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1 Segregation of *Tetragenococcus halophilus* and
2 *Zygosaccharomyces rouxii* using W₁/O/W₂ double
3 emulsion for use in mixed culture fermentation

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10

11 **Abstract**

12 Antagonism in mixed culture fermentation can result in undesirable metabolic activity and
13 negatively affect the fermentation process. Water-oil-water (W₁/O/W₂) double emulsions (DE)
14 could be utilized in fermentation for segregating multiple species and controlling their release
15 and activity. *Zygosaccharomyces rouxii* and *Tetragenococcus halophilus*, two predominant
16 microbial species in soy sauce fermentation, were incorporated in the internal W₁ and external
17 W₂ phase of a W₁/O/W₂, respectively. The suitability of DE for controlling *T. halophilus* and
18 *Z. rouxii* in soy sauce fermentation was studied in relation to emulsion stability and microbial
19 release profile. The effects of varying concentrations of *Z. rouxii* cells (5 and 7 log CFU/mL)

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20 and glucose (0%, 6%, 12%, 30% w/v) in the W₂ phase were investigated. DE stability was
21 determined by monitoring encapsulation stability (%), oil globule size, and microstructure with
22 fluorescence and optical microscopy. Furthermore, the effect of DE on the interaction between
23 *T. halophilus* and *Z. rouxii* was studied in Tryptic Soy Broth containing 10% w/v NaCl and
24 12% w/v glucose and physicochemical changes (glucose, ethanol, lactic acid, and acetic acid)
25 were monitored. DE destabilization resulted in cell release which was proportional to the
26 glucose concentration in W₂. Encapsulated *Z. rouxii* presented higher survival during storage
27 (~3 log). The application of DE affected microbial cells growth and physiology, which led to
28 the elimination of antagonism. These results demonstrate the potential use of DE as a delivery
29 system of mixed starter cultures in food fermentation, where multiple species are required to
30 act sequentially in a controlled manner.

31 Keywords: W₁/O/W₂ double emulsion; yeast encapsulation; *Tetragenococcus halophilus*;
32 *Zygosaccharomyces rouxii*; soy sauce; microbial antagonism.

33 **1. Introduction**

34 The utilization of mixed cultures in food fermentation is preferred over single culture since it
35 offers benefits such as improved flavor production and aroma complexity (Narvhus & Gadaga,
36 2003; Smit, Smit, & Engels, 2005), food safety (Freire, Ramos, & Schwan, 2015), and health
37 benefits (Chen et al., 2017). Each species of the mixed microbial community contributes to the
38 fermentation process individually. However, antagonistic interactions have been observed due
39 to production of growth-inhibitory compounds (Kemsawasd et al., 2015; Li & Liu, 2016),
40 changes in physicochemical properties of the substrate (e.g. pH) (Devanthi, Linforth, Onyeaka,
41 & Gkatzionis, 2018), competition for nutrients (Medina, Boido, Dellacassa, & Carrau, 2012),
42 and cell-to-cell contact-mediated inhibition (Nissen, Nielsen, & Arneborg, 2003). Such
43 interactions negatively affect the equilibrium in the mixed microbial community and

44 performance of each species. Therefore, a formulation is needed for controlling the delivery
45 and activity of these species, thus minimizing the effects of antagonistic interaction and
46 maximizing flavor development. Compared to free cells system in fermentation, microbial
47 encapsulation offers the benefits of higher cell density for faster fermentation and enhanced
48 tolerance against adverse conditions.

49 *Tetragenococcus halophilus* and *Zygosaccharomyces rouxii* are predominant lactic acid
50 bacteria (LAB) and yeast, respectively, during the second stage of soy sauce fermentation (or
51 brine fermentation) called *moromi* (van der Sluis, Tramper, & Wijffels, 2001). The activity of
52 these species is crucial since they produce key volatile compounds, taste active amino acids,
53 peptides, and sugars that contribute to the final flavor of soy sauce (Harada et al., 2016; Zhao,
54 Schieber, & Gänzle, 2016; Zhu & Tramper, 2013). However, our previous study has shown
55 antagonistic interactions between the two microbes that result in compromising flavor
56 development due to the rapid growth of *T. halophilus* producing lactic acid and acetic acid,
57 suppressing the alcoholic fermentation by yeast (Devanathi et al., 2018). On the other hand,
58 excessive alcohol production by *Z. rouxii* at the beginning of fermentation inhibits lactic acid
59 fermentation (Kusumegi, Yoshida, & Tomiyama, 1998).

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

68 Water-oil-water (W₁/O/W₂) double emulsion (DE) is a type of emulsion that contains two
69 aqueous compartments separated by an oil phase. Its multi-compartmentalized structure could
70 be used for delivering multiple starter cultures during fermentation, when minimum
71 interference between species is required. Segregation of multiple microbial species was
72 previously studied by Nissen et al. (2003) and Kemsawasd et al. (2015) during mixed culture
73 fermentation using dialysis tubing and double-compartment fermentation system separated by
74 cellulose membranes, respectively. Such compartmentalization was shown to reduce
75 antagonism caused by cell-to-cell contact and antimicrobial peptides secretion. Moreover,
76 stable mixed culture of *Lactococcus lactis* and *Bifidobacterium longum* was obtained during
77 continuous fermentation in two-reactor system by separately immobilizing the two strains in
78 κ-carrageenan/locust bean gum gel beads (Doleyres, Fliss, & Lacroix, 2004).

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

89 The aim of this study was to investigate the feasibility of DE as a delivery system of soy sauce
90 starter cultures, including its stability and release, effect on cell viability, and species-to-species
91 interaction under conditions relevant to moromi stage of soy sauce fermentation. Brine solution

92 and soybean oil were used as water and oil phases, respectively, in order to create a formulation
93 that reflects the moromi process. The effects of varying concentrations of *Z. rouxii* in the W₁
94 phase and glucose in the W₂ phase on DE stability and release profile were investigated and
95 the survival of the encapsulated *Z. rouxii* was monitored over storage. Also, the interaction
96 between *T. halophilus* and *Z. rouxii* was investigated by monitoring the microbiological and
97 physicochemical changes of the culture medium.

98 **2. Material and Methods**

99 *2.1 Materials*

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

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

115 2.2 Cultures preparation

116 *T. halophilus* was maintained on BHI agar supplemented with 10% (w/v) NaCl at 37 °C. Cells
117 were transferred into MRS broth containing 7% (w/v) NaCl followed by incubation for 36 h in
118 37 °C static incubator. Final cell concentration was adjusted to 10⁶ cells/mL. *Z. rouxii* was
119 maintained on YM agar with 5% (w/v) NaCl and incubated at 30 °C. *Z. rouxii* cells were
120 transferred into YM broth containing 5% (w/v) NaCl and incubated at 30 °C for 24 h with
121 agitation (150 rpm). Cells (10⁷ cells/mL) were harvested and washed by centrifuging at 10000
122 g for 15 min. In order to test the effect of initial cell concentration on emulsion stability, cell
123 concentrations were adjusted to 10⁸ cells/mL and 10⁶ cells/mL.

