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# Segregation of Tetragenococcus halophilus and Zygosaccharomyces rouxii using W<sub>1</sub>/O/W<sub>2</sub> double emulsion for use in mixed culture fermentation

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### 1 Segregation of *Tetragenococcus halophilus* and

## <sup>2</sup> Zygosaccharomyces rouxii using W<sub>1</sub>/O/W<sub>2</sub> double

<sup>3</sup> emulsion for use in mixed culture fermentation

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#### 11 Abstract

12 Antagonism in mixed culture fermentation can result in undesirable metabolic activity and 13 negatively affect the fermentation process. Water-oil-water  $(W_1/O/W_2)$  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 W<sub>1</sub> and external  $W_2$  phase of a  $W_1/O/W_2$ , respectively. The suitability of DE for controlling *T. halophilus* and 17 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 W<sub>2</sub> 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 W<sub>2</sub>. 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.

Keywords: W<sub>1</sub>/O/W<sub>2</sub> double emulsion; yeast encapsulation; *Tetragenococcus halophilus*; *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 interactions negatively affect the equilibrium in the mixed microbial community and 43

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 (Devanthi 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 over time due to alginate's sensitivity to high salt concentration present in the brine added 63 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 not compatible with the composition of soy sauce. 67

68 Water-oil-water  $(W_1/O/W_2)$  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.

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.

#### 98 2. Material and Methods

99 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).

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 37 °C static incubator. Final cell concentration was adjusted to 10<sup>6</sup> cells/mL. Z. rouxii was 118 119 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 120 agitation (150 rpm). Cells ( $10^7$  cells/mL) were harvested and washed by centrifuging at 10000 121 g for 15 min. In order to test the effect of initial cell concentration on emulsion stability, cell 122 concentrations were adjusted to  $10^8$  cells/mL and  $10^6$  cells/mL. 123

#### 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,  $W_1/O$  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  $W_1$ : 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 (10<sup>8</sup> cells/mL and 10<sup>6</sup> cells/mL) were used as 130  $W_1$ .

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:

137 
$$\Delta \pi = (C_i - C_e)RT \tag{1}$$

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.

140 DEs containing *T. halophilus* in the  $W_2$  were prepared by directly adding 2 mL of *T. halophilus* 141 (10<sup>6</sup> cells/mL) into the  $W_2$  after mixing process. For the study that investigates the effects of 142 DE on *T. halophillus* 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. halophillus* and *Z. rouxii* were inoculated at final 145 concentrations of 10<sup>6</sup> CFU/mL and 10<sup>5</sup> 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

Creaming volume (%) = (Creaming layer volume/Total volume of DE) x 100% (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 162 10x objective lens. Images were taken using Moticam 10 camera via Motic Images Plus video 163 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.

#### 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.

The encapsulation efficiency and encapsulation stability were determined by counting the number of the non-encapsulated *Z. rouxii* cells in the serum phase (W<sub>2</sub>). 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:

181 *Cell concentration (cell/mL) = (Total number of cells x 25 \times 10^4)/Number of squares (3)* 

182 Encapsulation efficiency (EE) and encapsulation stability (ES) were determined using the183 following equations:

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

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

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.

#### 195 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

204 hexylene glycol (Sigma Aldrich, United Kingdom) as an internal standard at final 205 concentration of 742 mg/L. Samples (1 µL) 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 µm 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

Each experiment was conducted in triplicate (N = 3) and the results are expressed as mean  $\pm$ 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.

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<sub>1</sub> phase of DEs with high encapsulation 221 efficiency (>99%; Figure 1a) regardless of low (10<sup>5</sup> CFU/mL) or high (10<sup>7</sup> 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<sub>2</sub> 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<sub>1</sub> phase after 30 days (Figure 2a). Such time dependent loss of inner W<sub>1</sub> phase could 229 occur due to coalescence between the W<sub>1</sub> droplets as well as coalescence between W<sub>1</sub> 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 W1 droplet's interface and displaces the lipophilic 233 surfactant (Span 80) molecules which causes an increase in coalescence events between the 234 W<sub>1</sub> 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<sub>2</sub> phase. Although the amount of W<sub>1</sub> 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<sub>1</sub> 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<sub>1</sub> 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

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.

256 The release profile was found to be influenced by the amount of glucose in the W<sub>2</sub> phase 257 (Figure 3b) and it followed a similar pattern to the loss in the W<sub>1</sub> phase (Figure 3a), by which 258 the complete loss in the W1 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<sub>1</sub> 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 263 264 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<sub>1</sub> phase were still noticeable by the end of storage. Although DE with 12% glucose was designed to be osmotically balanced ( $\Delta \pi$ 267 268 = 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.

