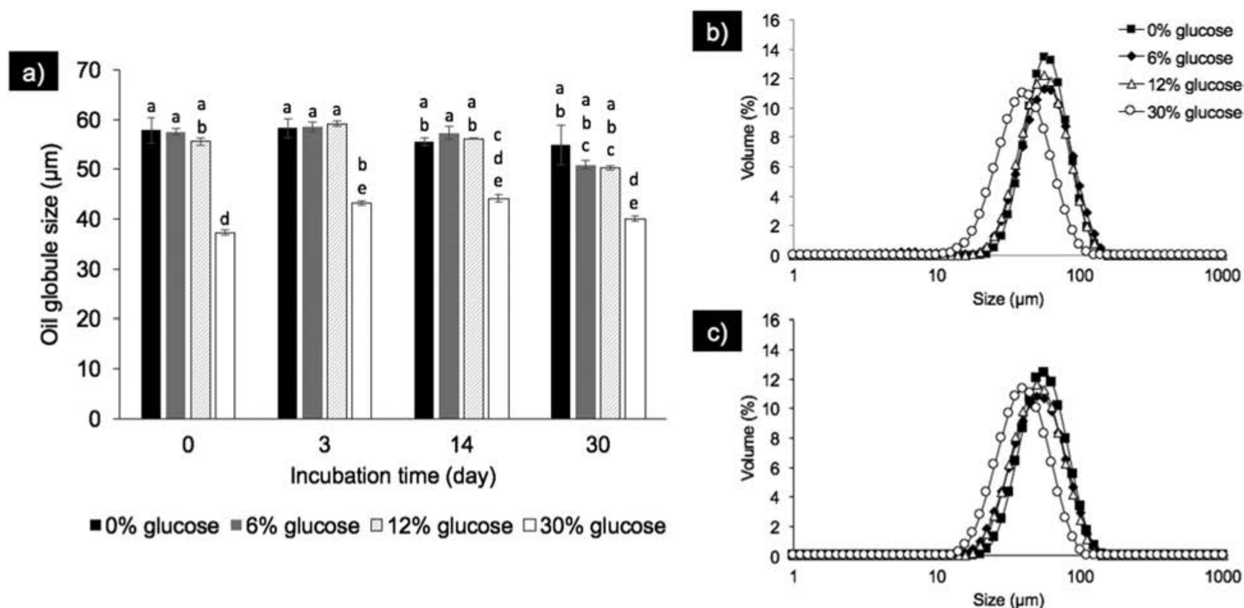

Fig. 4. DEs before and after 30 days of storage at 30 °C with different glucose concentrations in the external W_2 phase. (a) Average oil globule size; (b) Oil globule size distribution of DEs before storage and (c) after storage. Mean values with different letters are significantly different ($p < 0.05$).

results show that the stability of DE and release of *Z. rouxii* are influenced by the glucose concentration regardless of the osmotic pressure gradient between the two phases. However, the responses do not follow the same direction or linearity in all cases.

3.3. *Z. rouxii* cell viability after emulsification and during storage

To investigate the effect of emulsification and encapsulation on survival of *Z. rouxii*, cell viability was assessed immediately after encapsulation and during storage. The relative viability of *Z. rouxii* cells soon after the emulsification process was ~100% (Fig. 5), showing that the encapsulation technique as well as the surfactants used did not affect the yeast. This was reported in other studies for bacterial cells (El Kadri et al., 2015; Shima et al., 2006). Interestingly, the encapsulated cells viability remained high during 30 days of storage in the absence of nutrient (~2 log CFU/mL decrease), while no viable cells were detected in non-encapsulated cells by the end of incubation period (Fig. 5). The oil layer which functions as a barrier, might reduce mass transport and biological communication between the *Z. rouxii* cells and the environment and thus result in molecular gradient that could switch cells to the

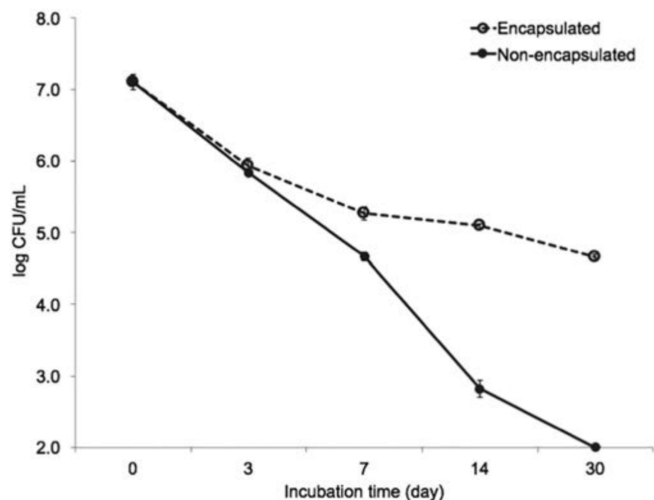

Fig. 5. Changes in *Z. rouxii* cells viability over 30 days of storage at 30 °C.

non-dividing resting state (G0) (Wang et al., 2008). Furthermore, the cells resistance towards environmental stress increases once it enters the resting state, including the ability to survive extended periods of starvation (Herman, 2002). It could be argued that *Z. rouxii* may have utilized the surfactants (PGPR and Tween80) as carbon sources (Luh, 1995), thus enabling the yeast to grow. However, no growth was observed when *Z. rouxii* was incubated with PGPR or Tween80 only (data not shown). These results indicate that encapsulation in DE is able to prolong life of *Z. rouxii* in the absence of nutrients.

3.4. The effects of encapsulation on *T. halophilus* and *Z. rouxii* interactions

Interaction between microbial species during fermentation would influence their growth which further affects the proportion of microbial population and their metabolic activity. In this study, the co-presence of *T. halophilus* and *Z. rouxii* resulted in antagonism as *T. halophilus* growth was inhibited, as indicated by a sharp decrease in *T. halophilus* cell count to undetectable level (< 2 log CFU/mL) at day 15 (Fig. 6c). This observation was in contrast to our previous study in which the growth inhibition was observed on *Z. rouxii* instead of *T. halophilus*, when both were co-present in a moromi model system (Devanthi et al., 2018). According to a study by Noda, Hayashi, and Mizunuma (1980), metabolite produced by *Pediococcus halophilus* (later reclassified as *T. halophilus*) during moromi fermentation can inhibit the growth of osmophilic *shoyu* yeasts such as *Saccharomyces rouxii* (later reclassified as *Z. rouxii*) and *Torulopsis versatilis*. However, a study by Inamori, Miyauchi, Uchida, and Yoshino (1984) showed that the growth inhibition in mixed cultures could occur to *P. halophilus* under aerobic conditions or *S. rouxii* under anaerobic conditions in static culture. Growth inhibition of *T. halophilus* in this study was possibly due to the aerobic conditions used during incubation. Also, inhibitory effect towards *Z. rouxii* which was previously reported, was observed in a digested liquid mixture of pre-cooked soybean and roasted wheat (Devanthi et al., 2018; Noda et al., 1980), while in this study interaction assay was performed in a synthetic broth medium. Furthermore, the presence of acetic acid in this study was unlikely to cause growth inhibition on *Z. rouxii* as previously reported (Kusumegi et al., 1998; Noda, Hayashi, & Mizunuma, 1982). It was suggested that acetic acid could interfere with proton expulsive activity of *Z. rouxii* for its halo-tolerance mechanisms, causing growth inhibition at NaCl concentration above 10%. In this study, we