124 2.3 DE preparation

125 The DEs were prepared using the 2-step emulsification method at ambient temperature by
126 using a high shear mixer (Silverson L5M). In the first step, W₁/O primary emulsion was
127 prepared by mixing sterile 10% (w/v) NaCl solution into the oil phase (soybean oil with 2% wt
128 PGPR) at W₁ : oil phase ratio of 20 : 80 at 1700 rpm for 2 min. For yeast encapsulation, *Z.*
129 *rouxii* suspensions in 10% (w/v) NaCl solution (10⁸ cells/mL and 10⁶ cells/mL) were used as
130 W₁.

131 In the second stage, W₁/O was re-emulsified in the continuous phase (W₂; sterile 10% (w/v)
132 NaCl in water with 1 % wt Tween80) at 2000 rpm for 1 min (W₁/O : W₂ ratio of 20 : 80). In
133 order to study the effect of glucose on the stability of DE and *Z. rouxii* release profile, various
134 concentrations of glucose (0%, 6%, 12%, and 30% w/v) were added to the W₂ in addition to
135 8.05% (w/v) NaCl (Table 1). The osmotic pressure gradient was calculated using Van't Hoff
136 equation as follows:

$$137 \quad \Delta\pi = (C_i - C_e)RT \quad (1)$$

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

140 DEs containing *T. halophilus* in the W_2 were prepared by directly adding 2 mL of *T. halophilus*
141 (10^6 cells/mL) into the W_2 after mixing process. For the study that investigates the effects of
142 DE on *T. halophilus* and *Z. rouxii* interaction, both microorganisms in DE or as free cells (as
143 a single or mixed cultures) were transferred into double concentrated TSB supplemented with
144 10% w/v NaCl and 12% w/v glucose. *T. halophilus* and *Z. rouxii* were inoculated at final
145 concentrations of 10^6 CFU/mL and 10^5 CFU/mL, respectively, followed by incubation in 30
146 °C static incubator for 30 days.

147 2.4 DE stability characterization

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

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

$$159 \quad \text{Creaming volume (\%)} = (\text{Creaming layer volume} / \text{Total volume of DE}) \times 100\% \quad (2)$$

160 **Microscopy observation:** DEs microstructure was observed by placing the samples onto
161 microscope slides followed by observation under a light microscope (Olympus BX50) with a
162 10x objective lens. Images were taken using Moticam 10 camera via Motic Images Plus video
163 acquisition software at 17fps.

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

170 2.5 *Determination of the encapsulation efficiency and encapsulation stability of DEs*

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

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

$$181 \quad \text{Cell concentration (cell/mL)} = (\text{Total number of cells} \times 25 \times 10^4) / \text{Number of squares} \quad (3)$$

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

$$184 \quad \text{Encapsulation efficiency (\%)} = ((N_0 - N_{w2})/N_0) \times 100\% \quad (4)$$

$$185 \quad \text{Encapsulation stability (\%)} = ((N_0 - N_{w2(t)})/N_0) \times 100\% \quad (5)$$

186 where N_0 is the number of free *Z. rouxii* cells initially added in the inner phase, while N_{w2} and
187 $N_{w2(t)}$ are the number of non-encapsulated *Z. rouxii* cells measured immediately after DEs were
188 formed and as a function of storage time, respectively.

189 2.6 *T. halophilus* and *Z. rouxii* cell enumeration

190 Viable cell counts were made by taking 0.1 mL of samples subjected to serial dilution in PBS
191 (phosphate buffered saline) buffer solution followed by plating on BHI agar supplemented with
192 7% (w/v) NaCl and 21.6 mg/L natamycin for *T. halophilus* and YM agar with the addition of
193 5% (w/v) NaCl and 100 mg/L chloramphenicol for *Z. rouxii*. Bacteria and yeast colonies were
194 counted after 2 days of incubation at 30 °C.

195 2.7 Physicochemical changes

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

201 GC analysis was performed using GC-2010 (Shimadzu, Japan), equipped with a flame
202 ionization detector (FID). Prior to analysis, samples were filtrated through 0.22 µm pore size
203 filter (Millex GP, Millipore, United Kingdom) and 300 µL of samples were added with 200 µL

204 hexylene glycol (Sigma Aldrich, United Kingdom) as an internal standard at final
205 concentration of 742 mg/L. Samples (1 μ L) were injected using auto sampler with split ratio
206 of 100:1 at 260 °C. Compound separation was done by using ZB-WAX plus column (30 m,
207 0.25 mm I.D., 0.25 μ m film thickness, Phenomenex, United States) and helium as the carrier
208 gas at a pressure of 104.99 kPa. The oven temperature was programmed at an initial
209 temperature of 30 °C for 5 min, followed by an increase to 50 °C at 4 °C/min (held for 5 min),
210 150 °C at 20 °C/min (held for 5 min), 200 °C at 10 °C/min (held for 5 min), and finally increase
211 to 220 °C at 4 °C/min. FID temperature was set to 300 °C.

212 2.8 *Statistics*

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

217 3. Results and discussion

218 3.1 *Encapsulation efficiency and stability during storage*

219 The amount of encapsulated *Z. rouxii* cells was monitored over storage (Figure 1). *Z. rouxii*
220 was successfully encapsulated in the internal W_1 phase of DEs with high encapsulation
221 efficiency (>99%; Figure 1a) regardless of low (10^5 CFU/mL) or high (10^7 CFU/mL) cell
222 concentrations. Relatively high encapsulation stability of DE was maintained up to 14 days of
223 storage (>75%), and significantly ($p < 0.05$) decreased at day 30 to 13.28% and 30.72%, for low
224 and high cell concentration, respectively. This observation was associated with the
225 fluorescence microscopy images (Figure 1b-d) in which non-encapsulated cells were
226 observable in the external W_2 phase at day 30. Furthermore, the stability of DE decreased over

227 time regardless of the presence and amount of encapsulated cells, as indicated by the loss in
228 inner W_1 phase after 30 days (Figure 2a). Such time dependent loss of inner W_1 phase could
229 occur due to coalescence between the W_1 droplets as well as coalescence between W_1 droplets
230 and the oil globule's interface (Chávez-Páez, Quezada, Ibarra-Bracamontes, González-Ochoa,
231 & Arauz-Lara, 2012). Ficheux et al. (1998) found that Tween80 migrates from the oil globule's
232 interface through the oil phase to the W_1 droplet's interface and displaces the lipophilic
233 surfactant (Span 80) molecules which causes an increase in coalescence events between the
234 W_1 droplet and the oil globule's interface leading to DE to become a single O/W emulsion. In
235 this study, such coalescence events may have occurred resulting in the release of hydrophilic
236 substances including *Z. rouxii* cells into the W_2 phase. Although the amount of W_1 phase
237 decreased, the average size (Figure 2b) and size distribution of the oil globules (Figure 2c-e)
238 were apparently preserved throughout storage and this might be attributed to coalescence
239 occurring between the W_1 droplets and the oil globule's interface as well as between the oil
240 globules. Such coalescence events have shown to increase the size of the interfacial film of the
241 oil globules despite loss in the W_1 phase maintaining the oil globule's size (Ficheux et al.,
242 1998). These results indicate the possibility to use such inherent instability of DE as a
243 mechanism for the release of *Z. rouxii* cells during fermentation.

