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1 Section: Microbial and Enzyme Technology

2

3 **Oil-in-water emulsions characterization by laser granulometry and impact on γ -**
4 **decalactone production in *Yarrowia lipolytica***

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18 **Abstract**

19 Oil-in-water emulsions composed of methyl ricinoleate (MR) or castor oil (CO) as the organic phase,
20 stabilized by Tween 80, are in the basis of the biotechnological production of γ -decalactone. *Yarrowia*
21 *lipolytica* was used due to its ability to grow on hydrophobic substrates and to carry out the
22 biotransformation. The characterization of oil droplets size distribution by laser granulometry was
23 performed under different oil concentrations. The impact of the presence of cells on droplets size was also
24 analyzed as well as the relevance of washing inoculum cells. Furthermore, the granulometric
25 characterization of the emulsions was related with γ -decalactone production and it was observed that, in
26 the presence of non-washed cells, the smaller droplets disappeared, using both oils, which increased γ -
27 decalactone concentration. This suggests that the access of cells to the substrate occurs by their adhesion
28 around larger oil droplets.

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34 **Keywords:** castor oil, γ -decalactone, droplet size distribution, emulsions, methyl ricinoleate

35

36 **Introduction**

37 The biotechnological production of γ -decalactone, a fruity-like aroma compound, results from the
38 biotransformation of ricinoleic acid and it can be carried out by several microorganisms, among which the
39 dimorphic yeast *Yarrowia lipolytica* (Aguedo et al. 2005a).

40 The medium usually used is composed by an oil-in-water emulsion stabilized by a non-ionic
41 surfactant, Tween 80. The oil is the source of ricinoleic acid, usually methyl ricinoleate (MR) or castor oil
42 (CO).

43 The growth of cells in a medium containing lipids occurs directly on droplets as their solubility in
44 water is limited (Gutierrez and Erickson 1977) and it is, thus, influenced by the size of the oil droplets
45 (Bakhuis and Bos 1969).

46 The interfacial surface of the oil droplets and consequently the surface between the two liquid phases
47 is a determining factor for the degradation of the hydrophobic substrate and, thus, for the cells, growth
48 and aroma production (Aguedo et al. 2005b). The lipidic droplets size is influenced by numerous
49 parameters which may be related to physical and chemical characteristics of the medium (pH, ionic
50 strength, presence of surfactants, etc) (Wilde 2000) or to the presence of microorganisms (inoculum
51 concentration, cells surface properties, etc). During the biotransformation of ricinoleic acid into γ -
52 decalactone, there is a direct contact between the cells surface and the substrate droplets which may occur
53 by adhesion of cells to the larger droplets surface or by adsorption of micro-droplets at the cells surface
54 (Bakhuis and Bos 1969).

55 Since the access of the cells to the substrate depends on the emulsion characteristics, emulsions were
56 characterized by determining the oil droplets size distribution, through the laser granulometry technique,
57 using both oils (MR and CO), at different concentrations and in the presence or absence of cells.
58 Moreover, since the cells surface properties may influence the interaction between the oil and cells,
59 different methodologies were used for inoculum cells preparation, according with Gomes et al. (2010).
60 This procedure had an impact on cells hydrophobic character and the effect on emulsion behaviour was
61 analyzed in the present work. In a last instance, the impact of each emulsion on γ -decalactone production
62 was also investigated.

63

64 **Materials and methods**

65 Microorganism, media and culture conditions

66 *Yarrowia lipolytica* W29 (ATCC20460) was cultured for 48 h on YPDA medium (30 g agar l⁻¹, 20 g
67 glucose l⁻¹, 20 g peptone l⁻¹, 10 g yeast extract l⁻¹) at 27 ° C and used to inoculate (to reach OD₆₀₀ of
68 ~0.25) a 500 ml baffled Erlenmeyer flask containing 200 ml glucose medium (YPD medium: glucose 20
69 g glucose l⁻¹, 20 g peptone l⁻¹, 10 g yeast extract l⁻¹). Flasks were shaken at 140 rpm and 27 °C for 19 h
70 until the cultures reached the late growth phase. This suspension was used to inoculate the
71 biotransformation medium.