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<sub>2</sub> 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<sub>2</sub> 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 289 290 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.

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; Figure 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, 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 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<sub>1</sub>. 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. halophillus 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, the co-presence of T. halophilus and Z. rouxii resulted in antagonism as T. halophilus growth 331 332 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 interfere with proton expulsive activity of Z. rouxii for its halo-tolerance mechanisms, causing 348 349 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.

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  $W_2$  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 molecular weight (Zhang et al., 2013). In this study, deleterious metabolite compounds 363 364 produced by Z. rouxii, might not be able to pass through the oil layer to the bulk medium (W<sub>2</sub> 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 towards T. halophilus was absent. T. halophilus might have exhibited physiological changes in 369 370 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

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.

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.

*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).

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, & Vuillemard, 1999), as well as a shift in metabolic pathway from homofermentative to heterofermentative, resulting in decreased lactic acid production 411 412 (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 W<sub>1</sub> and W<sub>2</sub> phase at the metabolic level. 417

#### 418 **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*. 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  $W_2$  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.

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#### 432 **6. References**

- Bergmaier, D., Champagne, C. P., & Lacroix, C. (2005). Growth and exopolysaccharide
  production during free and immobilized cell chemostat culture of *Lactobacillus rhamnosus* RW-9595M. *Journal of Applied Microbiology*, 98(2), 272–284.
- Chávez-Páez, M., Quezada, C. M., Ibarra-Bracamontes, L., González-Ochoa, H. O., & ArauzLara, J. L. (2012). Coalescence in double emulsions. *Langmuir*, 28(14), 5934–5939.
- 438 Chen, Y., Huang, Y., Bai, Y., Fu, C., Zhou, M., Gao, B., Wang, C., Li, D., Hu, Y., Xu, N.
- 439 (2017). Effects of mixed cultures of *Saccharomyces cerevisiae* and *Lactobacillus*440 *plantarum* in alcoholic fermentation on the physicochemical and sensory properties of
  441 citrus vinegar. *LWT Food Science and Technology*, 84, 753–763.
- 442 Devanthi, P. V. P., Linforth, R., Onyeaka, H., & Gkatzionis, K. (2018). Effects of co-443 inoculation and sequential inoculation of *Tetragenococcus halophilus* and

Zygosaccharomyces rouxii on soy sauce fermentation. Food Chemistry, 240, 1–8.

- 445 Doleyres, Y., Fliss, I., & Lacroix, C. (2004). Probiotics using immobilized cell technology.
  446 *Methods*, 145–150.
- 447 El Kadri, H., Gun, R., Overton, T. W., Bakalis, S., & Gkatzionis, K. (2016). Modulating the
- 448 release of *Escherichia coli* in double W<sub>1</sub>/O/W<sub>2</sub> emulsion globules under hypo-osmotic

449 pressure. *RSC Advances*, *6*(96), 93694–93706.

- 450 El Kadri, H., Overton, T., Bakalis, S., & Gkatzionis, K. (2015). Understanding and controlling
- 451 the release mechanism of *Escherichia coli* in double  $W_1/O/W_2$  emulsion globules in the
- 452 presence of NaCl in the  $W_2$  phase. *RSC Advances*, 5(127), 105098–105110.
- 453 Ficheux, M., Bonakdar, L., & Bibette, J. (1998). Some stability criteria for double emulsions.
  454 *Langmuir*, 14(11), 2702–2706.
- 455 Frasch-Melnik, S., Spyropoulos, F., & Norton, I. T. (2010). W<sub>1</sub>/O/W<sub>2</sub> double emulsions
  456 stabilised by fat crystals Formulation, stability and salt release. *Journal of Colloid and*457 *Interface Science*, *350*(1), 178–185.
- Freire, A. L., Ramos, C. L., & Schwan, R. F. (2015). Microbiological and chemical parameters
  during cassava based-substrate fermentation using potential starter cultures of lactic acid
  bacteria and yeast. *Food Research International*, *76*, 787–795.
- Garti, N. (1997). Progress in stabilization and transport phenomena of double emulsions in
  food applications. *Lebensmittel-Wissenschaft & Technologie*, *30*, 222–235.
- Hamada, T., Sugishita, M., Fukushima, Y., Tetsuro, F., & Motai, H. (1991). Continuous
  production of soy sauce by a bioreactor system. *Process Biochemistry*, 26, 39–45.