244 3.2 *The effect of glucose concentration on cell release and DE stability*

245 During moromi fermentation of soy sauce, *Z. rouxii* converts glucose into biomass and ethanol.
246 Changes in glucose concentration would alter the osmotic pressure balance between the two
247 phases of DE, therefore affecting its microstructure and encapsulation stability. For this reason,
248 the microstructure of DE (Figure 3a), *Z. rouxii* cells release profile (Figure 3b), and the oil
249 globule size (Figure 4a-c), were monitored by varying glucose concentration (0%, 6%, 12%,
250 and 30%) in the external W_2 phase, which created osmotic pressure gradient between W_1 and
251 W_2 phase, except for 12% which was designed to have balanced osmotic pressure (Table 1).

252 Prior to investigation, the ability of *Z. rouxii* to grow in glucose solution (5%) in the absence
253 of other nutrients was tested and the viable cells decreased by 2.53 log CFU/mL after 7 days
254 of incubation (data not shown). This aimed to ensure that the quantified cells during the release
255 study were solely due to release from the W_1 to W_2 phase and not the result of microbial growth.

256 The release profile was found to be influenced by the amount of glucose in the W_2 phase
257 (Figure 3b) and it followed a similar pattern to the loss in the W_1 phase (Figure 3a), by which
258 the complete loss in the W_1 phase was observed when maximum cell release occurred.
259 However, the DE instability and cell release rate were found to be driven by increasing amount
260 of glucose, rather than the osmotic pressure difference between the two phases. In the presence
261 of 30% glucose ($\Delta\pi = -24.84$ atm), the DE was transformed into O/W single emulsion due to
262 complete loss of the inner W_1 phase within 3 days, accompanied with a sharp increase in the
263 number of released cells which was followed by a plateau thereafter. Meanwhile, the release
264 of *Z. rouxii* cells in 6% glucose was gradual throughout storage and took place in a manner
265 comparable to control (0% glucose). The destabilization of DE containing 0% and 6% glucose
266 was reduced as the oil globules maintaining their inner W_1 phase were still noticeable by the
267 end of storage. Although DE with 12% glucose was designed to be osmotically balanced ($\Delta\pi$
268 = 0 atm), the DE microstructure was found to be more unstable compared to DEs with 0% ($\Delta\pi$
269 = 16.54 atm) and 6% glucose ($\Delta\pi = 8.28$ atm) as it was transformed into O/W single emulsion
270 by the end of storage. This also resulted in higher amount of cell release compared to DEs with
271 0% and 6% glucose. These results suggest that the faster release of *Z. rouxii* was associated
272 with increased destabilization of the DE.

273 The phenomena observed in this study are in contrast to the previous studies reporting the effect
274 of glucose in W_2 on osmotic pressure alteration of DE. The presence of glucose causes an
275 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 W₂ 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 W₂ 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 W₂ phase possessed the
295 lowest initial oil globule size ($37.28 \pm 0.74 \mu\text{m}$; 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 W₂ 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

301 size of oil globule during storage occurred in cases of 0%, 6%, and 12% glucose although these
302 responses were not statistically significant ($p < 0.05$) (Figure 4a). In contrast, DE containing
303 30% glucose showed significant ($P < 0.05$) increase in oil globule size at day 3 which then
304 stabilized until the end of storage period, although the oil globules lost their inner W_1 . This can
305 be attributed to the increase in coalescence events between the oil globules as it becomes less
306 stable in the presence of glucose. These results show that the stability of DE and release of *Z.*
307 *rouxii* are influenced by the glucose concentration regardless of the osmotic pressure gradient
308 between the two phases. However, the responses do not follow the same direction or linearity
309 in all cases.

310 3.3 *Z. rouxii* cell viability after emulsification and during storage

311 To investigate the effect of emulsification and encapsulation on survival of *Z. rouxii*, cell
312 viability was assessed immediately after encapsulation and during storage. The relative
313 viability of *Z. rouxii* cells soon after the emulsification process was ~100% (Figure 5), showing
314 that the encapsulation technique as well as the surfactants used did not affect the yeast. This
315 was reported in other studies for bacterial cells (El Kadri et al., 2015; Shima et al., 2006).
316 Interestingly, the encapsulated cells viability remained high during 30 days of storage in the
317 absence of nutrient (~2 log CFU/mL decrease), while no viable cells were detected in non-
318 encapsulated cells by the end of incubation period (Figure 5). The oil layer which functions as
319 a barrier, might reduce mass transport and biological communication between the *Z. rouxii*
320 cells and the environment and thus result in molecular gradient that could switch cells to the
321 non-dividing resting state (G0) (Wang et al., 2008). Furthermore, the cells resistance towards
322 environmental stress increases once it enters the resting state, including the ability to survive
323 extended periods of starvation (Herman, 2002). It could be argued that *Z. rouxii* may have
324 utilized the surfactants (PGPR and Tween80) as carbon sources (Luh, 1995), thus enabling the
325 yeast to grow. However, no growth was observed when *Z. rouxii* was incubated with PGPR or

326 Tween80 only (data not shown). These results indicate that encapsulation in DE is able to
327 prolong life of *Z. rouxii* in the absence of nutrients.

328 3.4 The effects of encapsulation on *T. halophilus* and *Z. rouxii* interactions.

329 Interaction between microbial species during fermentation would influence their growth which
330 further affects the proportion of microbial population and their metabolic activity. In this study,
331 the co-presence of *T. halophilus* and *Z. rouxii* resulted in antagonism as *T. halophilus* growth
332 was inhibited, as indicated by a sharp decrease in *T. halophilus* cell count to undetectable level
333 (< 2 log CFU/mL) at day 15 (Figure 6c). This observation was in contrast to our previous study
334 in which the growth inhibition was observed on *Z. rouxii* instead of *T. halophilus*, when both
335 were co-present in a moromi model system (Devanthi et al., 2018). According to a study by
336 Noda et al (1980), metabolite produced by *Pediococcus halophilus* (later reclassified as *T.*
337 *halophilus*) during moromi fermentation can inhibit the growth of osmophilic *shoyu* yeasts
338 such as *Saccharomyces rouxii* (later reclassified as *Z. rouxii*) and *Torulopsis versatilis*.
339 However, a study by Inamori, Miyauchi, Uchida, & Yoshino (1984) showed that the growth
340 inhibition in mixed cultures could occur to *P. halophilus* under aerobic conditions or *S. rouxii*
341 under anaerobic conditions in static culture. Growth inhibition of *T. halophilus* in this study
342 was possibly due to the aerobic conditions used during incubation. Also, inhibitory effect
343 towards *Z. rouxii* which was previously reported, was observed in a digested liquid mixture of
344 pre-cooked soybean and roasted wheat (Devanthi et al., 2018; Noda et al., 1980), while in this
345 study interaction assay was performed in a synthetic broth medium. Furthermore, the presence
346 of acetic acid in this study was unlikely to cause growth inhibition on *Z. rouxii* as previously
347 reported (Kusumegi et al., 1998; Noda et al., 1982). It was suggested that acetic acid could
348 interfere with proton expulsive activity of *Z. rouxii* for its halo-tolerance mechanisms, causing
349 growth inhibition at NaCl concentration above 10%. In this study, we did not observe any

350 decrease in *Z. rouxii* cells population which was possibly due to relatively low NaCl
351 concentration (10%) used in the medium.

