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Effect of Elevated Oxygen Concentration on the Yeast *Yarrowia lipolytica* for the Production of γ-Decalactones in Solid State Fermentation

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Abstract: To study the effect of elevated oxygen concentrations on β-oxidation for the production of lactones by *Yarrowia lipolytica* W29 in solid state fermentation (SSF), experiments using oxygen-enriched air, with different initial concentrations of oxygen ratio, were carried out. Growth kinetics using an oxygen ratio of 30% reached the stationary phase earlier than other conditions used. In addition, the production of γ-decalactone and 3-hydroxy-γ-decalactone reached the maximal concentrations of 270 mg L^{-1} and 1190 mg L^{-1}, respectively. Using higher initial oxygen ratios (40% and 50%), an incomplete growth inhibition occurred and resulted in a higher concentration of yeast at the stationary phase and a slightly higher 3-hydroxy-γ-decalactone accumulation. When oxygen-enriched air (oxygen ratio of 30%) was injected twice (at 0 and 20 h), 3-hydroxy-γ-decalactone reached a higher concentration (1620 mg L^{-1}) and it reached a very high concentration of 4600 mg L^{-1} in the condition of oxygen-enriched air injected many times (at 0, 20, 35, 48 and 60 h). This study suggested that oxygen is required for the production of 3-hydroxy-γ-decalactone in SSF. Oxygen may be consumed preferentially for long-chain fatty acid oxidations rather than at C10-level. Furthermore, the production of γ-decalactone may be improved by optimizing the growth conditions to reach a very high specific growth rate. A low oxygen availability in the system at the stationary growth phase led to an inhibition of γ-decalactone degradation. From the present work, an alternative system is proposed as a novel model to study the effect of elevated oxygen concentration in SSF.

Keywords: solid state fermentation; *Yarrowia lipolytica*; oxygen-enriched air; β-oxidation; castor oil; aroma production

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1. Introduction

The catabolism of hydroxylated fatty acid by yeast results in the accumulation of the popular flavoring compounds, γ-decalactones [1–4]. In submerged fermentation (SmF), as well as in solid state fermentation (SSF), the degradation of C18-hydroxylated fatty acids by *Y. lipolytica* resulted in the accumulation of four interesting lactones, γ-decalactone, 3-hydroxy-γ-decalactone, dec-2-en-4-olide and dec-3-en-4-olide [2,5–7]. The main lactone produced, 3-hydroxy-γ-decalactone, has no sensorial properties while the initially desired lactone was γ-decalactone, and the other two decenolides exhibit strong fruity notes and mushroom notes for dec-3-en-4-olide and dec-2-en-4-olide, respectively [5,8,9]. β-Oxidation is the main pathway of fatty acid degradation to produce theses lactones with yeast cells. *Y. lipolytica* has been known as a model organism for the metabolism of hydrophobic compounds [10]. In yeast, β-oxidation is known to be located in peroxisomes.

It is composed of four main reactions, two oxidations, a hydration and a cleavage reaction (Figure 1). Waché [9] suggested that the β-oxidation cycle could be divided into two parts, with intermediates exiting at two oxidation levels (oxidase and dehydrogenase). Genetic modification of metabolic pathways [11–15], as well as changes in environmental conditions [8,16–18], have been reported to greatly impact β-oxidation fluxes, with a particular effect on the accumulation of γ-decalactone and 3-hydroxy-γ-decalactone. Oxygen availability is the key parameter driving the extent of oxidation involved in the production of these lactones [2,6,8,9,16,17,19]. Recent works have proposed different processes based on the importance of aeration in β-oxidation pathways such as the control of K_La in a stirred tank bioreactor [16], in an airlift biofilm reactor [17] and in the use of a step-wise fed-batch culture [18] and airlift and stirred tank bioreactors [20]. To provide a tight contact between the co-substrate (oxygen) and yeast cells, a SSF process has been investigated recently for the production of lactones from castor oil as the substrate by using luffa sponge as an inert solid support [7]. In this work, different reactor types have been used to emphasize the importance of oxygen availability in the system. It has been reported that 3-hydroxy-γ-decalactone was produced up to 5 gL^{-1} after 70 h in a wide-mouth Erlenmeyer flask. However, the use of forced aeration reactors resulted in lactones stripping. The present work aims at investigating the effect of oxygen availability on the pathway of production of lactones in SSF in a system unrelated to forced aeration. Concerning that goal, an alternative SSF system was used based on oxygen-enriched air utilization in order to avoid the stripping of the volatile compounds by forced aeration.