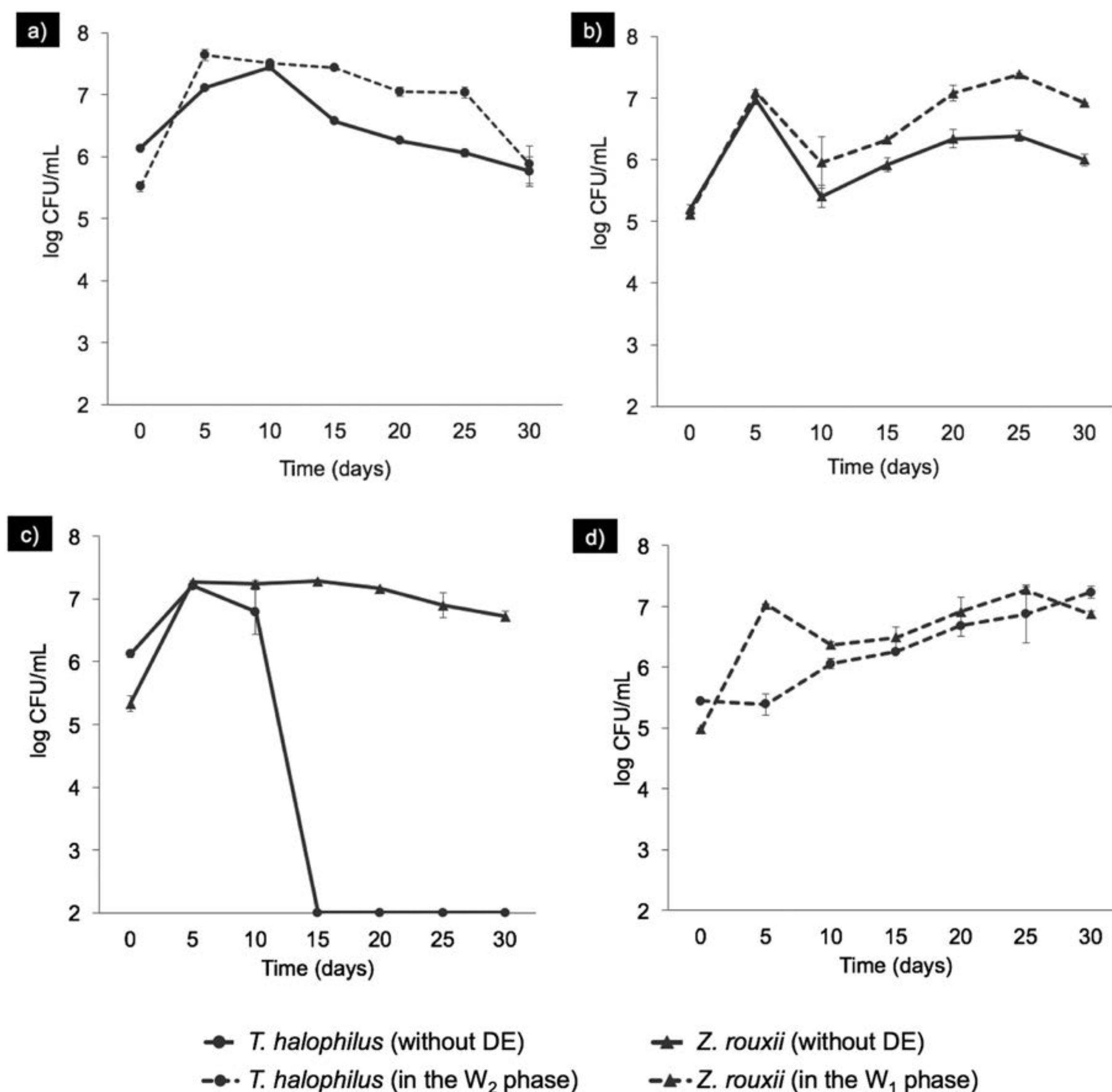

Fig. 6. Changes in viable cell number of (a) *T. halophilus*, (b) *Z. rouxii*, and mixed culture (c) without and (d) with DE, during 30 days of incubation at 30 °C.

did not observe any decrease in *Z. rouxii* cells population which was possibly due to relatively low NaCl concentration (10%) used in the medium.

The compartmentalization of *T. halophilus* and *Z. rouxii* in DE affected the growth kinetics in both single and co-culture. The growth of *T. halophilus* (Fig. 6a) and *Z. rouxii* (Fig. 6b) as single culture was slightly enhanced and the antagonism between *T. halophilus* and *Z. rouxii* was no longer observed when *Z. rouxii* was encapsulated in DE (Fig. 6d). *T. halophilus* was able to propagate steadily throughout the incubation period, reaching a final count of 7.23 log CFU/mL (Fig. 6d). The final cell counts of *Z. rouxii* in DE (6.87 log CFU/mL) did not differ significantly ($p < 0.05$) from non-DE culture (6.72 log CFU/mL), although a different growth pattern was observed, and its growth was not affected by the presence of *T. halophilus* in the W_2 phase. The oil layer functions as a physical barrier separating *T. halophilus* from *Z. rouxii*, thus minimizing antagonistic interaction between them. Also, the oil layer could serve as a selective membrane, allowing chemicals or molecules to diffuse in or out based on their molecular weight (Zhang et al., 2013). In this study, deleterious metabolite compounds produced by *Z. rouxii*, might not be able to pass through the oil layer to the bulk medium (W_2 phase) due to its molecular weight, thus minimizing its harmful effects towards *T. halophilus*. The ability of DE to gradually release the *Z. rouxii* into the bulk medium might also prevent detrimental effects towards *T. halophilus*. However, high *Z. rouxii* cell population was observed in the bulk medium due to their propagation after being released and yet the inhibitory effect towards *T. halophilus* was absent. *T. halophilus* might have exhibited physiological changes in the presence of DE, increasing its tolerance against inhibitory effect of *Z. rouxii*.

3.5. Physicochemical changes in DE during T. halophilus and Z. rouxii growth

To further understand how the presence of DE with single or mixed cultures can affect the interaction between the two microorganisms the physicochemical changes during fermentation were monitored. As seen in Fig. 7a–d, the presence of DE caused alteration in the metabolic activity of both microorganisms as a single or mixed cultures.

Glucose consumption (Fig. 7a) correlated with ethanol production (Fig. 7b). Glucose was exclusively consumed by *Z. rouxii*, therefore, ethanol was only produced in its presence. Both glucose consumption and ethanol production were accelerated when *Z. rouxii* was encapsulated. With mixed cultures, glucose was consumed in a gradual manner in the absence of DE which was accompanied by a slow

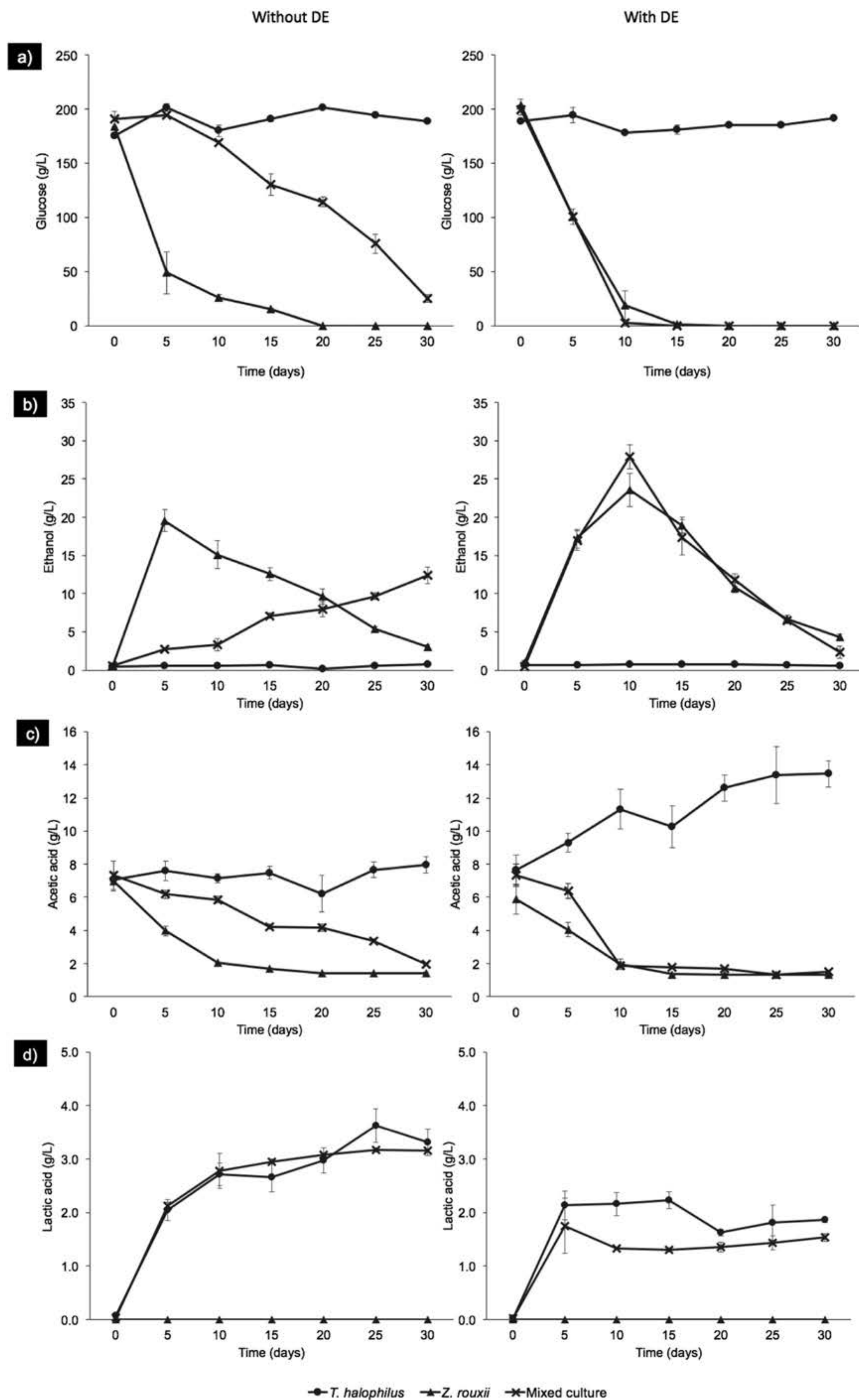

Fig. 7. Changes in (a) glucose; (b) ethanol; (c) acetic acid; and (d) lactic acid; during fermentation with and without DE.

production of ethanol, reaching maximum concentration of 12.39 g/L at day 30. In contrast, glucose was consumed faster in DE as it was depleted at day 10, associated with maximum ethanol production (27.94 g/L) which was comparable to concentrations in good quality soy sauce (Luh, 1995). Similar level of ethanol was also obtained in rapid fermentation of soy sauce described by Muramatsu, Sano, Uzuka, and Company (1993). Once glucose was depleted, the ethanol production was terminated and its concentration continuously decreased throughout the incubation period. Encapsulation seemed to delay glucose consumption by *Z. rouxii* as only half amount of glucose was consumed during the first 5 days when *Z. rouxii* was encapsulated. However, this led to prolonged ethanol production for up to 10 days, producing higher maximum concentration of ethanol (23.56 g/L) compared to non-encapsulated cells (19.57 g/L) with single culture.