352 The compartmentalization of *T. halophilus* and *Z. rouxii* in DE affected the growth kinetics in
353 both single and co-culture. The growth of *T. halophilus* (Figure 6a) and *Z. rouxii* (Figure 6b)
354 as single culture was slightly enhanced and the antagonism between *T. halophilus* and *Z. rouxii*
355 was no longer observed when *Z. rouxii* was encapsulated in DE (Figure 6d). *T. halophilus* was
356 able to propagate steadily throughout the incubation period, reaching a final count of 7.23 log
357 CFU/mL (Figure 6d). The final cell counts of *Z. rouxii* in DE (6.87 log CFU/mL) did not differ
358 significantly ($p < 0.05$) from non-DE culture (6.72 log CFU/mL), although a different growth
359 pattern was observed, and its growth was not affected by the presence of *T. halophilus* in the
360 W₂ phase. The oil layer functions as a physical barrier separating *T. halophilus* from *Z. rouxii*,
361 thus minimizing antagonistic interaction between them. Also, the oil layer could serve as a
362 selective membrane, allowing chemicals or molecules to diffuse in or out based on their
363 molecular weight (Zhang et al., 2013). In this study, deleterious metabolite compounds
364 produced by *Z. rouxii*, might not be able to pass through the oil layer to the bulk medium (W₂
365 phase) due to its molecular weight, thus minimizing its harmful effects toward *T. halophilus*.
366 The ability of DE to gradually release the *Z. rouxii* into the bulk medium might also prevent
367 detrimental effects toward *T. halophilus*. However, high *Z. rouxii* cell population was observed
368 in the bulk medium due to their propagation after being released and yet the inhibitory effect
369 towards *T. halophilus* was absent. *T. halophilus* might have exhibited physiological changes in
370 the presence of DE, increasing its tolerance against inhibitory effect of *Z. rouxii*.

371 3.5 Physicochemical changes in DE during *T. halophilus* and *Z. rouxii* growth

372 To further understand how the presence of DE with single or mixed cultures can affect the
373 interaction between the two microorganisms the physicochemical changes during fermentation

374 were monitored. As seen in Figures 7a-d, the presence of DE caused alteration in the metabolic
375 activity of both microorganisms as a single or mixed cultures.

376 Glucose consumption (Figure 7a) correlated with ethanol production (Figure 7b). Glucose was
377 exclusively consumed by *Z. rouxii*, therefore, ethanol was only produced in its presence. Both
378 glucose consumption and ethanol production were accelerated when *Z. rouxii* was
379 encapsulated. With mixed cultures, glucose was consumed in a gradual manner in the absence
380 of DE which was accompanied by a slow production of ethanol, reaching maximum
381 concentration of 12.39 g/L at day 30. In contrast, glucose was consumed faster in DE as it was
382 depleted at day 10, associated with maximum ethanol production (27.94 g/L) which was
383 comparable to concentrations in good quality soy sauce (Luh, 1995). Similar level of ethanol
384 was also obtained in rapid fermentation of soy sauce described by Muramatsu, Sano, Uzuka,
385 & Company (1993). Once glucose was depleted, the ethanol production was terminated and its
386 concentration continuously decreased throughout the incubation period. Encapsulation seemed
387 to delay glucose consumption by *Z. rouxii* as only half amount of glucose was consumed during
388 the first 5 days when *Z. rouxii* was encapsulated. However, this led to prolonged ethanol
389 production for up to 10 days, producing higher maximum concentration of ethanol (23.56 g/L)
390 compared to non-encapsulated cells (19.57 g/L) with single culture.

391 *T. halophilus* played a major role in both acetic acid (Figure 7c) and lactic acid (Figure 7d)
392 formation. In mixed culture, acetic acid concentration gradually decreased when *Z. rouxii* was
393 non-encapsulated, while the acetic acid concentration sharply decreased within the first 10 days
394 to 1.86 g/L when *Z. rouxii* was encapsulated. This was comparable to the amount of acetic acid
395 found in top-graded bottled soy sauces in China (Xu, 1990). However, the acetic acid
396 production by *T. halophilus* as single culture markedly increased by 1.7 fold in the presence of
397 DE although *T. halophilus* was non-encapsulated. In contrast, lactic acid production was

398 suppressed when DE was present, as the amount of lactic acid remained stable from day 5
399 onwards, and the suppression was more obvious in mixed cultures. The yield of lactic acid in
400 the presence of DE was about half of the bottled soy sauces in China (Xu, 1990). In contrast,
401 lactic acid increased exponentially in all non-DE systems, reaching almost twice the amount
402 of lactic acid produced in the presence of DE. The presence of DE might have caused a shift
403 in metabolic pathway of *T. halophilus* cells from homofermentative to heterofermentative, thus
404 decreasing the lactic acid yields (Krishnan, Gowda, Misra, & Karanth, 2001).

405 These results suggest that the presence of DE affects the physicochemical changes during *T.*
406 *halophilus* and *Z. rouxii* growth in both single and mixed culture. Changes in microbial cells
407 morphology and physiology due to immobilization have been reported in several studies
408 reviewed by Lacroix & Yildirim (2007), including increase in the production of insoluble
409 exopolysaccharides (Bergmaier, Champagne, & Lacroix, 2005), lactic acid (Lamboley,
410 Lacroix, Artignan, Champagne, & Vuillemand, 1999), as well as a shift in metabolic pathway
411 from homofermentative to heterofermentative, resulting in decreased lactic acid production
412 (Krishnan et al., 2001). The altered metabolic activity may have contributed to the elimination
413 of antagonism by reducing the production of inhibitory metabolites or enhancing the
414 production of metabolites essential for *T. halophilus* growth by *Z. rouxii*, as well as enhancing
415 cell adaptation towards changing environmental conditions. However, further investigation is
416 required to understand how the presence of DE affects the cells both in the W₁ and W₂ phase
417 at the metabolic level.

