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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)

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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.

Keywords: $W_1/O/W_2$ double emulsion; yeast encapsulation; *Tetragenococcus halophilus*; *Zygosaccharomyces rouxii*; soy sauce; microbial antagonism.

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 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 10000 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:

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

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. halophillus* 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. 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.

2.4 DE stability characterization

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 (λ = 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%.

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:

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

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 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 (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:

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

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

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 (Figure 1). *Z. rouxii* was successfully encapsulated in the internal W_1 phase of DEs with high encapsulation efficiency (>99%; Figure 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 (Figure 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 (Figure 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, Quezada, Ibarra-Bracamontes, González-Ochoa, & Arauz-Lara, 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 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 (Figure 2b) and size distribution of the oil globules (Figure 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 (Figure 3a), *Z. rouxii* cells release profile (Figure 3b), and the oil globule size (Figure 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 (Figure 3b) and it followed a similar pattern to the loss in the W_1 phase (Figure 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*

276 *versa* depending on the direction of the osmotic pressure gradient. This can destabilize the DE
277 resulting in morphological changes as well as the release of entrapped materials (Frasch-
278 Melnik, Spyropoulos, & Norton, 2010; Mezzenga, Folmer, & Hughes, 2004). However,
279 increased salt release proportional to the glucose concentration was also observed by Pawlik,
280 Cox, & Norton (2010). The authors suggested that PGPR was able to increase glucose
281 lipophilicity, therefore it became surface active. According to Garti (1997), the increasing
282 amount of lipophilic surfactant can increase the transport rate of water, surfactant, and water
283 soluble molecules even when there is no osmotic pressure gradient. The excess amount of
284 lipophilic emulsifier can increase the flux of water through reverse micelles formation. In the
285 present study, glucose might also behave as lipophilic emulsifier facilitating water movement
286 to the external W_2 phase through reverse micellar transport, which eventually led to release of
287 *Z. rouxii* cells since yeast cells are hydrophilic and therefore would preferentially reside within
288 the aqueous W_2 phase and not the oil phase. Furthermore, the release of *Z. rouxii* cells might
289 also be driven by bursting mechanism. According to El Kadri et al. (2016, 2015), osmotic
290 pressure balance alteration can lead to oil globule bursting which can be used to modulate the
291 release of bacterial cells. However, the release of *Z. rouxii* cells from DE might involve not
292 one but various mechanism and further investigation is required for a better understanding on
293 how *Z. rouxii* cells are being released.

294 DE prepared with the highest concentration of glucose (30%) in its W_2 phase possessed the
295 lowest initial oil globule size (37.28 ± 0.74 μm; Figure 4a), even though the mixing speed and
296 conditions during the two-step homogenizing process was maintained for all the formulations.
297 This is expected as the addition of glucose increases the viscosity of the W_2 phase which leads
298 to smaller oil globules to form (Khalid, Kobayashi, Neves, Uemura, & Nakajima, 2013).
299 Furthermore, it has been reported that glucose can further reduce the interfacial tension which
300 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 4a). In contrast, DE 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 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% (Figure 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 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 (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. halophillus 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 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 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 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 et al., 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 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* (Figure 6a) and *Z. rouxii* (Figure 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 (Figure 6d). *T. halophilus* was able to propagate steadily throughout the incubation period, reaching a final count of 7.23 log CFU/mL (Figure 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 toward *T. halophilus*. The ability of DE to gradually release the *Z. rouxii* into the bulk medium might also prevent detrimental 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 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 Figures 7a-d, the presence of DE caused alteration in the metabolic activity of both microorganisms as a single or mixed cultures.

Glucose consumption (Figure 7a) correlated with ethanol production (Figure 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 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, & 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 7c) and lactic acid (Figure 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 & 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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6. References

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554 Zhu, Y., & Tramper, J. (2013). Koji - where East meets West in fermentation. *Biotechnology*
555 *Advances*, *31*(8), 1448–1457.