T. halophilus played a major role in both acetic acid (Fig. 7c) and lactic acid (Fig. 7d) formation. In mixed culture, acetic acid concentration gradually decreased when *Z. rouxii* was non-encapsulated, while the acetic acid concentration sharply decreased within the first 10 days to 1.86 g/L when *Z. rouxii* was encapsulated. This was comparable to the amount of acetic acid found in top-graded bottled soy sauces in China (Xu, 1990). However, the acetic acid production by *T. halophilus* as single culture markedly increased by 1.7 fold in the presence of DE although *T. halophilus* was non-encapsulated. In contrast, lactic acid production was suppressed when DE was present, as the amount of lactic acid remained stable from day 5 onwards, and the suppression was more obvious in mixed cultures. The yield of lactic acid in the presence of DE was about half of the bottled soy sauces in China (Xu, 1990). In contrast, lactic acid increased exponentially in all non-DE systems, reaching almost twice the amount of lactic acid produced in the presence of DE. The presence of DE might have caused a shift in metabolic pathway of *T. halophilus* cells from homofermentative to heterofermentative, thus decreasing the lactic acid yields (Krishnan, Gowda, Misra, & Karanth, 2001).

These results suggest that the presence of DE affects the physicochemical changes during *T. halophilus* and *Z. rouxii* growth in both single and mixed culture. Changes in microbial cells morphology and physiology due to immobilization have been reported in several studies reviewed by Lacroix and Yildirim (2007), including increase in the production of insoluble exopolysaccharides (Bergmaier, Champagne, & Lacroix, 2005), lactic acid (Lamboley, Lacroix, Artignan, Champagne, & Vuillemard, 1999), as well as a shift in metabolic pathway from homofermentative to heterofermentative, resulting in decreased lactic acid production (Krishnan et al., 2001). The altered metabolic activity may have contributed to the elimination of antagonism by reducing the production of inhibitory metabolites or enhancing the production of metabolites essential for *T. halophilus* growth by *Z. rouxii*, as well as enhancing cell adaptation towards changing environmental conditions. However, further investigation is required to understand how the presence of DE affects the cells both in the W_1 and W_2 phase at the metabolic level.

4. Conclusion

The results in this study suggest that DEs could be a suitable formulation for the delivery of mixed starter cultures in soy sauce fermentation. *Z. rouxii* was successfully encapsulated in DE which enhanced survival during storage and eliminated antagonistic interaction with *T. halophilus*. The presence of DE altered the metabolic activity of the two species, which could have contributed to the elimination of antagonism. Although the initial encapsulation efficiency was high, it decreased over time due to DE instability and this could be utilized as a mechanism for gradual cell release depending on the glucose concentration in the W_2 phase. DE could offer a valuable tool for standardizing the microbial activity and aroma development in soy sauce fermentation. However, further study is needed for these observations to be validated in real soy sauce fermentation.

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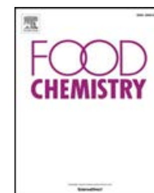

Water-in-oil-in-water double emulsion for the delivery of starter cultures in reduced-salt moromi fermentation of soy sauce

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ABSTRACT

This study investigated the application of water-oil-water ($W_1/O/W_2$) double emulsions (DE) for yeast encapsulation and sequential inoculation of *Zygosaccharomyces rouxii* and *Tetragenococcus halophilus* in moromi stage of soy sauce fermentation with reduced NaCl and/or substitution with KCl. *Z. rouxii* and *T. halophilus* were incorporated in the internal W_1 and external W_2 phase of DE, respectively. NaCl reduction and substitution promoted *T. halophilus* growth to 8.88 log CFU/mL, accompanied with faster sugar depletion and enhanced lactic acid production. Reducing NaCl without substitution increased the final pH (5.49) and decreased alcohols, acids, esters, furan and phenol content. However, the application of DE resulted in moromi with similar microbiological and physicochemical characteristics to that of high-salt. Principal component analysis of GC–MS data demonstrated that the reduced-salt moromi had identical aroma profile to that obtained in the standard one, indicating the feasibility of producing low-salt soy sauce without compromising its quality.

1. Introduction

Soy sauce is a traditional fermented seasoning that is popular in Asia and throughout the world, due to its intense umami taste and characteristic flavor. Soy sauce production process involves a 2-step fermentation process, called *koji* and *moromi.* Koji fermentation begins by mixing steam-cooked soybeans and roasted wheat flour with spores of mold, such as *Aspergillus oryzae* or *Aspergillus sojae*, and, after 3 days of incubation, a compact mass is formed due to mycelium growth (Zhu & Tramper, 2013). The resulting koji is then immersed in brine solution, typically containing 18–22% NaCl to initiate the second step of fermentation and produce moromi mash, and left to ferment for approximately 6 months. During this stage, a wide range of microbial species grow spontaneously and produce numerous flavor compounds, which are essential to the organoleptic properties of the final product. *Tetragenococcus halophilus* and *Zygosaccharomyces rouxii* have been considered as the most predominant osmophilic lactic acid bacteria (LAB) and yeast, respectively, and play major roles in the aroma formation (Röling, Timotius, Prasetyo, Stouthamer, & Van Verseveld, 1994). The use of brine with high NaCl concentration in moromi fermentation is important to control undesirable microorganisms and improve the flavor profile and texture of the final product (Song, Jeong, & Baik, 2015a). However, high NaCl content contributes to excessive sodium intake, which has been reported to increase risk of hypertension, cardiovascular disease, and renal dysfunction (Kremer, Mojet, & Shimojo, 2009). Furthermore, the World Health Organization (WHO) recommends a limitation of average daily intake of sodium to 2 g, which is equivalent to 5 g of salts (WHO, 2012). As a consequence, producing soy sauce with low NaCl content without compromising its quality and consumer acceptability is a challenge, and low salt soy sauce products are now available. Soy sauce production with reduced NaCl has been investigated by different approaches. Moromi fermentation in the absence of NaCl was possible by autolyzing koji under high temperature prior to fermentation (Muramatsu, Sano, Uzuka, & Company, 1993). Nevertheless, the absence of salt during fermentation may result in the growth of spoilage microorganisms and the quality of final product can differ from the original. Salt reduction during moromi fermentation could result in lower content of essential acids, alcohols, and esters, and higher acidity content (Song et al., 2015a). Such problems could be counteracted by the addition of mixed cultures of indigenous yeast species (Song et al., 2015a) as well as combining LAB and yeasts (Singracha, Niamsiri, Visessanguan, Lertsiri, & Assavanig, 2017).

However, a recent study showed that the final aroma profile in moromi fermentation was compromised due to antagonism between co-inoculated *Tetragenococcus halophilus* and *Zygosaccharomyces rouxii* while their sequential inoculation could improve the aroma complexity (Devanthi, Linforth, Onyeaka, & Gkatzionis, 2018). The application of

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sequential inoculation of mixed cultures has been reported to improve flavor quality of fermented foods and beverages. Modulation of the inoculation time was found to be key in achieving the desired quality of apple cider (Ye, Yue, & Yuan, 2014). Furthermore, in whey fermentation, sequential inoculation of *Kluyveromyces lactis* B10 and *Torulaspora delbrueckii* B14 after 48 h improved volatile compounds production (e.g. alcohols and esters) (Andrade, Melo, Genisheva, Schwan, & Duarte, 2017). Higher production of 3-sulfanylhexyl acetate (3SHA) and 3SH (3-sulfanyl-1-hexanol), which are the most important volatiles in Sauvignon blanc aroma, has been achieved with sequential culture of *T. delbrueckii* and *S. cerevisiae*.

A formulation is needed to control the sequential delivery and activity of microbial cultures in soy sauce fermentation. Water-in-oil-in-water ($W_1/O/W_2$) double emulsions (DE) have been studied in recent years for their ability to encapsulate hydrophilic substances, including bacteria for protection and controlled release. Their multi-compartmentalized structure is created by dispersing a water-in-oil (W_1/O) emulsion in another aqueous phase (W_2). Recent studies have focused on probiotic bacteria encapsulation in DE for enhancing survival during digestion (Eslami, Davarpanah, & Vahabzadeh, 2016; Shima, Morita, Yamashita, & Adachi, 2006). The instability of DE structure can be used to modulate the release of bacterial cells by utilizing changes in osmotic balance (El Kadri, Gun, Overton, Bakalis, & Gkatzionis, 2016; El Kadri, Overton, Bakalis, & Gkatzionis, 2015) as they would occur during fermentation. A previous study demonstrated that the inherent DE instability acted as a mechanism for gradual release of *Z. rouxii*, which could be linked to changes in glucose concentration in the medium (Devanthi, El Kadri, Bowden, Spyropoulos, & Gkatzionis, 2018).

In this study, the application of DE for the encapsulation and sequential delivery of *T. halophilus* and *Z. rouxii* cultures was tested in conditions reflecting moromi fermentation with reduced NaCl content and/or substitution with KCl. The stability of DE in moromi was examined by monitoring its microstructure, oil globules size, and distribution. Furthermore, microbial population and physicochemical changes as well as volatile compounds formation were monitored.

2. Materials and methods

2.1. Materials, chemicals, and microorganisms

Soy and wheat flour were purchased from a local retailer (UK). *Aspergillus oryzae* 126,842 was purchased from Centre for Agriculture and Biosciences International (Egham, 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. Sodium chloride (NaCl, extra pure) was purchased from Acros Organics (Fairlawn, NJ). Microbiological growth media used were Czapex Dox Agar (CDA; Oxoid Ltd., Basingstoke, UK), Brain Heart Infusion agar (BHI, Oxoid Ltd., UK), de Man, Rogosa, and Sharpe broth (MRS broth, Oxoid Ltd., UK), Yeast Malt agar (YM agar, Sigma-Aldrich, Gillingham, UK), Yeast Malt broth (YM broth, Sigma-Aldrich, UK). Bacteria and yeast growth were controlled using chloramphenicol (Oxoid Ltd., UK) and natamycin (Sigma-Aldrich, UK), respectively. 1-Octen-3-ol (purity ≥ 98%) was purchased from Sigma-Aldrich. Soybean oil (Alfa Aesar, Heysham, UK) was used as the oil phase of the DE. Polysorbate 80 (Tween 80, Sigma-Aldrich, United Kingdom) and polyglycerol polyricinoleate (PGPR, Danisco A/S, Copenhagen, Denmark) were used as water and oil soluble emulsifiers, respectively.