418 **4. Conclusion**

419 The results in this study suggest that DEs could be a suitable formulation for the delivery of
420 mixed starter cultures in soy sauce fermentation. *Z. rouxii* was successfully encapsulated in DE
421 which enhanced survival during storage and eliminated antagonistic interaction with *T.*

422 *halophilus*. The presence of DE altered the metabolic activity of the two species, which could
423 have contributed to the elimination of antagonism. Although the initial encapsulation efficiency
424 was high, it decreased over time due to DE instability and this could be utilized as a mechanism
425 for gradual cell release depending on the glucose concentration in the W₂ phase. DE could offer
426 a valuable tool for standardizing the microbial activity and aroma development in soy sauce
427 fermentation. However, further study is needed for these observations to be validated in real
428 soy sauce fermentation.

429 **5. Acknowledgements**

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431 Finance for funding this work as part of a doctorate program.

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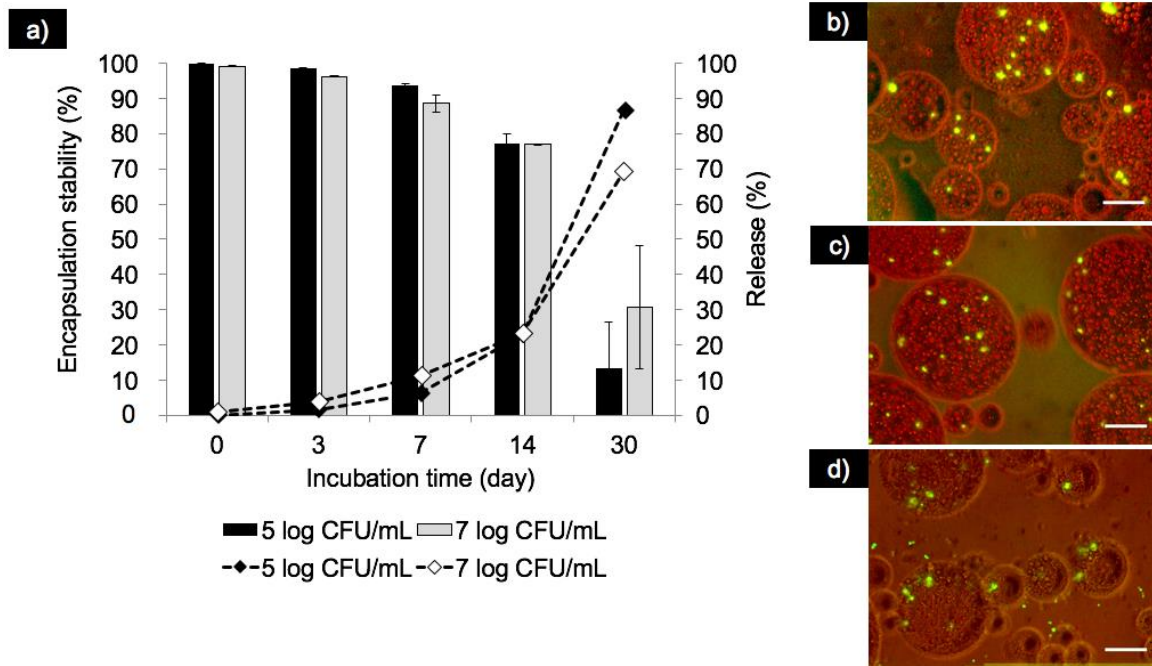
556 Table 1. Formulation of $W_1/O/W_2$ double emulsions (DE) with varying glucose concentrations

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		
			0%	16.54	$W_1 > W_2$
10%	8.05%	0%	6%	8.28	$W_1 > W_2$
			12%	0	$W_1 = W_2$
			30%	-24.84	$W_1 < W_2$

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

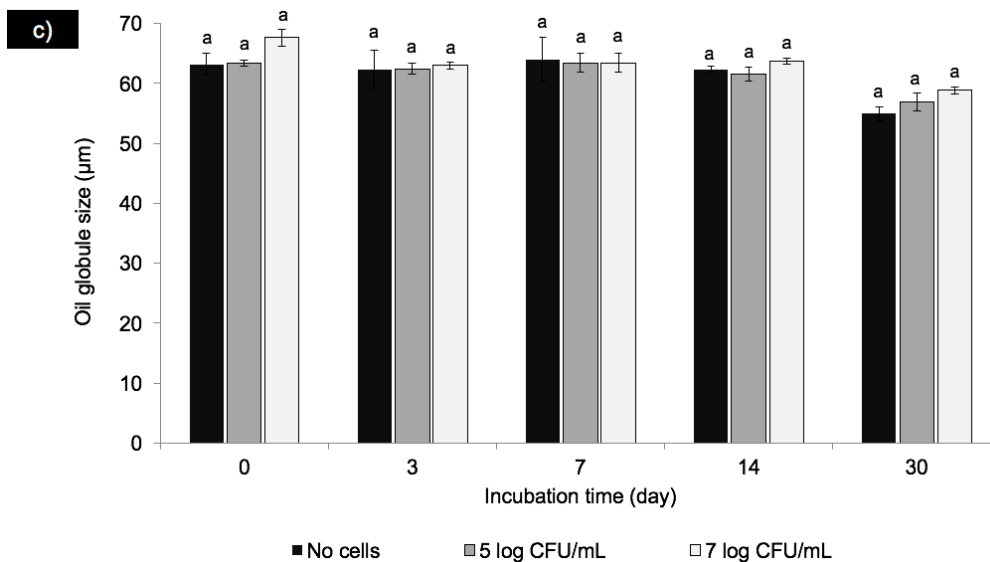
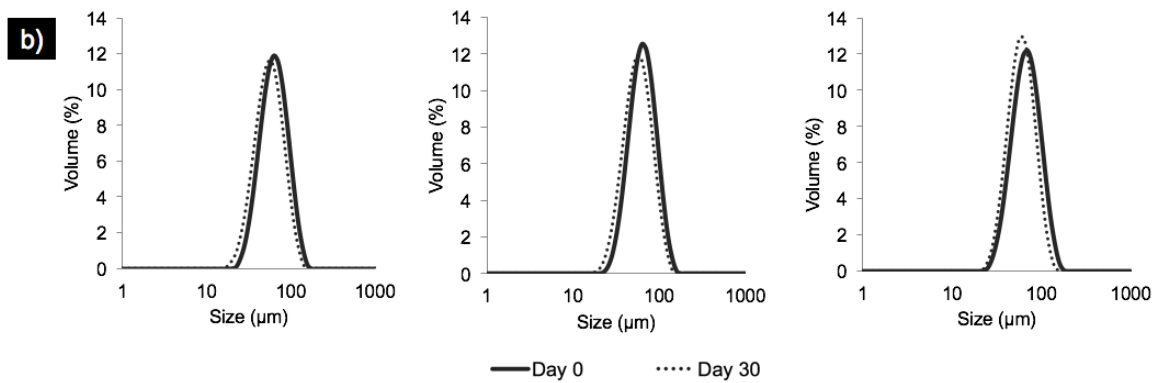
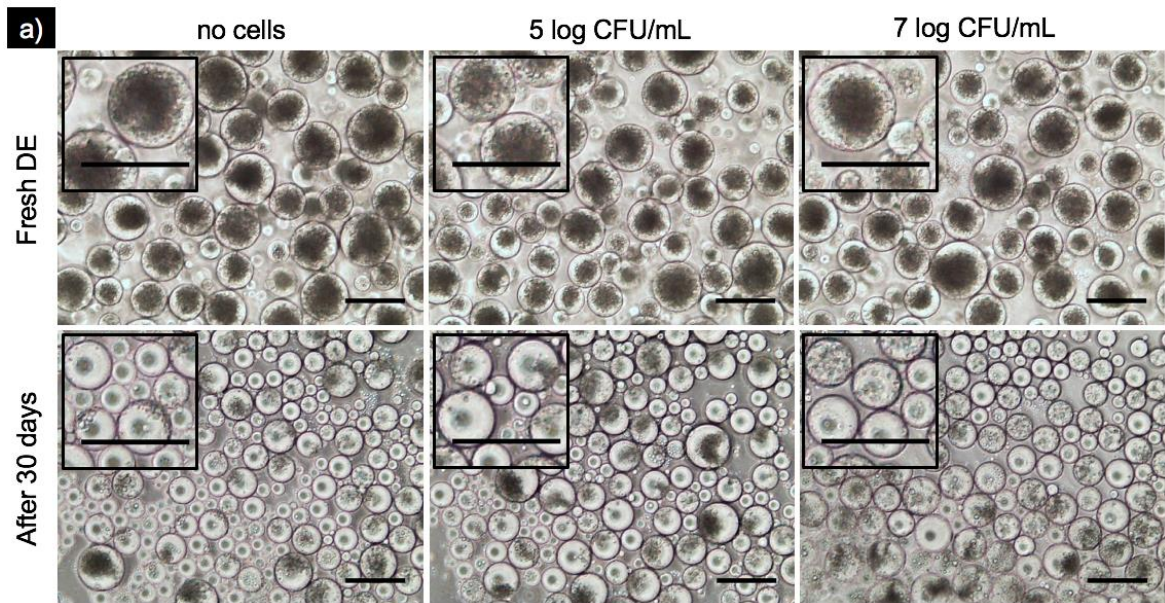
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571 Figure 2. DEs with no cells, 5 log CFU/mL, and 7 log CFU/mL before and after 30 days of