556 Table 1. Formulation of $W_1/O/W_2$ double emulsions (DE) with varying glucose concentrations

557 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	$W_1<W_2$

558

559

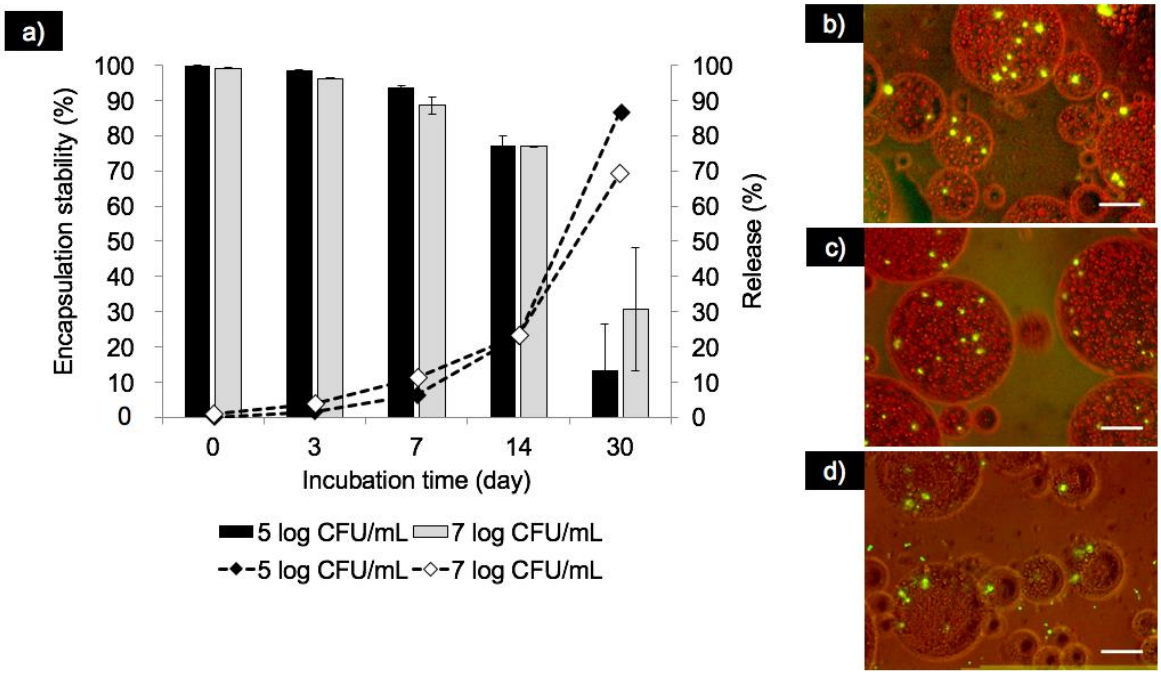
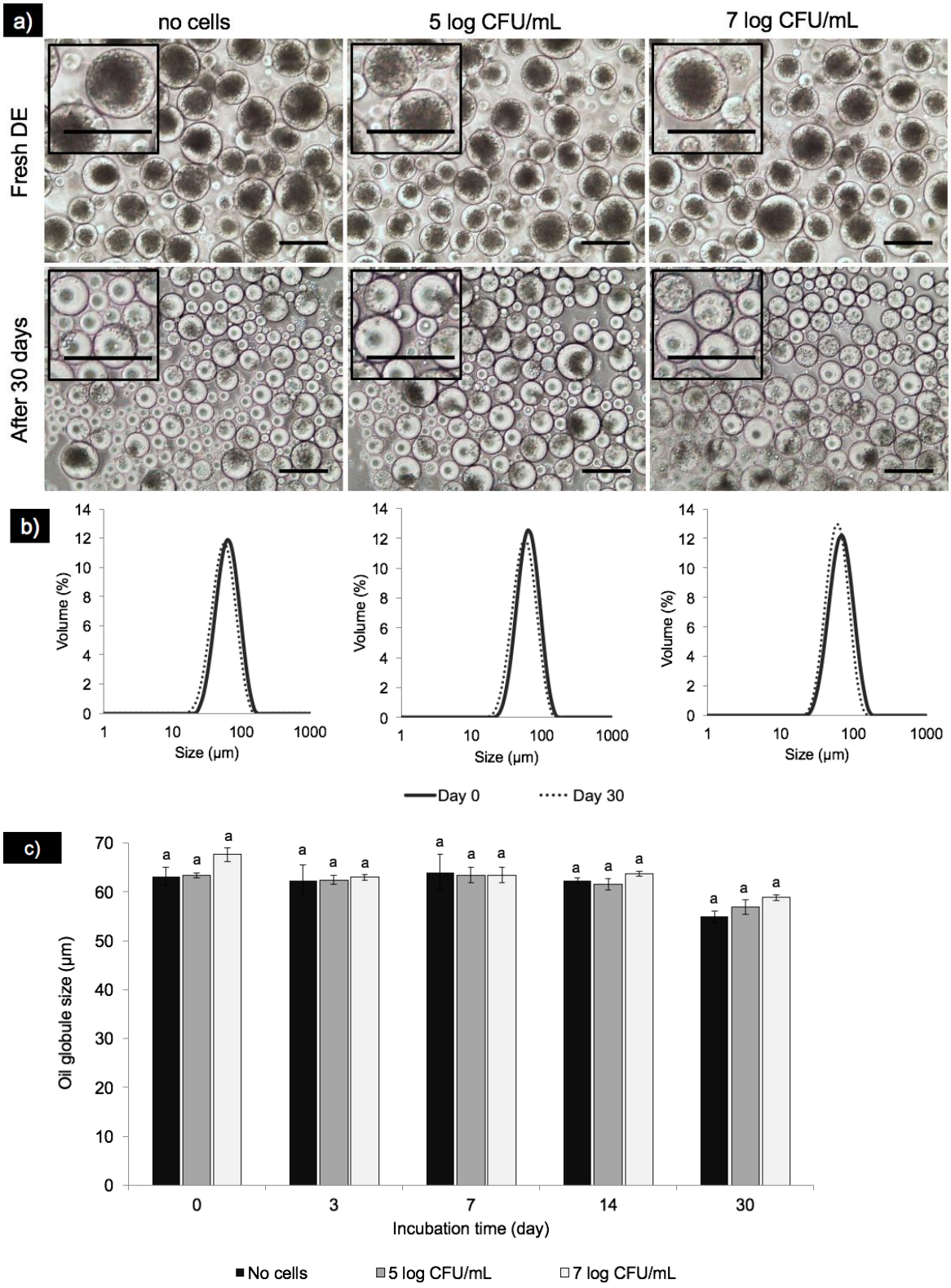