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. Briefly, 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, UK) 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 hemocytometer 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. DE preparation

The DEs were prepared using a 2-step emulsification method at ambient temperature by using a high shear mixer (Silverson L5M). In the first step, W_1/O primary emulsion was prepared by mixing sterile 6% (*w/v*) NaCl solution into the oil phase (soybean oil with 2% wt PGPR) at W_1:oil phase ratio of 20:80 at 1700 rpm for 2 min. For yeast encapsulation, *Z. rouxii* suspension in 6% (*w/v*) NaCl solution (10^7 cells/mL) was used as W_1.

In the second stage, W_1/O was re-emulsified in the continuous phase (W_2; sterile 6% (*w/v*) NaCl in water with 1% wt Tween 80) at 2000 rpm for 1 min ($W_1/O:W_2$ ratio of 20:80). The final concentration of encapsulated *Z. rouxii* cells was $\sim 10^5$ cells/mL. DEs containing *T. halophilus* in the W_2 were prepared by directly adding 2 mL of *T. halophilus* (10^6 cells/mL) into the W_2 after the mixing process.

2.4. Soy sauce fermentation

2.4.1. Koji preparation

Koji was prepared using the modified method of Su, Wang, Kwok, and Lee (2005). Soy and wheat flour were sterilized 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* spore to a final concentration of 10^5 spores/g substrate (Chou & Ling, 1998). The inoculated substrates were transferred into sterile Petri dishes (d:140 mm) and incubated at 30 °C for 3 days.

2.4.2. Moromi preparation

Different types of brine (18% *w/v* NaCl; 6% *w/v* NaCl and 12% *w/v* KCl; 6% *w/v* NaCl) were added to the koji with ratio of 1:5 (koji:brine) to create moromi $A_{[18\%]}$, $B_{[6:12\%]}$, and $C_{[6\%]}$ respectively, followed by inoculation as shown in Fig. 1. Moromi $A_{[18\%]}$ and $B_{[6:12\%]}$ were simultaneously inoculated with *T. halophilus* and *Z. rouxii*. Three different moromi C were prepared according to the inoculation method of *Z. rouxii*. Moromi $C1_{[6\%]}$ was simultaneously inoculated with *T. halophilus and Z. rouxii*, while moromi $C2_{[6\%]}$ and $C3_{[6\%]}$ were inoculated with *Z.*

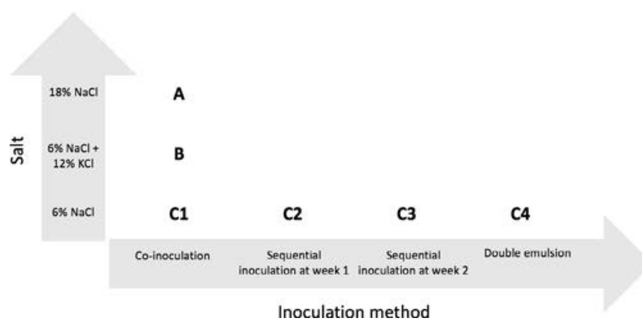

Fig. 1. Set of moromi samples varying in salt composition and inoculation method.

rouxii after 1 week and 2 weeks, respectively. Moromi $C4_{[6\%]}$ was inoculated with DE (10% *v/v*) containing *T. halophilus* and *Z. rouxii*, which had been incorporated in its W_2 and W_1 phase, respectively, prior to inoculation. The inoculated moromi mashes were then incubated at 30 °C for 4 weeks and samples were taken at Week 0, 1, 2, 3, and 4. *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. In order to study the effect of koji:brine ratio on DE stability, koji was mixed with 18% *w/v* NaCl solution with koji:brine ratio of 1:3, 1:5, and 1:7 followed by incubation at 30 °C for 7 days.

2.5. Rheological measurements

Rheological characterization of moromi was done by measuring the viscosity of koji mixed with varying concentrations of brine solution (18% NaCl *w/v*). The viscosity was measured for moromi containing koji:brine ratio of 1:3, 1:5, 1:7 and brine only at 30 °C using AR-G2 rheometer (TA instruments, New Castle, DE) on a parallel plate geometry (d: 40 mm). The apparent viscosity was measured over a shear rate range of 0.1–100 s^{-1}. Briefly, 1 mL of sample was placed between the cone and the plate, and measurement was started immediately. In total, 30 data points were recorded at 10-s intervals during the shearing. Shear stress was determined as a function of shear rate. Data were fitted to power-law model (Barnes, Hutton, & Walters, 1989):

$$\eta = K\cdot\gamma^{n-1} \quad (1)$$

where; η refers to viscosity (Pa·s), K to consistency coefficient (Pa·s^n), γ to shear rate (s^{-1}), and n to flow behavior index (dimensionless).

2.6. Physicochemical analysis

Soy mash samples were centrifuged at 15000*g* for 15 min at ambient temperature. 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), ethanol, and l-glutamic acid were analyzed using 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).

2.7. Volatile compound 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, Thermo Fisher UK) and the vials were sealed with magnetic cap (20 mm diameter, 5 mm center, PTFE/Silicone Liner; Grace Alltech). Samples were allowed to equilibrate at 22 °C for 30 min before analysis. Three replicates were prepared for all samples.

The volatiles extraction was performed using a 1-cm Stableflex fiber coated with 50/30 µm divinylbenzene-Carboxen on polydimethylsiloxane bonded to a flexible fused silica core (Supelco, Bellefonte, PA). It was conditioned for 90 min at 300 °C in the injection port. The fiber was pushed out of the housing and inserted into the vials through the center of the vial cap. The penetration depth was fixed at 22 mm. The extraction was carried out by exposing the fiber to the headspace for 10 min at 40 °C. For all analyses, desorption time was set to 10 min at 230 °C.

Chromatography was carried out using a Trace GC Ultra gas chromatography (Thermo Electron Corporation, Hemel Hempstead, UK) equipped with a polar column ZB-Wax (30 m × 0.25 mm I.D.; film thickness: 1 µm) from Phenomenex (Torrance, CA). Mass spectrometry (MS) was performed with a DSQ mass spectrometer (Thermo Electron Corporation, Hemel Hempstead, UK)). GC–MS parameters were set according to a previous study (Gkatzionis, Linforth, & Dodd, 2009): The temperature of the injection port was 230 °C. Helium was employed as the carrier gas, at a constant pressure of 17 psi. The oven temperature program was as follows: an initial temperature of 40 °C was maintained for 2 min, increasing at a rate of 8 °C/min to a final temperature of 220 °C. The transfer line from the gas chromatograph to the mass spectrometer was held at 250 °C. The mass spectrometer was operated in positive ionization electron impact mode (EI+) at 70 eV. The detector was operated in scan mode (2 scans/s) scanning from m/z 20 to 250. Source temperature was 200 °C.

Compounds were identified by comparing their retention times and mass spectra with those of standards or their retention indices (RI) with those published in databases and their mass spectra with the National Institute of Standards and Technology (NIST) mass spectral library using XCalibur Software (Thermo Electron Corporation, UK). The signal intensity for each compound was expressed relative to the signal observed when the headspace above a 0.1 µg/mL 1-octen-3-ol solution was sampled.

2.8. DE stability characterization

DE samples were placed onto the microscope slides and the microstructure was observed under a light microscope (Olympus BX50) with a 10× objective lens. Images were taken using a Moticam 10 camera *via* Motic Images Plus video acquisition software at 17fps. The oil droplets size distribution of DE was determined from microscopic images using image analysis software (ImageJ), by measuring the diameter of at least 500 oil droplets from 3 different samples of DE.

2.9. Statistical analysis

Microbial cell enumeration, physicochemical tests, and volatile compounds analysis were conducted in triplicate and repeated in two independent experiments. The results were presented as mean ± standard deviation. Significant differences among means were tested by one-way analysis of variances (ANOVA) using IBM SPSS Statistics Software Version 21 at $p < 0.05$ and Tukey's test was applied for means comparison. Principal component analysis (PCA) was performed using XLSTAT™ version 2015.6.01.24027 (Addinsoft, New York, NY) to reduce the dimensionality of the dataset and show the differences in volatile compounds among the soy sauce samples. Observations/variables were chosen as data format and Pearson's correlation matrix was used as PCA type.

3. Results and discussion

3.1. The effect of viscosity on the stability of DE in moromi

DEs were formulated using ingredients relevant to moromi constituents and soybean oil was used as the oil phase. Since the reduced-salt moromi contained 6% NaCl, the internal W_1 and external W_2 phase of DE also contained 6% NaCl. This aimed to balance the osmotic pressure between the two phases, thus reducing instability of DE due to water movement across the oil phase (Mezzenga, Folmer, & Hughes, 2004).