572 storage at 30 °C under iso-osmotic condition. (a) Optical micrographs; (b) Oil globule size

573 distribution; (c) Average oil globule size. Scale bar: 100 μm . Mean values with different letters
574 are significantly different ($p < 0.05$).

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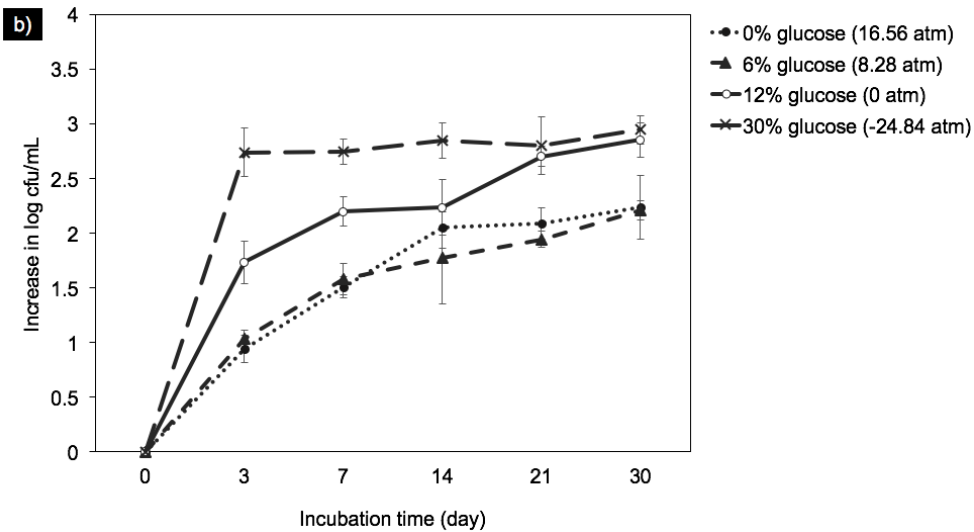
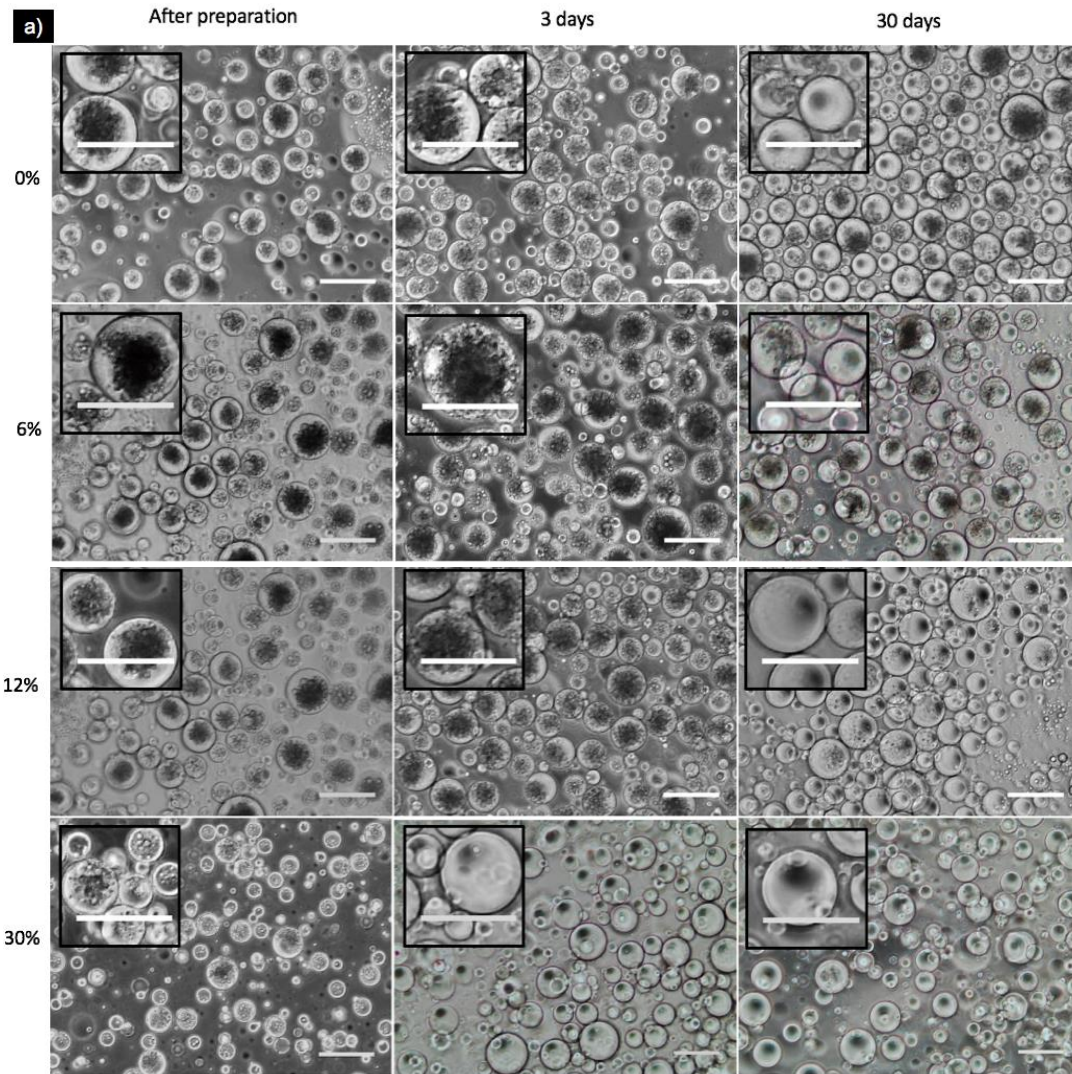
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599 Figure 3. Double emulsion with 0%, 6%, 12%, and 30% glucose in the W₂ phase after
600 preparation, 3, and 30 days of storage at 30 °C. (a) Optical micrographs of W₁/O/W₂. (b) *Z.*
601 *rouxii* cell release profile into W₂ phase. Scale bar: 100 μm.