Figure 1. (a) Changes in the percentage of entrapped (bar chart) and released (line chart) *Z. rouxii* cells in DEs prepared under iso-osmotic conditions over 30 days of storage at 30 °C. (b) Fluorescence microscopy images of the entrapped *Z. rouxii* cells at day 0, (c) day 7, and (d) day 30. Scale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

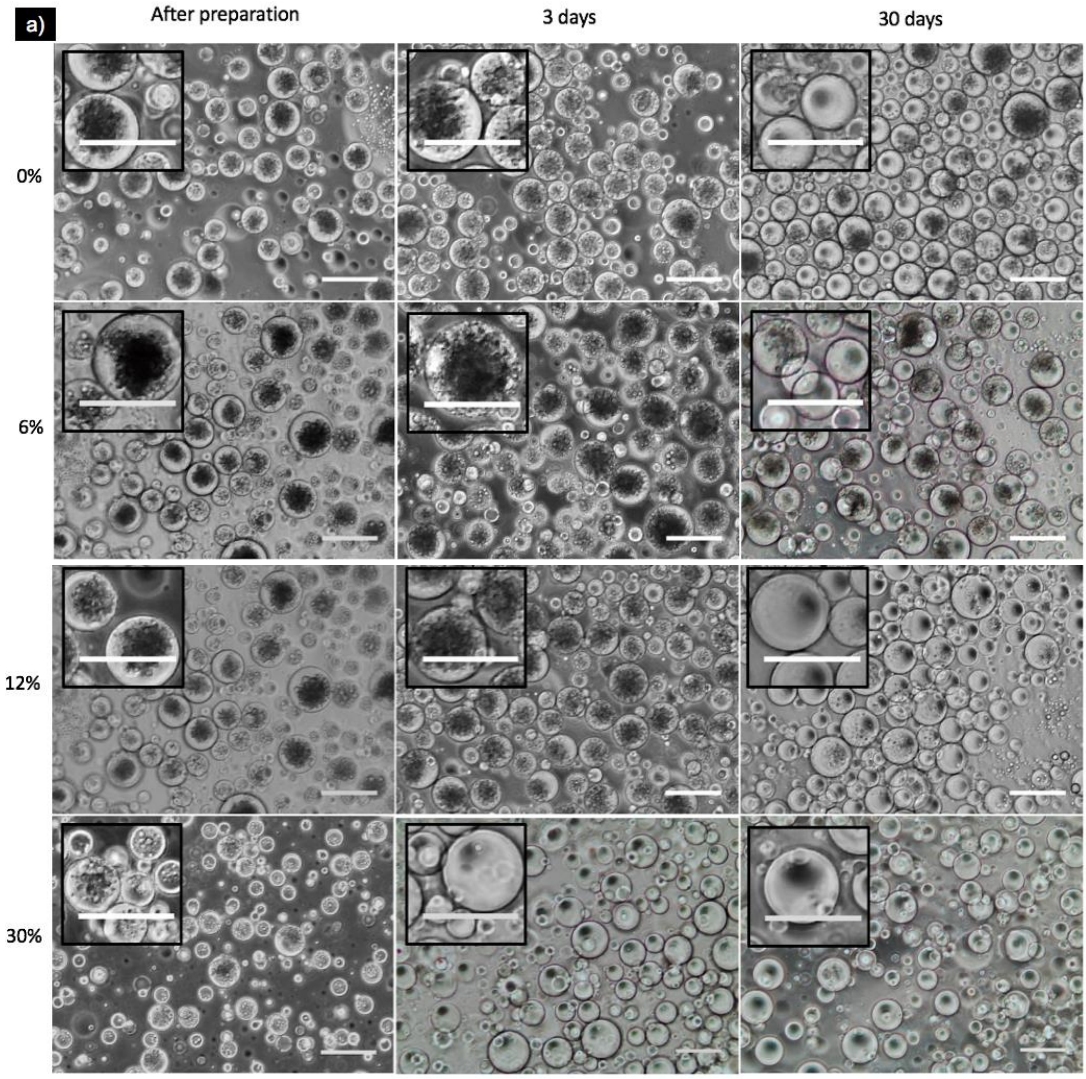


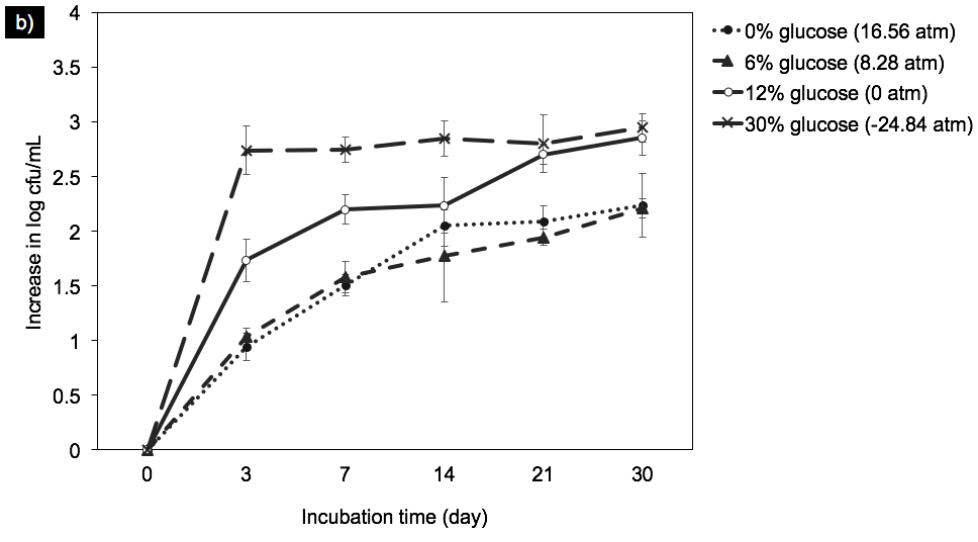
570

571 Figure 2. DEs with no cells, 5 log CFU/mL, and 7 log CFU/mL before and after 30 days of

572 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 ($p<0.05$).


a)
After preparation
3 days
30 days
0%
6%
12%
30%


b)
Increase in log cfu/mL
Incubation time (day)
0% glucose (16.56 atm)
6% glucose (8.28 atm)
12% glucose (0 atm)
30% glucose (-24.84 atm)

598

Figure 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.