72 Two sources of ricinoleic acid were used: methyl ricinoleate (MR) and castor oil (CO) at different
73 concentrations. The composition of the biotransformation medium was: 6.7 g YNB (yeast nitrogen base)
74 with amino acids l⁻¹, 2.5 g NH₄Cl l⁻¹, from 10 to 50 g MR or CO l⁻¹ and from 1 to 5 g Tween 80 l⁻¹. Two
75 methodologies were tested for inoculation of the biotransformation medium: with or without the cell
76 washing step. In the first case, cells from the YPD medium were washed three times in sterile water,
77 centrifuged (6000 g, 5 min) and transferred to the biotransformation medium. In the second, the
78 components of the biotransformation medium were directly added to the YPD medium containing the
79 cells (Gomes et al. 2010).

80 All chemicals were purchased from Sigma-Aldrich except for CO, which was purchased from José M.
81 Vaz Pereira (Sintra, Portugal) and MR, which was kindly supplied by Stéarinerie Dubois (Boulogne,
82 France).

83

84 γ -Decalactone extraction and quantification

85 γ -Decalactone was extracted from 2 ml broth with 2 ml diethyl ether. The organic phase was analyzed by
86 GC as described in previous work (Gomes et al. 2010).

87

88 Laser granulometry

89 The droplet size distribution of MR and CO emulsions was evaluated using a Malvern Mastersizer laser
90 granulometer (Model S2-01, Malvern Instruments, Malvern, UK), based on the Fraunhofer approximation
91 (Agrawal et al. 1991).

92 Emulsions with MR and CO were analyzed with and without cells precultured during 19 h on YPD
93 medium. For the essays without cells, the emulsions were shaken during 1.25 h prior to analysis. For the
94 experiments with cells, the emulsions were shaken for 1 h before inoculation and then for more 15 min,

95 with cells, before analysis. A cellular suspension prepared in sterile water was also analyzed, in order to
96 determine the size of the yeast cells. All assays were performed in triplicate.

97

98 **Results and discussion**

99 The granulometric analyses of the emulsified MR and CO media, without cells, indicated the existence of
100 two distinct droplet populations (Figure 1). In the population with smaller dimension, the droplets size
101 remained practically with the same mean value among all MR (1.5 μm) and CO (1.9 μm) concentrations
102 tested. In the larger size population, the mean droplets diameter increased from 61 μm to 96 μm , with the
103 increase in MR concentration, and from 27 μm to 64 μm , with the increase in CO concentration.
104 Moreover, there was an increase in percentage of larger droplets with the oil concentration increase.

105 (Figure 1)

106 Aguedo et al. (2003) have also characterized an emulsion composed by 10 g MR l^{-1} . The results
107 obtained by these authors differ from those presented in this work since they have reported the existence
108 of three distinct droplet populations with mean sizes of 0.1 μm , 3 μm and 43 μm , in the MR emulsion.
109 These divergences may be justified by a variation in the concentration of Tween 80 (0.2 g l^{-1} vs. 1 g l^{-1} , in
110 their work and in the present one, respectively). Thus, it seems that the use of a higher concentration of
111 surfactant has an impact upon the MR droplets size, since the smaller droplets (0.1 μm) disappeared and a
112 population of larger droplets emerged: 61 μm in this study, compared with 43 μm in their work.

113 Prior to the determination of droplets size in the presence of cells, the cells size distribution was
114 evaluated by suspending the cells in sterile water. A population with mean values of 5.4 μm was detected,
115 corresponding to an oval morphology (Lopes et al. 2007).