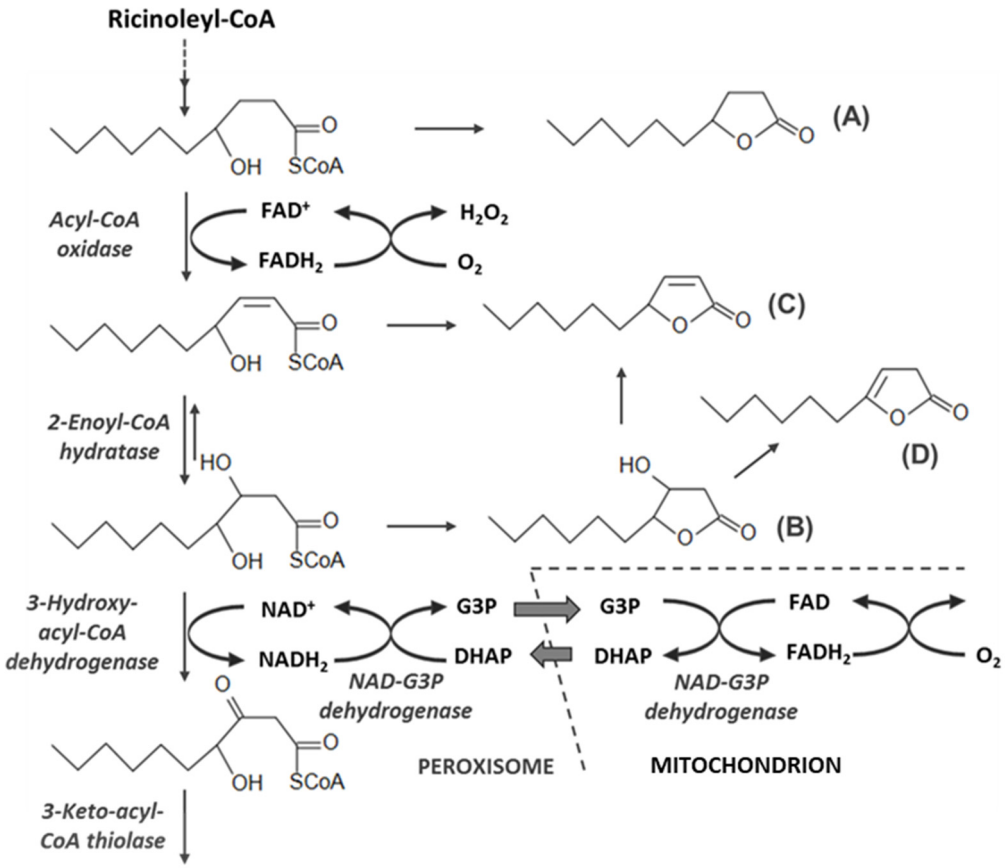

Figure 1. β-Oxidation loop at the C10 level of the degradation of ricinoleyl-CoA, enzymatic activities, co-substrate (oxygen) cofactor requirements and reaction products. (**A**) γ-decalactone; (**B**) 3-hydroxy-γ-decalactone; (**C**) dec-2-en-4-olide; (**D**) dec-3-en-4-olide.

2. Materials and Methods

2.1. Solid Support, Biotransformation Medium, Strain and Inoculum Preparation

Luffa sponge was used as the solid support in this study. Luffa sponge was obtained from the dried fruit of Luffa free of seeds (origin Cambodia). It was cut into a rectangular prism shape (approximately 1 × 1 × 0.5 cm). Biotransformation medium comprising 20 g/L of castor oil, 2 g/L of Tween 80 and 6.7 g/L of YNB (yeast nitrogen base) with amino acid was used. The strain used in this study was *Y. lipolytica* W29 (ATCC 20460). The pretreatment procedures of solid support and inoculation preparation have been described in Try et al. [7].

2.2. Solid State Fermentation Conditions

The solid support was humidified by the haft of biotransformation media prepared and then autoclaved at 121 °C for 15 min. It was then adjusted to a final moisture content of 70% with yeast-cell-inoculated biotransformation media. SSF process was carried out in 24 mL bottles filled with 2 g luffa sponge support impregnated with yeast-cell-inoculated biotransformation media. SSF bottles were then injected with the oxygen-enriched air containing different conditions of oxygen ratio. They were then placed upside-down during incubation in an incubator (Laboandco, Marolles-en-Brie, France) at 27 °C. Two categories of oxygen-enriched air injection were carried out: (a) air containing different ratios of oxygen (20; 30; 40 and 50%), injection at 0 h of SSF process and (b) air containing 30% of oxygen ratio, (i) injection at 0 and 20 h and (ii) injection at 0, 20, 35, 48 and 60 h.