In order to describe the relationship between the viscosity of moromi and DE stability, moromi formulations with different viscosities were tested by varying the ratio of koji:brine (1:3, 1:5, and 1:7). The Power-Law model was used to describe the flow curves of the moromi. The rheological parameters of this model are presented in Table S1. All the moromi formulations exhibited non-Newtonian behavior at shear rates ranging between 0.1 and 100 s^{-1} at 30 °C (Fig. S1a). Moreover, the plot of the viscosity against shear rate of the koji and brine mixtures yielded a flow index (n) of less than 1 (shear

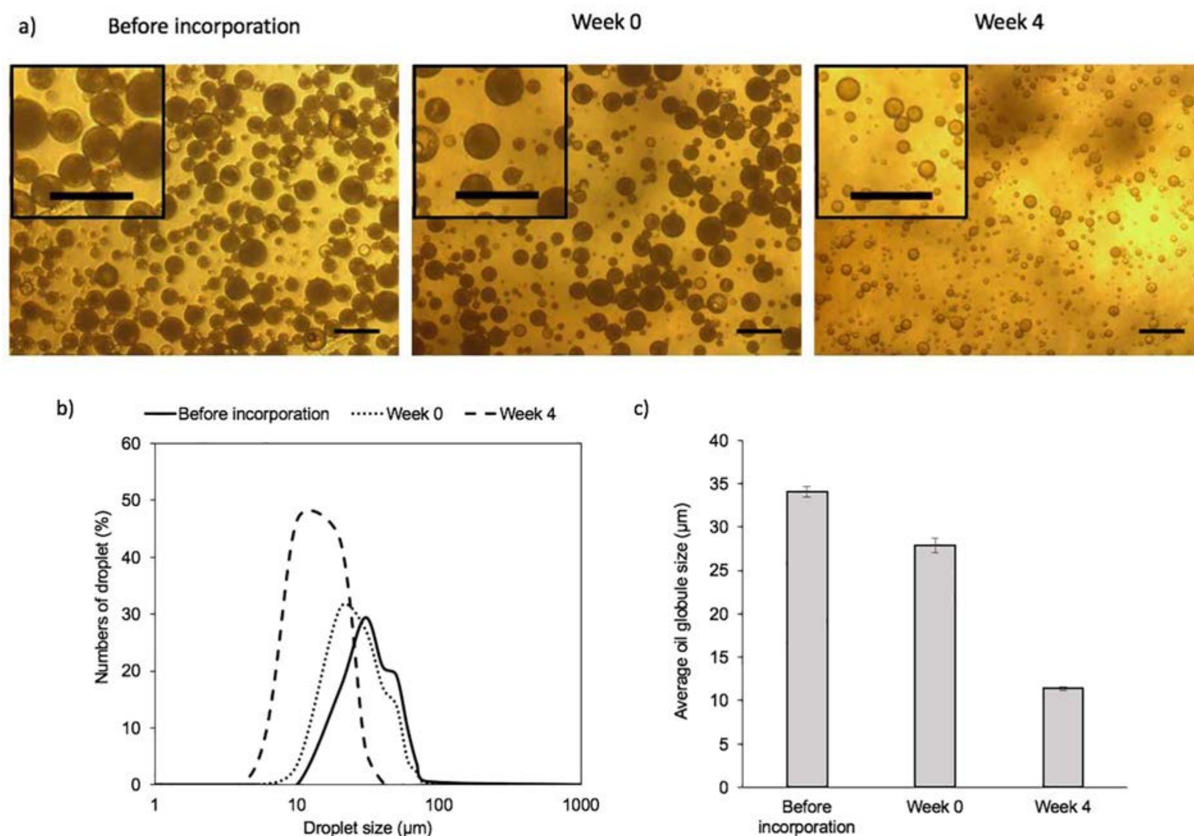

Fig. 2. (a) Optical micrograph of $W_1/O/W_2$ DE before and after incorporation into moromi, and after 4 weeks of fermentation. Scale bar: 100 μm. (b) Oil globule size distribution before and after fermentation. (c) Average oil globule size before and after fermentation.

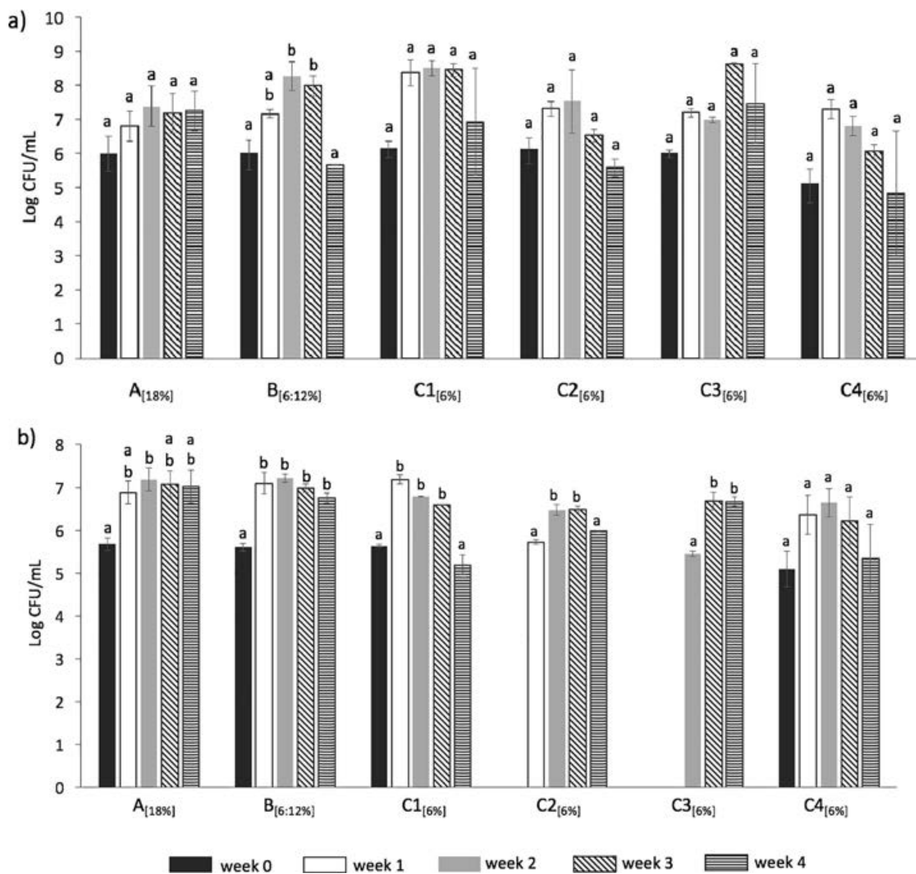

Fig. 3. Changes in population of (a) *T. halophilus* and (b) *Z. rouxii* during fermentation of low and high salt moromi at 30 °C. The samples contained co-inoculated *T. halophilus* and *Z. rouxii* in 18% NaCl ($A_{[18\%]}$), 6% NaCl and 12% KCl ($B_{[6:12\%]}$), 6% NaCl ($C1_{[6\%]}$), and sequentially inoculated *T. halophilus* and *Z. rouxii* at week 1 ($C2_{[6\%]}$), week 2 ($C3_{[6\%]}$), or with DE ($C4_{[6\%]}$). The addition time of *Z. rouxii* cells for sequential inoculation is indicated by the asterisk mark (*). Means within the same group with different letters (a, b, c) are significantly different ($p < 0.05$).

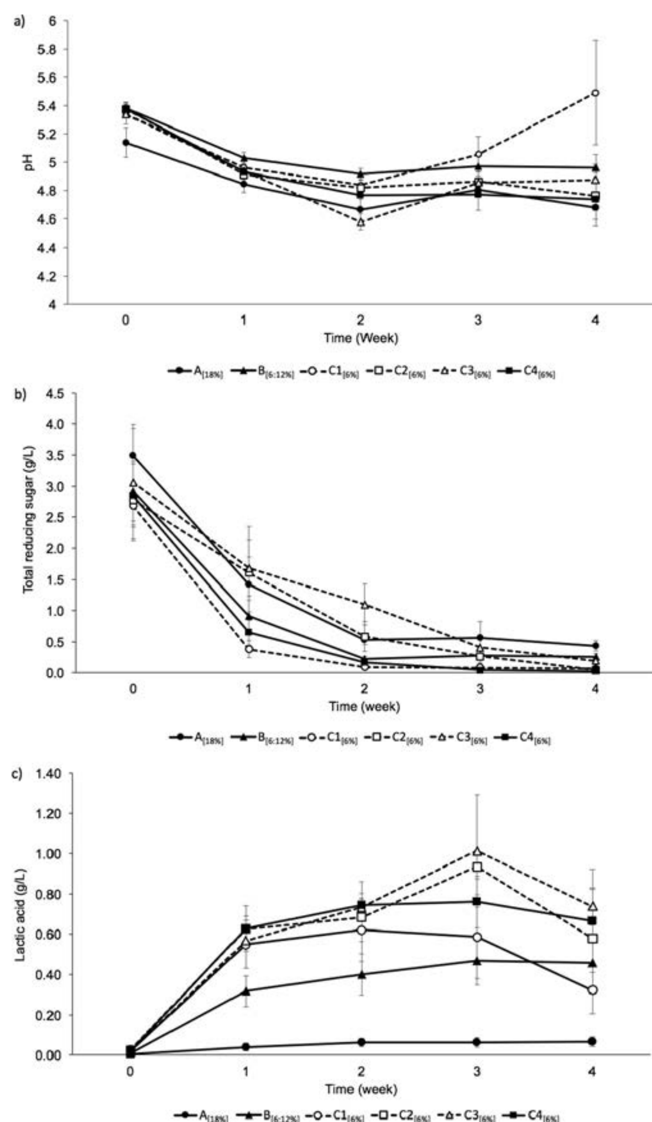

Fig. 4. Changes in (a) pH, (b) total reducing sugar, (c) lactic acid, (d) ethanol, and (e) glutamic acid during fermentation of low and high salt moromi at 30 °C.

thinning), indicating that their flow behavior had a non-Newtonian profile. Similar non-Newtonian behavior has been reported for semi-solids of similar composition to koji which could be attributed to the presence of high molecular weight components, such as proteins or dextrin (Manohar, Manohar, & Rao, 1998).