465	Harada, R., Yuzuki, M., Ito, K., Shiga, K., Bamba, T., & Fukusaki, E. (2016). Influence of
466	yeast and lactic acid bacterium on the constituent profile of soy sauce during fermentation.
467	Journal of Bioscience and Bioengineering, 123(2), 203–208.

- 468 Herman, P. K. (2002). Stationary phase in yeast. *Current Opinion in Microbiology*, *5*, 602–
  469 607.
- 470 Herman, P. K. (2002). Stationary phase in yeast. *Current Opinion in Microbiology*, *5*, 602–
  471 607.
- 472 Inamori, K., Miyauchi, K., Uchida, K., & Yoshino, H. (1984). Interaction between Pediococcus
- *halophilus* and *Saccharomyces rouxii* (Microorganisms involved in *shoyu* moromi
  fermentation. Part I). *Nippon Nougeikagaku Kaishi*, 58(8), 771–777.
- Kemsawasd, V., Branco, P., Almeida, M. G., Caldeira, J., Albergaria, H., & Arneborg, N.
  (2015). Cell-to-cell contact and antimicrobial peptides play a combined role in the death
  of *Lachanchea thermotolerans* during mixed-culture alcoholic fermentation with *Saccharomyces cerevisiae. FEMS Microbiology Letters*, 362(14), 1–8.
- Khalid, N., Kobayashi, I., Neves, M. A., Uemura, K., & Nakajima, M. (2013). Preparation and
  characterization of water-in-oil-in-water emulsions containing a high concentration of Lascorbic acid. *Bioscience, Biotechnology, and Biochemistry*, 77(6), 1171–1178.
- 482 Krishnan, S., Gowda, L. R., Misra, M. C., & Karanth, N. G. (2001). Physiological and
  483 morphological changes in immobilized *L. plantarum* NCIM 2084 cells during repeated
  484 batch fermentation for production of lactic acid. *Food Biotechnology*, *15*(3), 193–202.
- Kusumegi, K., Yoshida, H., & Tomiyama, S. (1998). Inhibitory effects of acetic acid on
  respiration and growth of *Zygosaccharomyces rouxii*. *Journal of Fermentation and*

487 *Bioengineering*, 85(2), 213–217.

- 488 Lacroix, C., & Yildirim, S. (2007). Fermentation technologies for the production of probiotics
  489 with high viability and functionality. *Current Opinion in Biotechnology*, *18*(2), 176–183.
- 490 Lamboley, L., Lacroix, C., Artignan, J. M., Champagne, C. P., & Vuillemard, J. C. (1999).
- 491 Long-term mechanical and biological stability of an immobilized cell reactor for
  492 continuous mixed-strain mesophilic lactic starter production in whey permeate.
  493 *Biotechnology Progress*, 15(4), 646–654.
- Li, X., & Liu, S. Q. (2016). Antagonism between *Saccharomyces cerevisiae* and *Williopsis mrakii* and significance for mixed yeast alcoholic beverage fermentations. *International Journal of Food Science and Technology*, *51*(3), 656–663.
- 497 Luh, B. S. (1995). Industial production of soy sauce. *Journal of Industrial Microbiology*, 14,
  498 467–741.
- Medina, K., Boido, E., Dellacassa, E., & Carrau, F. (2012). Growth of non-Saccharomyces
  yeasts affects nutrient availability for Saccharomyces cerevisiae during wine
  fermentation. International Journal of Food Microbiology, 157(2), 245–250.
- Mezzenga, R., Folmer, B. M., & Hughes, E. (2004). Design of double emulsions by osmotic
   pressure tailoring. *Langmuir*, 20(9), 3574–3582.
- Muramatsu, S., Sano, Y., Uzuka, Y., & Company, T. S. (1993). Rapid fermentation of soy
  sauce. ACS Symposium Series, 200–210.
- Narvhus, J. a., & Gadaga, T. H. (2003). The role of interaction between yeasts and lactic acid
  bacteria in African fermented milks: A review. *International Journal of Food Microbiology*, 86, 51–60.

509	Nissen, P., Nielsen, D., & Arneborg, N. (2003). Viable Saccharomyces cerevisiae cells at high
510	concentrations cause early growth arrest of non-Saccharomyces yeasts in mixed cultures
511	by a cell - cell contact-mediated mechanism. Yeast, 20(4), 331–341.

512 Noda, F., Hayashi, K., & Mizunuma, T. (1980). Antagonism between osmophilic lactic acid

bacteria and yeasts in brine fermentation of soy sauce. *Applied and Environmental Microbiology*, 40(3), 452–457.