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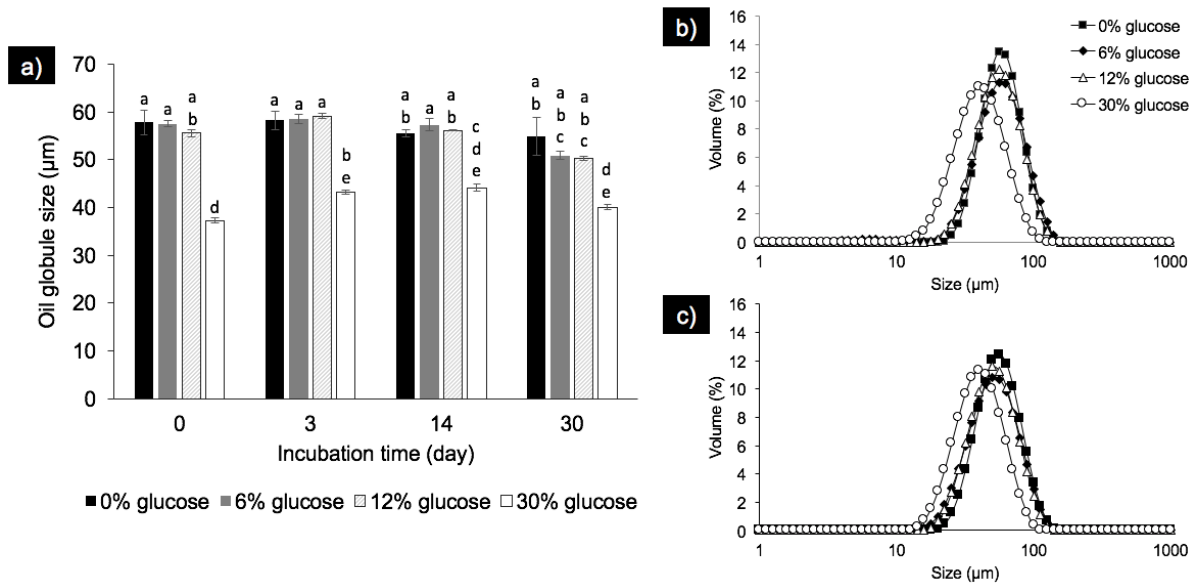
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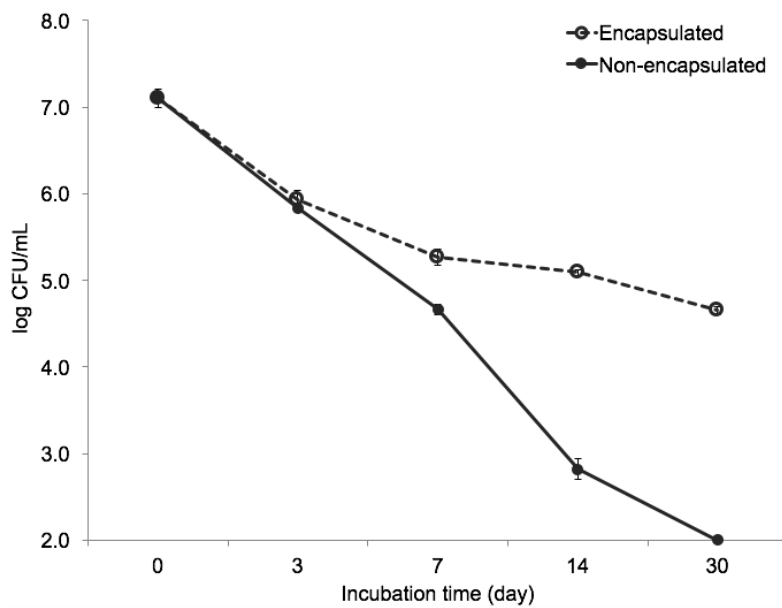
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632 Figure 4. DEs before and after 30 days of storage at 30 °C with different glucose concentrations
633 in the external W₂ phase. (a) Average oil globule size; (b) Oil globule size distribution of DEs
634 before storage and (c) after storage. Mean values with different letters are significantly different
635 (p<0.05).