DE maintained its microstructure after 4 weeks of fermentation (Fig. 2a). However, the oil globule size significantly decreased from 27.88 µm to 11.40 µm (Fig. 2b and c). This could be attributed to the high viscosity of the moromi system. The viscosity increased when the amount of brine added was decreased (Fig. S1a). After incorporation into the moromi system, the DE stability was determined by observing its microstructure (i.e. inner W_1 phase) using microscopy and monitoring the oil globule size. The initial oil globule size (31.84 µm) decreased immediately after incorporation into the moromi slurry and during storage (Fig. S1b and S1c). However, the decrease in koji:brine 1:3 was more noticeable compared to those with higher fractions of brine. By the end of storage, the oil globule size of DE in koji: brine 1:3, 1:5, and 1:7 was 6.84 µm, 18.02 µm, and 15.29 µm, respectively. Moreover, all the oil globules in koji:brine 1:3 completely lost their inner phase, while in koji:brine 1:5 and 1:7, the DE structure was maintained (Fig. S1d). These data indicate that DEs were destabilized in the moromi system; however, the destabilization was not proportional to the viscosity of the moromi.

3.2. The effect of salt reduction and inoculation sequence on the growth of T. halophilus and Z. rouxii

Salt concentration is a significant parameter that determines soy sauce fermentation process by affecting microbial growth. High salt concentration is typically used in soy sauce fermentation, in order to suppress the growth of undesirable microorganism as well as improving the organoleptic properties of the final product. *T. halophilus* growth was suppressed during the first 2 weeks of fermentation (from 6.30 log CFU/mL to 4.17 log /mL) when 18% NaCl ($A_{[18\%]}$) was present in moromi (Fig. 3a). Meanwhile, its growth was significantly enhanced when part of the NaCl was replaced with KCl ($B_{[6:12\%]}$) and maintained high viability, reaching 7.88 log CFU/mL. Interestingly, the growth of *T. halophilus* in $A_{[18\%]}$ recovered after 2 weeks and exceeded $B_{[6:12\%]}$ by the end of incubation. In any case, the growth was higher at the lowest salt concentration ($C1_{[6\%]}$, $C2_{[6\%]}$, $C3_{[6\%]}$) throughout the fermentation, where the cell count sharply increased to 8.49 log /mL within the first week and remained stable throughout the incubation period. Although *T. halophilus* is an osmophilic LAB that can tolerate up to 26% NaCl, it grows best at 5 to 10% *w/v* (Taniguchi et al., 1988). Therefore, raising the NaCl concentration can increase the osmotic stress, reducing the ability of *T. halophilus* to grow (Kobayashi et al., 2004). This indicated that *T. halophilus* could not grow immediately after inoculation in the presence of high NaCl concentration, as previously described by Taniguchi et al. (1988).

The growth of *T. halophilus* under reduced-salt environment was enhanced when it was simultaneously inoculated with *Z. rouxii* ($C1_{[6\%]}$) compared to sequential inoculation ($C2_{[6\%]}$, $C3_{[6\%]}$) and gradual release in DE ($C4_{[6\%]}$). The addition of *Z. rouxii* from the early stage of fermentation might have supplied a variety of metabolites such as pyruvate, amino acids, and vitamins, which are essential for the early stage of bacterial growth (Devanthi et al., 2018; Sudun, Wulijideligen, Arakawa, Miyamoto, & Miyamoto, 2013).

Z. rouxii was not affected significantly by salt concentration during the first 3 weeks of fermentation. However, low-salt moromi ($C1_{[6\%]}$) suffered a decrease in its population at Week 4, in contrast to the enhanced growth of *T. halophilus*. *Z. rouxii* is typically added to enhance flavor and aroma formation in soy sauce production through alcoholic fermentation (Van Der Sluis, Tramper, & Wijffels, 2001; Wah, Walaisri, Assavanig, Niamsiri, & Lertsiri, 2013). In a previous study by Singracha et al. (2017), the addition of *Z. rouxii* in combination with *T. halophilus* and *Pichia guilliermondii* was shown to increase the total population of lactic acid bacteria and yeast in reduced-salt moromi fermentation. Since *Z. rouxii* grows optimally at low pH, *Z. rouxii* would be better added at the later stage of fermentation, once moromi is acidified due to organic acids production by *T. halophilus*. In the present study, *Z. rouxii* sequential inoculation ($C2_{[6\%]}$and $C3_{[6\%]}$) and gradual release in DE ($C4_{[6\%]}$) did not have significant effect on growth, as this seemed to depend primarily on the salt formulation and less on inoculation sequence (Fig. 3b).

3.3. Physicochemical changes during fermentation

The changes in pH, reducing sugar, lactic acid, ethanol, and glutamic acid were measured to monitor the fermentation progress, as they are associated with the growth of microorganisms (Fig. 4). Besides increasing in population during soy sauce fermentation, LAB also utilize and convert carbohydrates into organic acids, which can bring the pH down. Reduction in pH can also occur due to the accumulation of free fatty acids, amino acids, and peptides containing carbonylic side chains, resulting from other microbial activities and raw materials hydrolysis (Hoang et al., 2016; Van Der Sluis et al., 2001; Yanfang et al., 2009). As shown in Fig. 4a, pH of all moromi samples decreased from ~5.3 to final pH of ~4.8, which was similar to values reported in previous

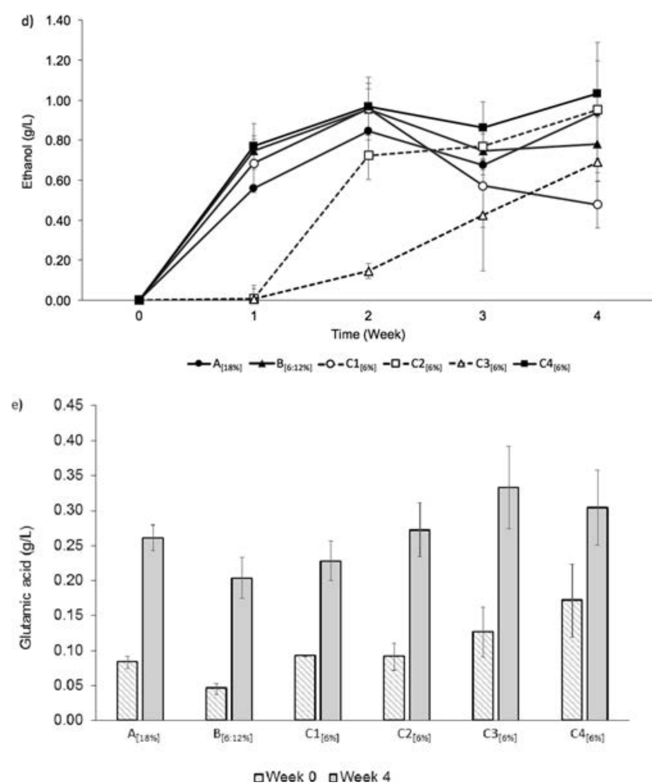

Fig. 4. (*continued*)

studies of traditional Korean (Song, Jeong, & Baik, 2015b) and reduced-salt soy sauce (Singracha et al., 2017). The pH decreased within two weeks and then remained constant throughout the fermentation period, except for $C1_{[6\%]}$, where pH increased to 5.49. The reduction in pH was associated with the increase in the lactic acid amount produced by *T. halophilus* (Fig. 4c). Although lactic acid production was greatly suppressed by 18% NaCl, the reduction in pH was unaffected, which could be due to production of other organic acids. Although *T. halophilus* is known as homofermentative, some strains are regarded as heterofermentative and they are able to produce acetic acid (Justé et al., 2012). Moreover, homofermentative strains of *T. halophilus* are reported to undergo mixed acid fermentation under certain growth conditions (Röling & van Verseveld, 1997).

The production of lactic acid was significantly lower in the presence of high salt concentration, and high sodium content had a greater impact on the suppression (Fig. 4c). In low salt concentration, microorganisms are able to perform faster metabolic activity, therefore producing higher amount of acids (Hoang et al., 2016). In the present study, lactic acid production in reduced-salt moromi was enhanced when the inoculation of *Z. rouxii* was modulated, sequentially or gradually by using DE. In co-inoculation, *Z. rouxii* might have changed the physicochemical properties of the substrate, which could suppress the fermentation of lactic acid by *T. halophilus*, as reported in a previous study (Devanthi et al., 2018).

Reducing sugar is important during fermentation as it serves as a carbon source for microbial growth as well as flavor and aroma formation. The initial content of total reducing sugar in all moromi samples ranged from 2.68 to 3.49 g/L and it constantly decreased throughout the incubation period (Fig. 4b), which was in agreement with the previous study by Zhang, Zhou, Cui, Huang, and Wu (2016). The reduction patterns were comparable regardless of salt concentration and sequence of inoculation. During fermentation, reducing sugar is consumed by microbes or possibly reacts with free amino acids during the Maillard reaction (Kim & Lee, 2008). Since the fungal amylase, which breaks down the polysaccharide into simple sugars, was heat-inactivated prior to the moromi stage, the amount of reducing sugar was expected to decrease over time. The reducing sugar content in moromi decreased faster when low salt concentration ($B_{[6:12\%]}$, $C1_{[6\%]}$, and $C4_{[6\%]}$) was used. This could be attributed to faster metabolic activity of the microbes, which also corresponded to higher *T. halophilus* population and lactic acid production (Hoang et al., 2016). Furthermore, the reducing sugar content decreased at a slower rate when *Z. rouxii* was inoculated sequentially after 1 or 2 weeks of fermentation, but not when DE was used. This was expected since *Z. rouxii* is the main user of sugar for biomass and ethanol production (Devanthi et al., 2018). The activity of the released *Z. rouxii* cells might have caused faster sugar depletion in DE ($C4_{[6\%]}$).