116 (Figure 2)

117 When cells were present at around $1.5 \times 10^9 \text{ ml}^{-1}$ in the MR and CO emulsions, there were still two
118 populations (in some cases, three) with different particle sizes (Figures 3 and 4). However, drops with
119 1.5-1.9 μm size disappeared in all emulsions. This disappearance, due to the presence of cells, may be
120 explained by the adhesion of smaller substrate droplets to the cells surface and to the higher number of
121 cell particles that would overshadow the smallest oil droplets (Aguedo et al. 2003). Nevertheless, the
122 population of cells remained with a mean size of approximately 5.4 μm . The existence of this population
123 was also reported by Aguedo et al. (2003).

124 (Figures 3 and 4)

125 When washed cells were used (Figure 3), a population had a mean oil droplet size of 0.9 μm ,
126 although in a small percentage number, in all experiments and for both oils. This decrease in the oil
127 droplets mean size with the addition of *Y. lipolytica* washed cells was also stated by Waché et al. (2000),
128 when using emulsions composed by 5 g MR l^{-1} and 1 g Tween 80 l^{-1} .

129 Another population, with a mean droplet size comprised between 32 μm and 40 μm , was also noticed
130 in all experiments and for both substrates, although with a larger number of particles in CO emulsions.
131 Aguedo et al. (2003) also described the existence of a population of MR droplets with a mean size of 42
132 μm , in emulsions with *Y. lipolytica* washed cells.

133 In the experiments involving non-washed cells (Figure 4), as the concentration of MR increased, the
134 size of oil droplets also increased slightly, ranging from 40 μm to 69 μm , except for the experiment with
135 50 g MR l^{-1} , which presented a mean size of 52 μm .

136 When comparing the experiments with washed and non-washed cells, using both oils, one can observe
137 that the smallest oil droplets disappeared (except for the case with 50 g CO l^{-1} , where the existence of a
138 small population of oil droplets with a mean size of 0.8 μm was noticed). This disappearance is probably
139 related to the higher hydrophobicity of non-washed cells, as proved in previous work (Gomes et al. 2010),
140 allowing droplets to adsorb to their surface, turning its presence not measurable.

141 Table 1 shows that the accumulation of γ -decalactone is directly dependent on the MR concentration,
142 with the greater production of aroma occurring when the substrate concentration is higher. The
143 accumulation of γ -decalactone was also greater when non-washed cells are used. In this situation, the MR
144 droplets size is higher (the smaller population with 0.9 μm disappeared), suggesting that higher particle
145 sizes are more favorable to the production of the aroma, indicating that the adhesion of cells to the
146 substrate occurs by the linkage of cells around larger oil droplets (Bakhuis and Bos 1969). In fact, cells
147 possess a much smaller size than the oil droplets and the number of cell particles is far superior to the
148 number of oil particles, which seems to corroborate the hypothesis proposed.

149 (Table 1)

150 With respect to the use of CO and non-washed cells, the size of oil droplets varied from 34 μm to 46
151 μm , for the larger population (Figure 4).

152 When castor oil is used as substrate of the process, there is no aroma production when washed cells
153 are used in the biotransformation. When using non-washed cells, the most adequate oil concentration to
154 be employed, in terms of overall productivity, is 30 g l^{-1} , suggesting that the most favorable droplets size

155 corresponds to a diameter of approx. 46 μm , the larger size determined among all CO emulsions. This
156 corroborates the hypothesis previously suggested about larger particle sizes being more favorable to the
157 aroma production.

158 The main differences obtained in the aroma production under different operating conditions cannot be
159 attributed solely to the size of oil droplets; the quantity of available substrate for cells to use in the
160 biotransformation, as well as to the cells surface properties, which determines how the substrate is
161 assimilated, need to be taken into account. Gomes et al. (2010) verified, by means of the MATH test, that
162 there was a greater adhesion of non-washed cells to hexadecane, than of washed cells. These differences
163 point out that non-washed cells are more compatible with the substrate, which might also explain the
164 divergences obtained in the production of γ -decalactone, apart from the differences in the oil droplets
165 size.

166

167 **Conclusion**

168 This study aimed to characterize the oil droplets size distribution on different oil-in-water emulsions,
169 composed by different concentrations of methyl ricinoleate or castor oil and stabilized by a non-ionic
170 surfactant. The impact of the presence of cells on droplets size was shown, as well as the relevance of a
171 washing step of the inoculum cells.