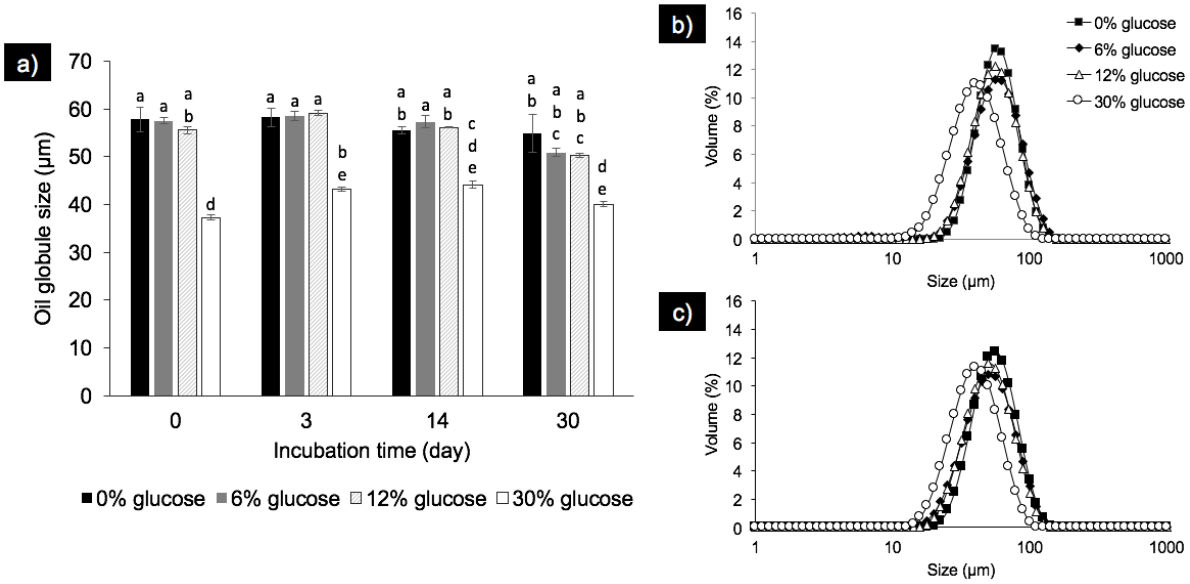


Figure 4. DEs before and after 30 days of storage at 30 °C with different glucose concentrations in the external W_2 phase. (a) Average oil globule size; (b) Oil globule size distribution of DEs before storage and (c) after storage. Mean values with different letters are significantly different ($p<0.05$).

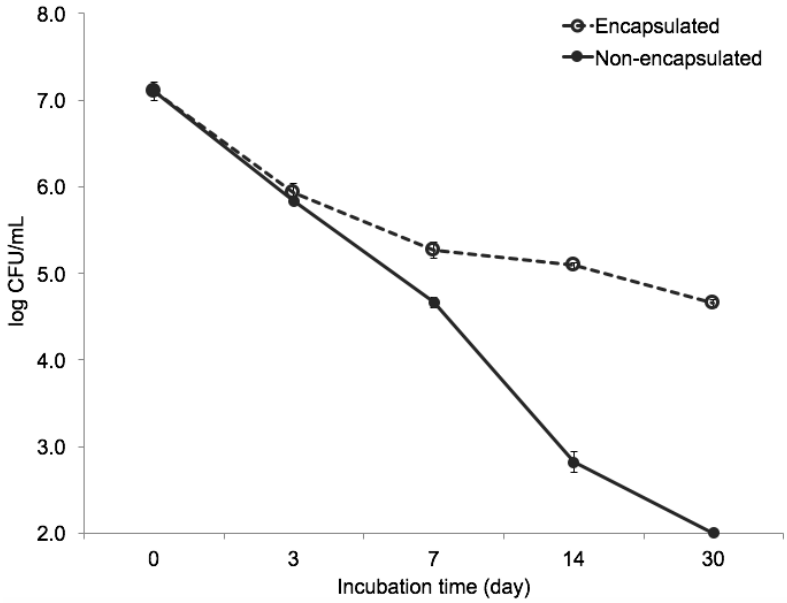


Figure 5. Changes in *Z. rouxii* cells viability over 30 days of storage at 30°C.

