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

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Abstract

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

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

Introduction

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

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

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

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

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

Materials and methods

Microorganism, media and culture conditions

Yarrowia lipolytica W29 (ATCC20460) was cultured for 48 h on YPDA medium (30 g agar l⁻¹, 20 g 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 ~0.25) a 500 ml baffled Erlenmeyer flask containing 200 ml glucose medium (YPD medium: glucose 20 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 until the cultures reached the late growth phase. This suspension was used to inoculate the biotransformation medium.

Two sources of ricinoleic acid were used: methyl ricinoleate (MR) and castor oil (CO) at different concentrations. The composition of the biotransformation medium was: 6.7 g YNB (yeast nitrogen base) 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 methodologies were tested for inoculation of the biotransformation medium: with or without the cell washing step. In the first case, cells from the YPD medium were washed three times in sterile water, centrifuged (6000 g, 5 min) and transferred to the biotransformation medium. In the second, the components of the biotransformation medium were directly added to the YPD medium containing the cells (Gomes et al. 2010).

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

γ -Decalactone extraction and quantification

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

Laser granulometry

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

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

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

Results and discussion

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

(Figure 1)

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

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

(Figure 2)

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

(Figures 3 and 4)

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

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

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

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

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

(Table 1)

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

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

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

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

Conclusion

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

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

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

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

Acknowledgments

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

		Washed cells			Non-washed cells		
	[Oil] (g l ⁻¹)	Maximum mean droplet size (μm)	[γ -Decalactone] (mg l ⁻¹)	$Y_{P/S}$ (mg g ⁻¹)	Mean droplet size (μm)	[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

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

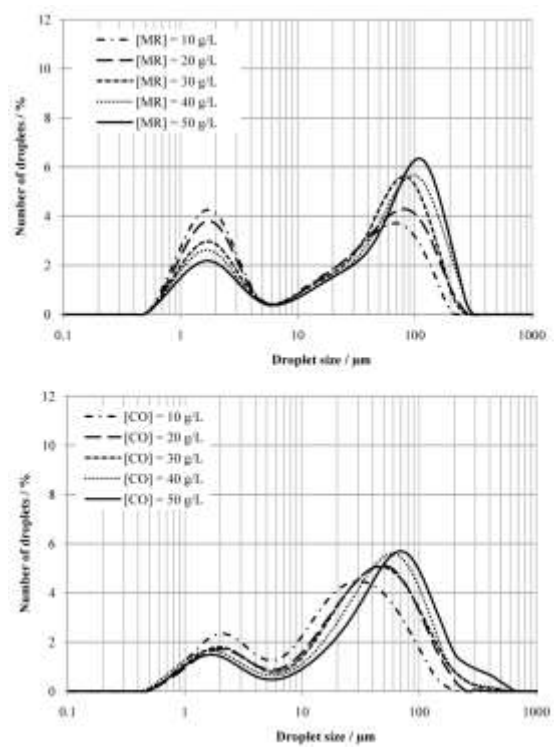
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231 **Fig. 4** Methyl ricinoleate (MR) and castor oil (CO) droplets size distribution (in μm) in emulsions
232 with non-washed cells, related to the number of particles (in %). Data are the mean of three
233 independent experiments

234

235 Fig. 1

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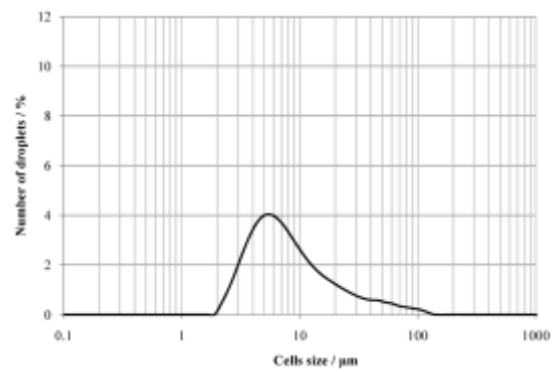


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239 **Fig. 2**

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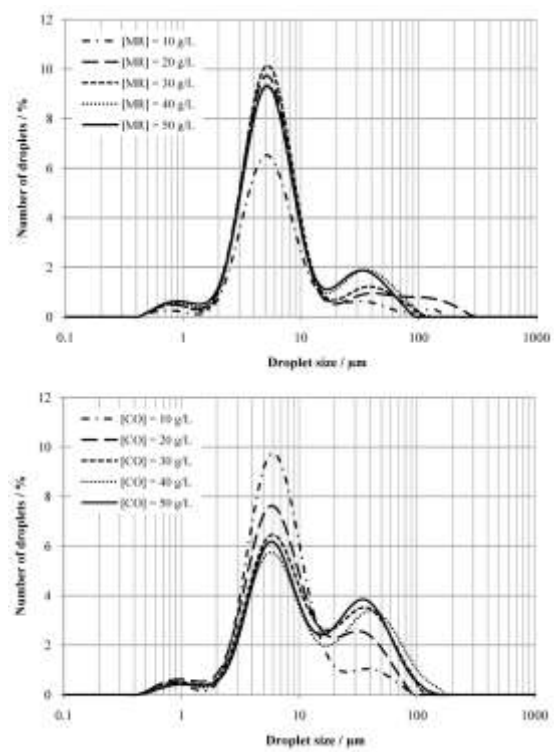


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243 **Fig. 3**

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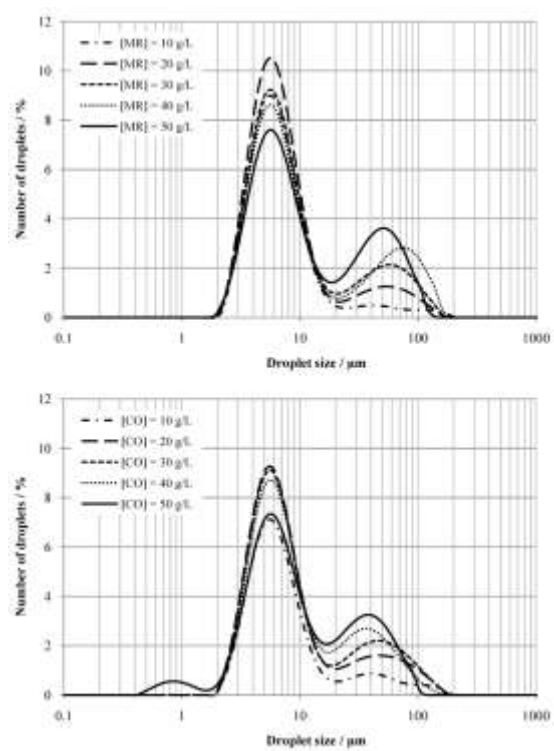


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247 **Fig. 4**

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