172 In the experiments without cells, an increase in both oil concentrations led to an enlargement of the
173 mean oil droplets size. In the presence of cells, there are two or three populations with different particle
174 sizes. However, when cells were washed, a smaller population of both oil droplets was detected.

175 Moreover, the granulometric characterization of the emulsions was related with γ -decalactone
176 accumulation profiles and allowed to conclude that larger oil droplets favors the aroma production,
177 suggesting that, in this case, the access of cells to the substrate occurs by their adhesion around larger oil
178 droplets.

179 The obtained results are an important contribution for a better understanding of the factors affecting
180 the assimilation of hydrophobic compounds by *Yarrowia lipolytica*, clearly demonstrating the impact of
181 substrate concentration, as well as of cells, on droplet size distribution and, consequently, on γ -
182 decalactone production.

183

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187

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212 176-181

213 **Table 1** Mean droplet size (highest), maximum γ -decalactone accumulation and yield in respect to
 214 the substrate ($Y_{P/S}$) achieved in biotransformation media with different MR or CO concentrations
 215 and with washed or non-washed cells
 216

		Washed cells			Non-washed cells		
	[Oil] (g l ⁻¹)	Maximum mean droplet size (μ m)	[γ -Decalactone] (mg l ⁻¹)	$Y_{P/S}$ (mg g ⁻¹)	Mean droplet size (μ m)	[γ -Decalactone] (mg l ⁻¹)	$Y_{P/S}$ (mg g ⁻¹)
MR	10	32.4	173	17.3	5.4; 39.8	434	43.4
	30	39.8	284	9.5	5.4; 60.2	724	24.1
	50	34.7	471	9.4	5.4; 52.5	997	19.9
CO	10	39.8	0	0	5.4; 39.8	1371	137.1
	30	34.7	0	0	5.4; 45.7	1839	61.3
	50	34.7	0	0	5.4; 39.8	1339	26.8

217

218 **Figure Captions**

219

220 **Fig. 1** Methyl ricinoleate (MR) and castor oil (CO) droplets size distribution (in μm) in emulsions
221 without cells, related to the number of particles (in %). Data are the mean of three independent
222 experiments

223

224 **Fig. 2** Cells size distribution (in μm) in sterile water, related to the number of particles (in %).
225 Data are the mean of three independent experiments

226

227 **Fig. 3** Methyl ricinoleate (MR) and castor oil (CO) droplets size distribution (in μm) in emulsions
228 with washed cells, related to the number of particles (in %). Data are the mean of three
229 independent experiments

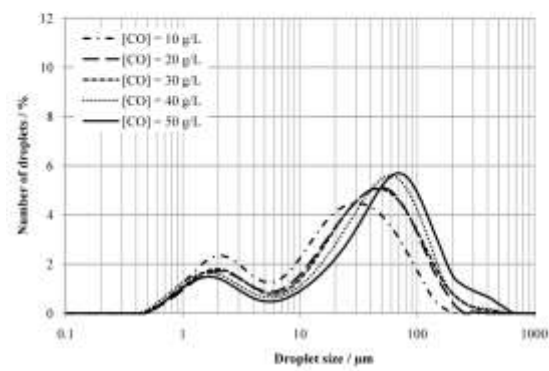
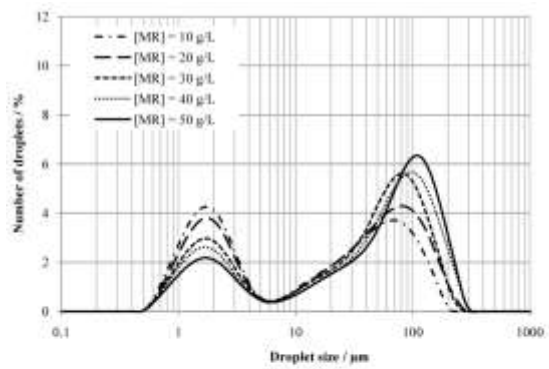
230