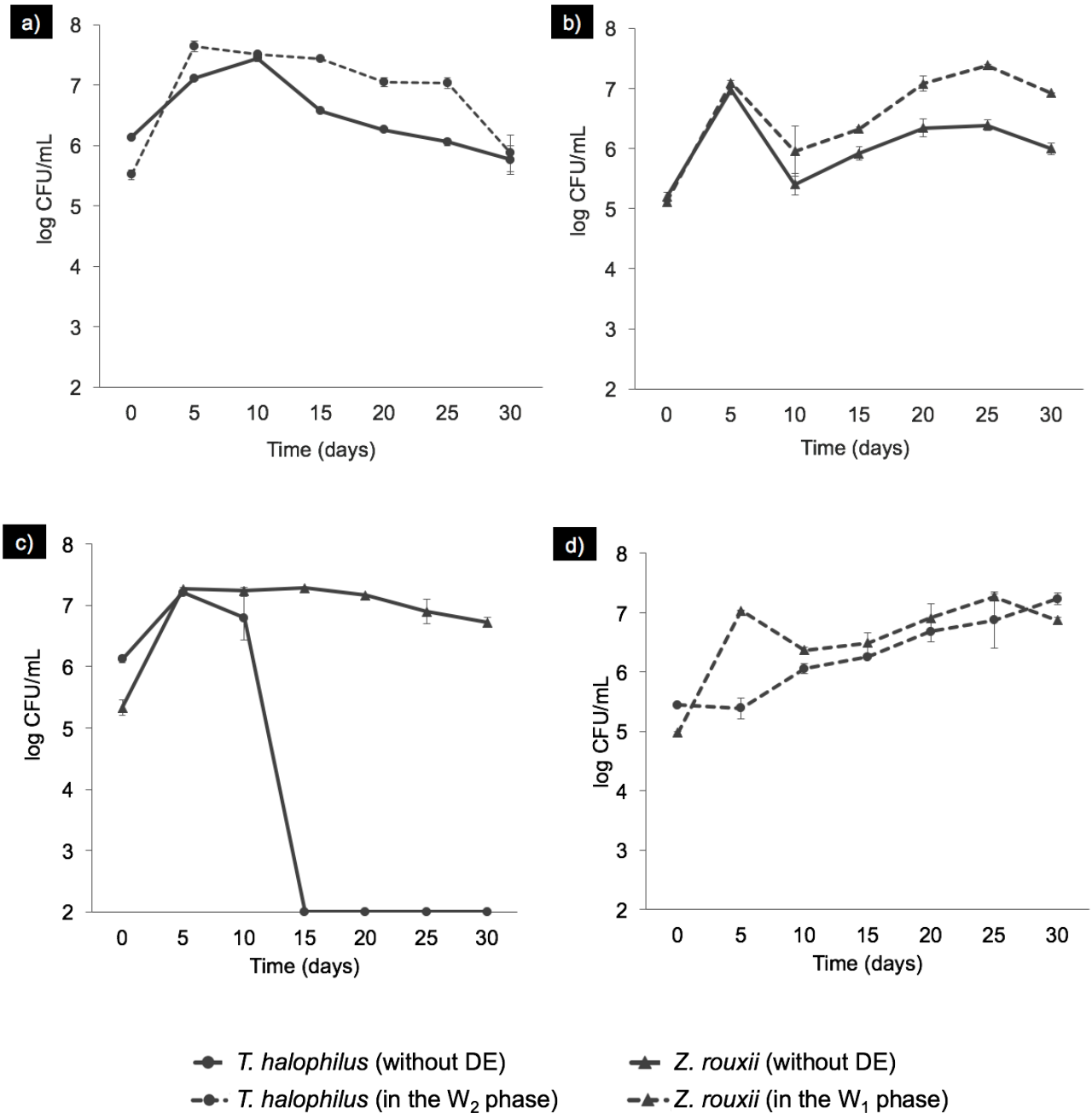
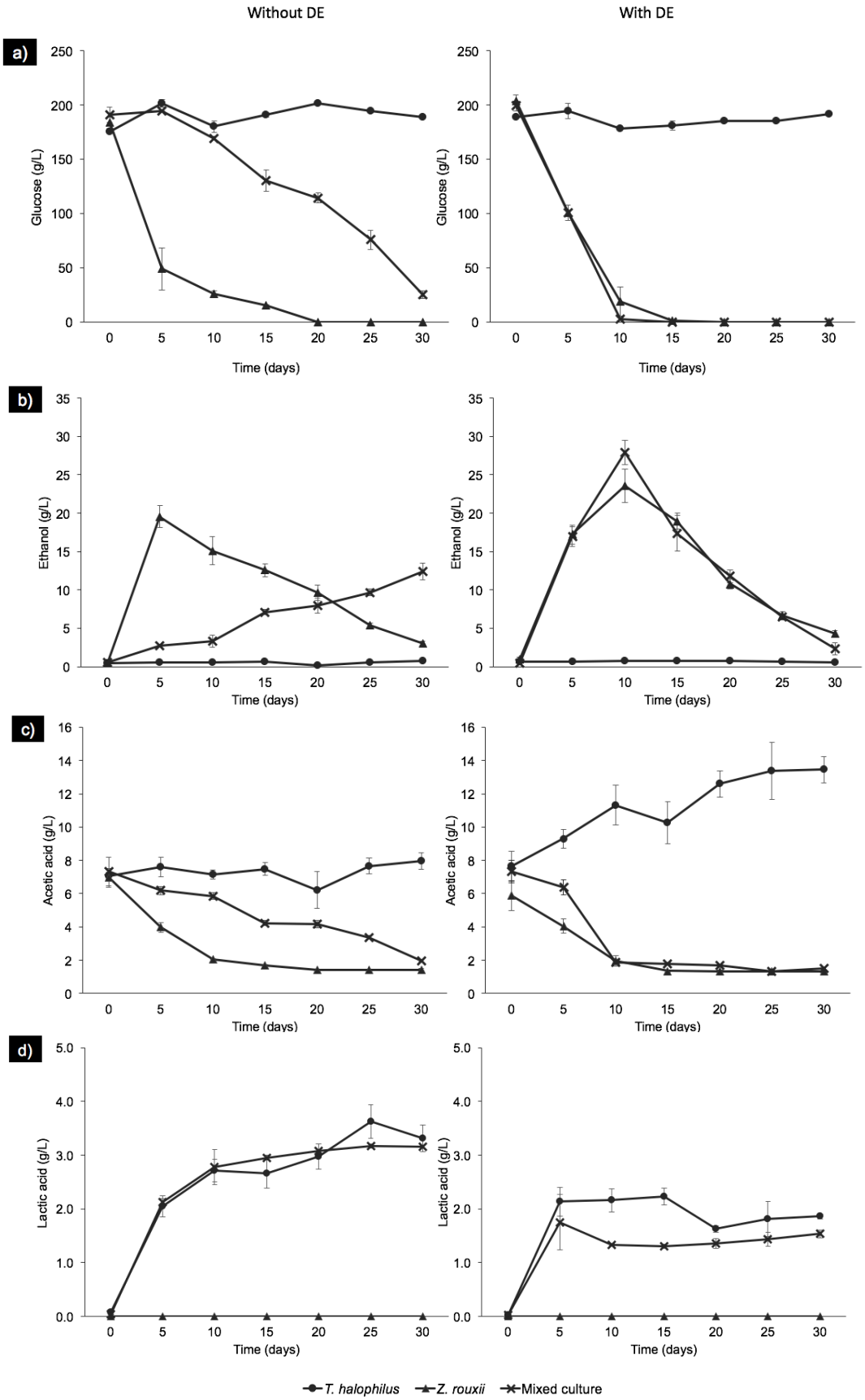


Figure 6. Changes in viable cell number of (a) *T. halophilus*, (b) *Z. rouxii*, and mixed culture (c) without and (d) with DE, during 30 days of incubation at 30°C.



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

651