Ethanol production was highly affected by variation in salt concentration and sequence of inoculation (Fig. 4d). In low-salt moromi ($C1_{[6\%]}$), the amount of ethanol constantly decreased after 2 weeks of fermentation compared to a high concentration of salt ($A_{[18\%]}$and $B_{[6:12\%]}$). However, the decrease in ethanol production was compensated when *Z. rouxii* was added simultaneously ($C2_{[6\%]}$ and $C3_{[6\%]}$) or using DE ($C4_{[6\%]}$). Interestingly, ethanol production with a similar pattern to $A_{[18\%]}$ and B and at highest concentration was achieved when *Z. rouxii* was encapsulated in DE.

Z. rouxii is known to produce extracellular glutaminase, which is a proteolytic enzyme that converts l-glutamine derived from soy protein to l-glutamic acid (Iyer & Singhal, 2008; Kashyap, Sabu, Pandey, Szakacs, & Soccol, 2002). Unlike the glutaminase produced by koji mold, *Z. rouxii* glutaminase is more tolerant against high salinity. l-Glutamic acid is essential for improving the flavor of the final product since it contributes to the “umami” taste of the soy sauce. Therefore, high activity of glutaminase is desirable, in order to increase the production of l-glutamic acid. As shown in Fig. 4e, the amount of glutamic acid increased after the fermentation process and the final concentration of glutamic acid between samples did not differ significantly ($p > 0.05$).

3.4. Formation of volatile compounds

A total of 38 volatile compounds was detected in the moromi samples using SPME-GC/MS, including 15 alcohols, 5 acids, 8 aldehydes, 4 esters, 1 furan, 1 phenol, 3 ketones, and 1 alkene (Table 1). Alcohol was found to be the most abundant compound in all samples, comprising more than 90% of the total volatiles, as previously found in high-salt liquid state fermentation, low-salt solid-state fermentation, and Koikuchi soy sauce (Feng et al., 2015).

Salt reduction ($C1_{[6\%]}$) was shown to have a great influence on the volatiles production in moromi, especially alcohols (Table 1). Yeasts contribute to the formation of alcohols through the reduction of related aldehydes (Sun, Jiang, & Zhao, 2010; Van Der Sluis et al., 2001). Lowering salt concentration to 6% *w/v* ($C1_{[6\%]}$) significantly ($p < 0.05$) enhanced the production of 2,4-dimethyl-3-pentanol, 2,6-dimethyl-4-heptanol, 3-methyl-1-butanol, 5-nonanol, and phenylethyl alcohol. On the other hand, the production of ethanol and propanol was reduced in low salt concentration ($C1_{[6\%]}$), which was in agreement with the previously studied reduced-salt Korean soy sauce (Song et al., 2015a). Partial salt substitution with KCl ($B_{[6:12\%]}$) did not affect the production of most volatile compounds, except for 2-furanmethanol, 2-methoxy-5-methyphenol, and 2-methyl-1-propanol which were significantly ($p < 0.05$) lower compared to sample $A_{[18\%]}$. In previous studies reported by Sasaki (1996) and Jansen, Veurink, Euverink, and Dijkhuizen (2003), the production of higher alcohols, including phenylethyl alcohol, 3-methyl-1-butanol, 1-propanol, and 2-methyl-1-propanol, was found to decrease with an increase of NaCl concentration. However, the amounts of 1-propanol and 2-methyl-1-propanol decreased under reduced NaCl conditions ($B_{[6:12\%]}$). This might have arisen from decreasing uptake of the related amino acid by yeast, since these compounds are mainly produced by *Z. rouxii* from their corresponding branched-chain amino acids *via* the Ehrlich pathway (Van Der Sluis et al., 2001). The method of inoculation was found to affect the

Table 1
Aroma compounds found in moromi after 4 weeks of fermentation in low and high salt concentration. The values are relative to the peak area observed when the headspace above a 0.1 μg/mL 1-octen-3-ol solution was analyzed. Each value is based on three replicates.

Compound	LRI1	Day 30																	
		A[18%]			B[6:12%]			C1[6%]			C2[6%]			C3[6%]			C4[6%]		
		mean		SD	mean		SD	mean		SD	mean		SD	mean		SD	mean		SD
Alcohols																			
1-octen-3-ol	1466	0.039	a	0.009	0.020	a	0.002	0.037	a	0.006	0.086	b	0.035	0.037	a	0.007	0.039	a	0.011
2-ethyl-1-hexanol	1508	0.029	a	0.003	0.028	ab	0.008	0.014	abc	0.007	0.025	abc	0.011	0.012	bc	0.003	0.010	c	0.003
2-furanmethanol	1690	0.084	a	0.006	0.046	b	0.003	0.039	b	0.002	0.089	a	0.011	0.051	b	0.004	0.085	a	0.016
2-methyl-1-propanol	1115	0.385	a	0.055	0.186	bc	0.019	0.204	b	0.070	0.159	bc	0.030	0.055	c	0.012	0.185	bc	0.081
2,4-dimethyl-3-pentanol	1395	0.013	a	0.001	0.019	a	0.001	0.298	b	0.203	0.021	a	0.004	0.025	a	0.003	0.009	a	0.001
2,6-dimethyl-4-heptanol	1506	0.031	ab	0.010	0.005	a	0.001	0.090	b	0.025	0.032	ab	0.006	0.081	b	0.049	0.013	a	0.006
3-methyl-3-buten-1-ol	1271	0.026	a	0.004	0.021	a	0.002	0.019	a	0.004	0.004	b	0.001	0.006	b	0.001	0.006	b	0.001
3-methyl-1-butanol	1225	25.061	a	3.033	18.089	ac	1.609	40.297	b	8.876	19.532	ac	3.675	9.445	c	1.787	21.703	a	2.603
5-nonanol	1473	0.002	a	0.000	0.001	a	0.000	0.006	b	0.003	0.005	ab	0.002	0.008	b	0.002	0.002	a	0.000
ethanol	950	33.709	a	2.159	27.538	ab	1.129	14.398	b	1.106	39.284	a	3.380	16.496	b	1.616	36.572	a	12.076
1-heptanol	1473	0.042	ab	0.006	0.016	a	0.002	0.016	a	0.002	0.015	a	0.006	0.007	a	0.001	0.066	b	0.033
1-hexanol	1371	0.071	a	0.012	0.033	ab	0.002	0.032	ab	0.010	0.046	ab	0.018	0.014	b	0.002	0.072	a	0.032
methanol	915	0.449	a	0.025	0.511	a	0.065	0.488	a	0.088	0.604	a	0.082	0.661	a	0.110	0.523	a	0.159
phenylethyl alcohol	1957	2.425	a	0.371	2.059	ac	0.121	3.801	b	0.352	2.010	ac	0.193	1.408	c	0.535	1.658	ac	0.467
1-propanol	1057	0.376	ac	0.030	0.281	ab	0.023	0.180	b	0.030	0.468	cd	0.057	0.204	b	0.038	0.547	d	0.088
acids																			
4-methyl-2-oxovaleric acid	1478	0.016	ac	0.005	0.006	a	0.000	0.040	b	0.006	0.012	ac	0.004	0.024	c	0.010	0.007	a	0.000
2-methylpropanoic acid	1596	0.113	a	0.046	0.057	ab	0.025	0.000	b	0.000	0.025	bc	0.007	0.086	ac	0.037	0.040	bc	0.004
3-methylbutanoic acid	1699	0.400	ab	0.153	0.205	a	0.081	0.008	a	0.012	0.116	a	0.103	0.776	b	0.323	0.158	a	0.043
acetic acid	1481	0.161	ab	0.098	0.041	ab	0.040	0.000	a	0.000	0.212	ab	0.055	0.300	b	0.133	0.312	b	0.178
propionic acid	1565	0.010	a	0.010	0.018	a	0.023	0.015	a	0.025	0.061	a	0.090	0.045	a	0.058	0.108	a	0.107
aldehydes																			
2-methylbutanal	929	0.009	a	0.002	0.001	a	0.000	0.026	a	0.027	0.009	a	0.009	0.006	a	0.005	0.004	a	0.001
2-methylpropanal	824	0.026	a	0.009	0.009	a	0.002	0.127	b	0.057	0.027	a	0.022	0.019	a	0.001	0.011	a	0.003
3-methylbutanal	934	0.083	a	0.013	0.012	a	0.002	0.091	a	0.063	0.109	a	0.114	0.102	a	0.101	0.040	a	0.013
benzaldehyde	1568	0.021	a	0.004	0.034	ab	0.001	0.013	a	0.001	0.060	b	0.030	0.023	a	0.003	0.022	a	0.001
furfural	1500	0.014	ab	0.002	0.010	ab	0.001	0.009	a	0.000	0.015	b	0.003	0.010	ac	0.001	0.014	bc	0.003
hexanal	1104	0.001	a	0.001	0.002	a	0.001	0.001	a	0.000	0.003	a	0.001	0.001	a	0.000	0.002	a	0.002
pentanal	1001	0.014	a	0.007	0.015	a	0.007	0.015	a	0.004	0.029	a	0.010	0.023	a	0.007	0.024	a	0.006
propanal	807	0.004	a	0.000	0.005	a	0.001	0.003	a	0.001	0.004	a	0.002	0.006	a	0.001	0.005	a	0.003
esters																			
2-phenylethyl acetate	1860	0.316	ac	0.054	0.136	b	0.006	0.094	b	0.024	0.378	c	0.071	0.163	b	0.061	0.191	ab	0.040
ethyl acetate	906	0.196	a	0.046	0.079	a	0.026	0.073	a	0.023	0.100	a	0.077	0.019	a	0.009	0.245	a	0.189
ethyl propionate	975	0.032	ab	0.008	0.006	a	0.000	0.010	ab	0.005	0.011	ab	0.009	0.003	a	0.002	0.038	b	0.024
isoamyl acetate	1141	0.256	a	0.074	0.029	a	0.006	0.878	b	0.280	0.115	a	0.119	0.044	a	0.015	0.243	a	0.121
furan																			
3-acetyl-2,5-dimethylfuran	1450	0.186	a	0.061	0.001	bc	0.000	0.001	b	0.000	0.069	c	0.014	0.009	bc	0.001	0.000	b	0.000
phenol																			
2-methoxy-5-methylphenol	1614	0.258	a	0.081	0.001	b	0.000	0.001	b	0.000	0.169	a	0.035	0.008	b	0.001	0.001	b	0.001
ketone																			
3-methyl-2-pentanone	1037	0.004	a	0.001	0.009	ac	0.001	0.032	b	0.011	0.000	a	0.000	0.018	bc	0.006	0.004	a	0.003
acetoin	1318	0.656	a	0.039	0.451	a	0.043	1.624	b	0.572	0.066	a	0.013	0.227	a	0.146	0.175	a	0.103
acetone	829	0.671	a	0.088	0.334	a	0.082	0.507	a	0.084	0.405	a	0.107	0.614	a	0.179	0.453	a	0.303
Others																			
D-limonene	1217	0.006	a	0.003	0.001	a	0.001	0.004	a	0.004	0.002	a	0.001	0.183	a	0.179	0.008	a	0.006