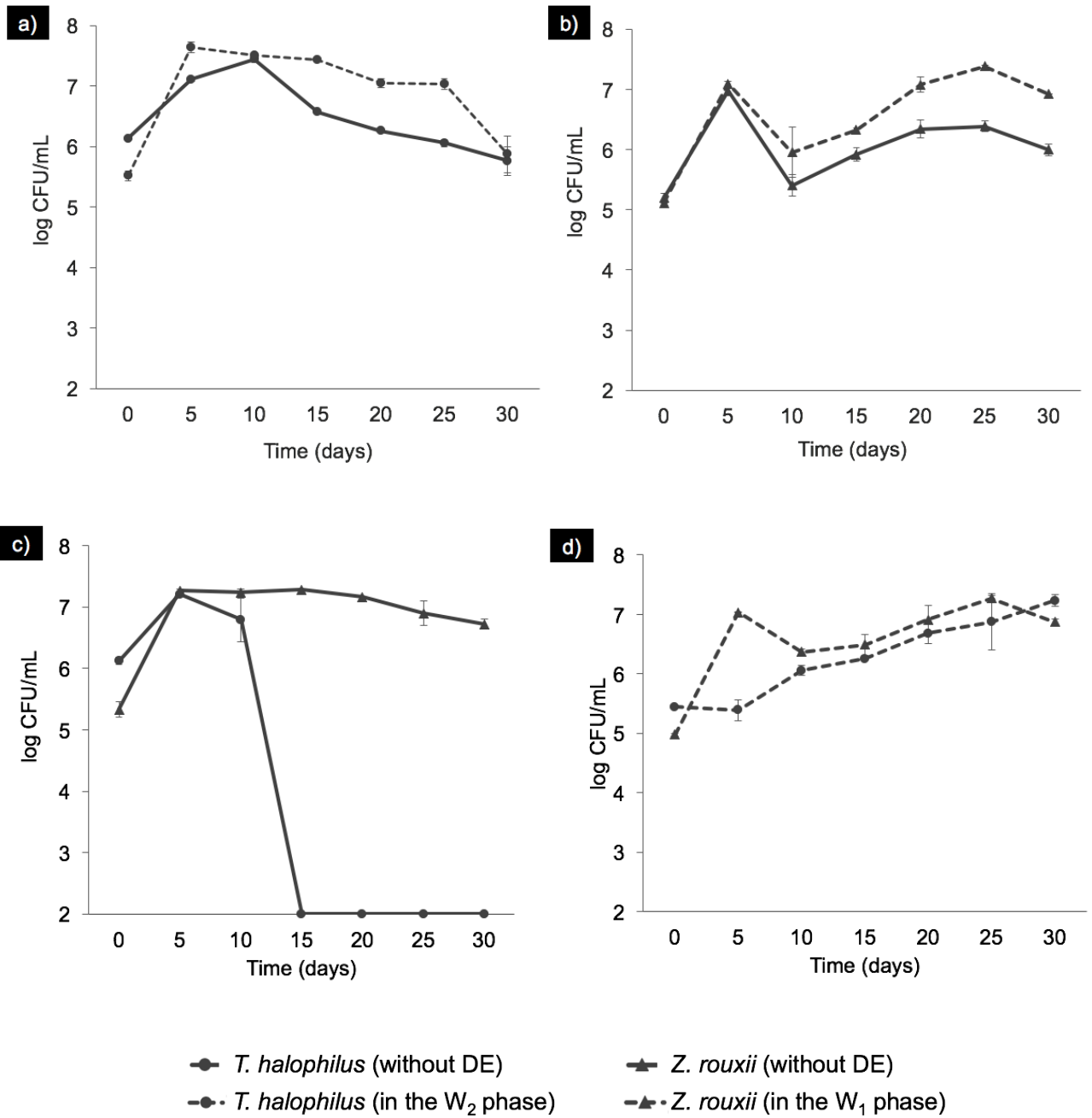
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Figure 5. Changes in *Z. rouxii* cells viability over 30 days of storage at 30°C.

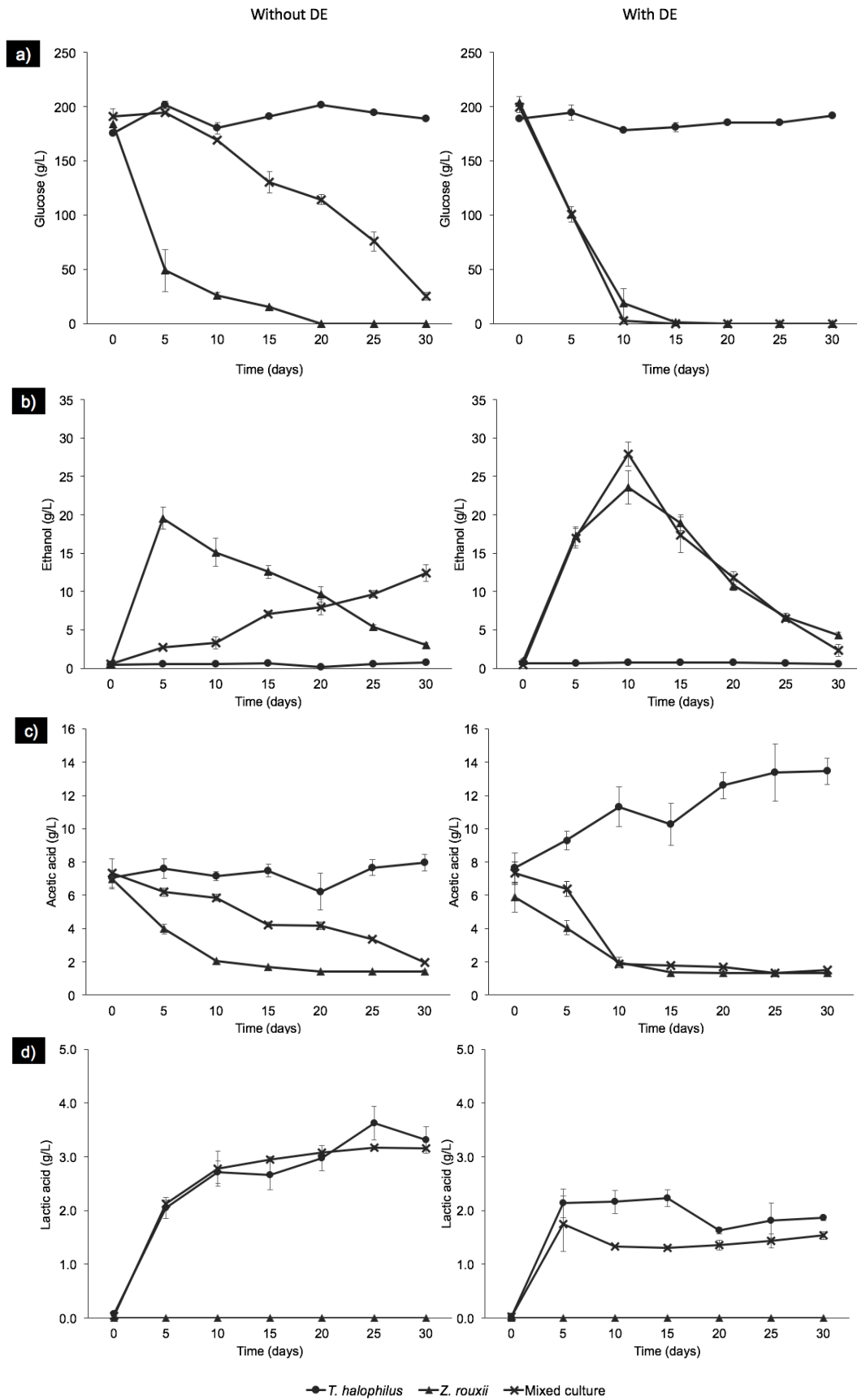
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645 Figure 6. Changes in viable cell number of (a) *T. halophilus*, (b) *Z. rouxii*, and mixed culture
646 (c) without and (d) with DE, during 30 days of incubation at 30°C.

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