231 **Fig. 4** Methyl ricinoleate (MR) and castor oil (CO) droplets size distribution (in μm) in emulsions
232 with non-washed cells, related to the number of particles (in %). Data are the mean of three
233 independent experiments

234

235 Fig. 1

236

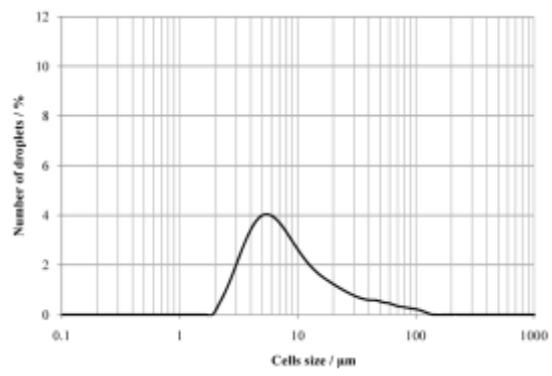


237

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239 Fig. 2

240

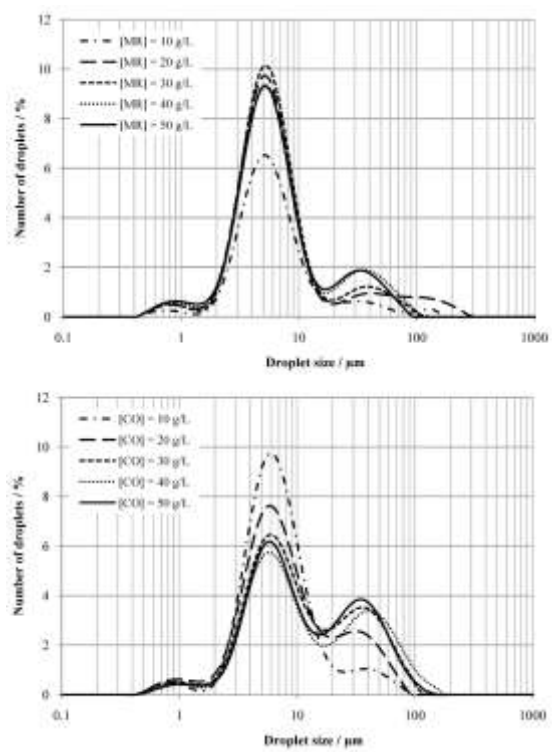


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243 Fig. 3

244

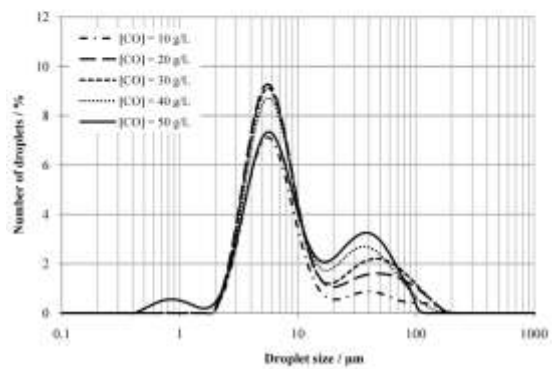
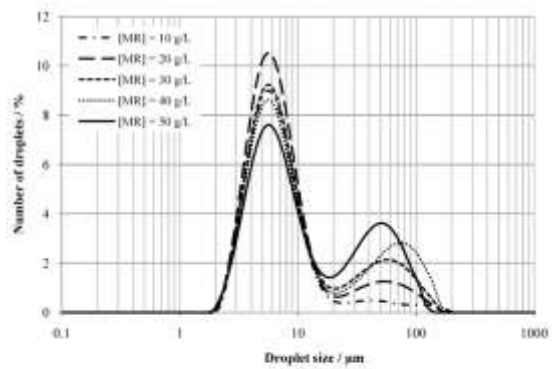


245

246

247 Fig. 4

248



249