- Noda, F., Hayashi, K., & Mizunuma, T. (1982). Influence of pH on inhibitory activity of acetic
  acid on osmophilic yeasts used in brine fermentation of soy sauce. *Applied and Environmental Microbiology*, 43(1), 245–246.
- Pawlik, A., Cox, P. W., & Norton, I. T. (2010). Food grade duplex emulsions designed and
  stabilised with different osmotic pressures. *Journal of Colloid and Interface Science*, *352*(1), 59–67.
- 521 Pimentel-González, D. J., Campos-Montiel, R. G., Lobato-Calleros, C., Pedroza-Islas, R., &
  522 Vernon-Carter, E. J. (2009). Encapsulation of *Lactobacillus rhamnosus* in double
  523 emulsions formulated with sweet whey as emulsifier and survival in simulated
  524 gastrointestinal conditions. *Food Research International*, 42(2), 292–297.
- Rodríguez-Huezo, M. E., Estrada-Fernández, A. G., García-Almendárez, B. E., LudeñaUrquizo, F., Campos-Montiel, R. G., & Pimentel-González, D. J. (2014). Viability of *Lactobacillus plantarum* entrapped in double emulsion during Oaxaca cheese
  manufacture, melting and simulated intestinal conditions. *LWT Food Science and Technology*, 59(2), 768–773.
- Shima, M., Morita, Y., Yamashita, M., & Adachi, S. (2006). Protection of *Lactobacillus acidophilus* from the low pH of a model gastric juice by incorporation in a W/O/W

emulsion. *Food Hydrocolloids*, 20(8), 1164–1169.

- Smit, G., Smit, B. A., & Engels, W. J. M. (2005). Flavour formation by lactic acid bacteria and
  biochemical flavour profiling of cheese products. *FEMS Microbiology Reviews*, 29(3),
  591–610.
- van der Sluis, C., Mulder, A. N. T., Grolle, K. C. F., Engbers, G. H. M., ter Schure, E. G.,
  Tramper, J., & Wijffels, R. H. (2000). Immobilized soy-sauce yeasts: development and
  characterization of a new polyethylene-oxide support. *Journal of Biotechnology*, *80*(2),
  179–188.
- van der Sluis, C., Tramper, J., & Wijffels, R. H. (2001). Enhancing and accelerating flavour
  formation by salt-tolerant yeasts in Japanese soy-sauce processes. *Trends in Food Science and Technology*, *12*(9), 322–327.
- Wang, B., Liu, P., Jiang, W., Pan, H., Xu, X., & Tang, R. (2008). Yeast cells with an artificial
  mineral shell: Protection and modification of living cells by biomimetic mineralization. *Angewandte Chemie International Edition*, 47(19), 3560–3564.
- 546 Xu, Y. (1990). Advances in the soy sauce industry in China. *Journal of Fermentation and*547 *Bioengineering*, 70(6), 434–439.
- 548 Zhang, Y., Ho, Y. P., Chiu, Y. L., Chan, H. F., Chlebina, B., Schuhmann, T., You, L., Leong,
- 549 K. W. (2013). A programmable microenvironment for cellular studies via microfluidics550 generated double emulsions. *Biomaterials*, *34*(19), 4564–4572.
- Zhao, C. J., Schieber, A., & Gänzle, M. G. (2016). Formation of taste-active amino acids,
  amino acid derivatives and peptides in food fermentations A review. *Food Research International*, 89, 39–47.

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

556	Table 1. Formulation	of $W_1/O/W_2$ double of	emulsions (DE)	with varying g	lucose concentrations
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N	aCl W2	Glucose W1	$\Delta \pi$ (atm) W <sub>2</sub>		Molar concentration of solute	
			0%	16.54	W1>W2	
10%	8 05%	0%	6%	8.28	W <sub>1</sub> >W <sub>2</sub>	
1070	0.0570		12%	0	$\mathbf{W}_1 = \mathbf{W}_2$	
			30%	-24.84	$W_1 \!\!<\!\! W_2$	

557	in the	$W_2$	phase	and	directions	of	osmotic	pressure	gradients.
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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.



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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599	Figure 3. Double emulsion with 0%, 6%, 12%, and 30% glucose in the $W_2$ phase after
600	preparation, 3, and 30 days of storage at 30 °C. (a) Optical micrographs of $W_1/O/W_2$ . (b) Z.
601	<i>rouxii</i> cell release profile into $W_2$ phase. Scale bar: 100 $\mu$ m.
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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.



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.



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