[1]LRI: linear retention indices of the compounds relative to an alkane series.
Means within the same row with different letters (a, b, c) are significantly different ($p < 0.05$).
$A_{[18\%]}$: Co-inoculation; 18% NaCl.
$B_{[6:12\%]}$: Co-inoculation; 6% NaCl and 12% KCl.
$C1_{[6\%]}$: Co-inoculation; 6% NaCl.
$C2_{[6\%]}$: Sequential inoculation starting at Week 1; 6% NaCl.
$C3_{[6\%]}$: Sequential inoculation starting at Week 2; 6% NaCl.
$C4_{[6\%]}$: Inoculation with DE; 6% NaCl.

production of most alcohols in the reduced-salt moromi. Moromi with similar flavor pattern to those containing high salt concentrations ($A_{[18\%]}$ and $B_{[6:12\%]}$) was achieved when *Z. rouxii* was added sequentially at Week 1 ($C2_{[6\%]}$) or using DE ($C4_{[6\%]}$). The addition of *Z. rouxii* at Week 2 resulted in significantly ($p < 0.05$) lower amounts of 2-furanmethanol, 3-methyl-1-butanol, ethanol, 1-heptanol, 1-hexanol, and 1-propanol. This result corresponds to the ethanol measurement during fermentation by using enzymatic reaction (Fig. 4d).

Salt reduction was also found to affect the production of several acids. The amount of 4-methyl-2-oxovaleric acid was enhanced in reduced-salt moromi, only when *Z. rouxii* was added simultaneously. Meanwhile, 2-methylpropanoic acid, which contributes to cheese/fatty odor, was found to be significantly lower in all reduced-salt moromi samples. However, noticeably higher amount of 2-methylpropanoic acid was detected when *Z. rouxii* was added at Week 2. The production of some acids, including 3-methylbutanoic acid (cheese/sweet) and

acetic acid (sour/vinegar-like odor), was found to be enhanced when *Z. rouxii* inoculation was delayed for 2 weeks. Acetic acid production was also similar when DE was used. These acids have been reported as the highest odor-active compounds in Chinese soy sauce (Feng et al., 2014). Among these acids, 2-methylpropanoic acid and 3-methylbutanoic acid are formed *via* branched-chain α-keto acid catabolism (Song et al., 2015b)

Aldehydes contribute to nutty and malty aroma in soy sauce (Feng et al., 2015). In the present study, most aldehyde compounds were not affected by salt reduction, except for 2-methylpropanal which was significantly enhanced in reduced-salt moromi when mixed cultures were added simultaneously ($C1_{[6\%]}$). This branched-chain aldehyde is considered as an important flavor compound, perceived as malty, chocolate-like, with low taste threshold (Smit, Engels, & Smit, 2009). It is generated from branched-chain amino acid valine *via* Strecker degradation or microbial activity, which then can be converted to its corresponding alcohol (2-methyl-1-propanol) and/or acid (2-methylpropanoic acid) (Ardö, 2006; Song et al., 2015a). The effect of modulating the inoculation time of *Z. rouxii* on aldehydes formation was hardly seen, except for benzaldehyde (burnt sugar/sweet) and furfural (bread/sweet), which were significantly enhanced in $C2_{[6\%]}$ and $C4_{[6\%]}$, respectively.

Replacing NaCl with KCl decreased the amount of 2-phenylethyl acetate, which contributes to honey, rosy odor. However, this could be compensated for by adding *Z. rouxii* at Week 1 of the fermentation process. In our previous study, it was found that the production of 2-phenylethyl acetate could be enhanced by adding *Z. rouxii* sequentially rather than simultaneously (Devanthi et al., 2018). *Z. rouxii* enhances the production of esters (Van Der Sluis et al., 2001), although the production of isoamyl acetate (banana aroma) was significantly enhanced in reduced-salt moromi. This was only observed in $C1_{[6\%]}$, while the amount of isoamyl acetate in $C2_{[6\%]}$, $C3_{[6\%]}$, and $C4_{[6\%]}$ was similar to that at high salt concentration ($A_{[18\%]}$and $B_{[6:12\%]}$).

The only furan and phenol compounds detected in all moromi samples were 3-acetyl-2,5-dimethylfuran and 2-methoxy-5-methylphenol, respectively. These were produced in negligible amount when either salt or NaCl were reduced, except when *Z. rouxii* was added at Week 1. Several ketones, such as 3-methyl-2-pentanone and acetoin, were produced at significantly higher concentrations in reduced-salt moromi. The amounts of these compounds were similar to moromi containing high salt ($A_{[18\%]}$ and $B_{[6:12\%]}$) when *Z. rouxii* was added sequentially, with or without DE.

3.5. Principal component analysis

PCA analysis was conducted, in order to gain more understanding on the relationship between the fermentation conditions and profiles of volatile compounds. The first (PC1) and second principal component (PC2) accounted for 30.60% and 21.43% of the total variance, respectively (Fig. 5a and b). The PCA score plot demonstrates distinct separation of some moromi samples (Fig. 5a). In the case of co-inoculated samples, low salt moromi sample ($C1_{[6\%]}$) was differentiated from high salt moromi sample ($A_{[18\%]}$) while reduced NaCl sample (substituted with 12% KCl; B) was positioned in the middle of PC1. This indicates that salt reduction affected the aroma profile of moromi. Replacing part of NaCl with KCl ($B_{[6:12\%]}$) was associated with lower content of 2-furanmethanol, 2-methyl-1-propanol, 2-phenylethyl acetate, 3-acetyl-2,5-dimethylfuran, and 2-methoxy-5-methylphenol. $C1_{[6\%]}$ was associated with high amounts of 3-methyl-1-butanol, phenylethyl alcohol, 2,4-dimethyl-3-pentanol, 2,6-dimethyl-4-heptanol, isoamyl acetate, 2-methylpropanal, 4-methyl-2-oxovaleric acid, 3-methyl-2-pentanone, and acetoin (Fig. 5b).

The method of inoculation was found to affect the aroma profiles, and adding *Z. rouxii* encapsulated in DE or sequentially after 1 week matched the aroma profile obtained with high salt concentration. This was not the case when *Z. rouxii* was added sequentially after 2 weeks of fermentation. Clustering of samples $A_{[18\%]}$, $C2_{[6\%]}$, and $C4_{[6\%]}$was influenced by compounds such as 2-furanmethanol, 2,4-dimethyl-3-pentanol, 2,6-dimethyl-4-heptanol, 3-methyl-1-butanol, 5-nonanol, ethanol, 1-hexanol, methanol, phenylethyl alcohol, 4-methyl-2-oxovaleric acid, 3-methylbutanoic acid, acetic acid, propionic acid, 2-methylbutanal, 2-methylpropanal, 3-methylbutanal, furfural, hexanal, pentanal, propanal, ethyl acetate, ethyl propionate, isoamyl acetate, 3-methyl-2-pentanone, acetoin, acetone, and d-limonene.

Fig. 5. (a) PCA score plot of six moromi samples after 4-week fermentation. The scores are based on three replicates of each sample. The identical symbols represent triplicate measurements. (b) PCA loading plot of the aroma compounds detected in moromi after 4-week fermentation.

4. Conclusion

Salt reduction could affect microbial growth and physicochemical changes during moromi fermentation. Low salt concentrations could promote *T. halophilus* growth and enhance lactic acid production. However, the final overall aroma balance differed from the original soy sauce fermented with high salt concentration, indicated by lower

content of some alcohols, acids, esters, furan, and phenol. The use of DE for delivering the mixed cultures of *T. halophilus* and *Z. rouxii* in reduced-salt moromi could compensate for such changes by promoting the formation of some essential volatile compounds, including alcohols (e.g., 2-furanmethanol and ethanol) and esters (e.g., 2-phenylethyl acetate). This indicates the possibility of producing soy sauce in a low salt environment with a volatile profile pattern identical to the original high-salt soy sauce. The results obtained in this study provide the soy sauce industry with a new technique for standardizing the microbial activity and aroma development, which also offers health benefits to the consumers, due to low salt content in the final product. However, since modulating the release has a great impact on the aroma formation, further study is needed in order to tailor the physicochemical properties of DE, therefore enabling the cell release in a more controlled manner.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2018.03.022.

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