2.3. Injection System of Oxygen-Enriched Air

A simple injection system was used in this work (Figure 2). A source of compressed air and an oxygen bottle were connected with two different float flow-rate meters (glass as float material 2.53 g mL^{-1}, N054-17G for compressed air and N013-88G for oxygen) in order to adjust the oxygen content in the flow stream of air before sending to the thermoregulated water bath, the air filter, the humidifier and oxygen-enriched air distributor via silicon tubes. From the air distributor, needles (Terumo®, Terumo Group, Tokyo, Japan, 0.8 mm of diameter by 50 mm) were connected to inject the oxygen-enriched air (70% of relative humidity) through a cotton plug into SSF bottles. Oxygen-enriched air injection was carried out over 1 min into each SSF bottles with a flow rate of 0.5 L min^{-1} using needles. SSF bottles with cotton plugs were then covered using three layers of parafilm (Parafilm® M, Sigma-Aldrich, Saint-Quentin Fallavier, France), holed using rubber bands and placed in position upside down. The empty bottles were also injected with the oxygen-enriched air in different conditions to control the loss of oxygen during fermentation. No significant oxygen loss was detected.

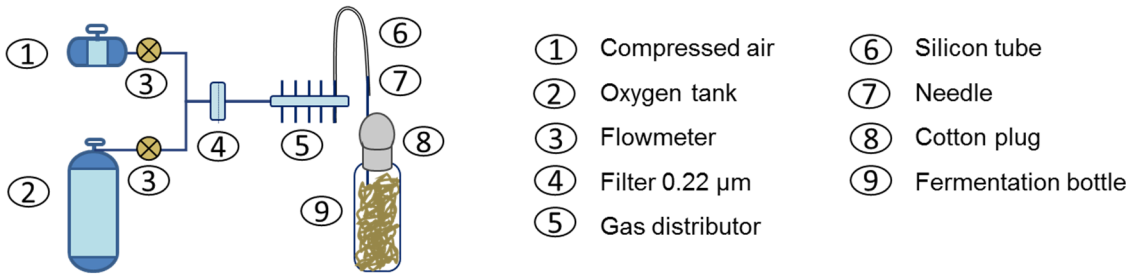

Figure 2. Scheme of air containing different concentrations of oxygen-enriched air injection system.

2.4. Gases Measurement and Biomass Estimation

The gases measurement of oxygen and carbon dioxide were carried out using a gases analyzer (CheckMate 9900 O_2/CO_2, PBI-Dansensor A/S, PBI Dansensor, Ringsted, Denmark). Biomass estimation was carried out following indirect method by measuring the production of carbon dioxide. A correlation between carbon dioxide (%) and yeast cell concentrations (cells g^{-1} of dried weight) was obtained from the direct measurement using

the biomass extraction method previously described in Try et al. [7]. It corresponded to $y = 2.10^7x + 10^8$ with a R^2 of 0.9684 and was used for biomass determination in this study.

2.5. Extraction and Quantification of Lactones

The solid support has been prepared once and then distributed to the different experimental vials to make sure the substrate, biomass and solid support were homogeneous. Three vials have been dedicated for each point of analysis. Three independent experiments were carried out for the study presented in this manuscript. Analyses were carried out on each SSF bottle. γ-Decalactone (≥98%, Sigma–Aldrich France, Saint-Quentin Fallavier, France) was used for analytic development. An amount of 50 µL of γ-undecalactone (internal standard, ≥98%, Sigma–Aldrich France, Saint-Quentin Fallavier, France) solubilized in absolute ethanol was added to reach a final concentration of 100 mg L^{-1} in solvent. An amount of 10 mL of diethyl-ether was then added for solid–liquid extraction [21]. The detailed extraction method was previously described in Try et al. [7]. The analyses were carried out using a coupled gas chromatograph PerkinElmer (Clarus 500, Villebon-sur-Yvette, France)/FID detector with an Elite-Wax ETR column (60.0 m by 250 µm by 0.25 µm) with nitrogen as the carrier gas at a linear flow rate of 4.3 mL min^{-1}. The split injector (split ratio, 7.1:1) temperature was set to 250 °C. The oven temperature was programmed to increase from 60 °C to 145 °C at a rate of 5 °C min^{-1}, then from 145 °C to 215 °C at a rate of 2 °C/min and finally from 215 °C to 250 °C at a rate of 10 °C min^{-1}.

2.6. Modelling of Cells Growth, Oxygen Consumption and Lactone Productions

A logistic differential equation was use to estimate growth kinetics (Equation (1)) as well as oxygen consumption and lactone production kinetics (Equation (2)) in different conditions of oxygen ratio. The least squares method with solver function in Microsoft Excel was used for the optimization of model parameters:

$$\frac{dX}{dt} = \mu_{max}\left(1 - \frac{X}{X_{max}}\right)X \quad (1)$$

$$\frac{dP}{dt} = q_{max}\left(1 - \frac{P}{P_{max}}\right)X \quad (2)$$

where X, μ_{max} and X_{max} are the concentration (cells g^{-1} of dried weight), the specific growth rate (h^{-1}) and the maximal concentration of biomass (cells g^{-1} of dried weight), respectively. P, q_{max} and P_{max} are the concentrations of lactones (mg L^{-1}) or oxygen consumed (mg L^{-1} of air), the specific production rates of lactones or the specific consumption rates of oxygen (mg mg^{-1} cell h^{-1}), and the maximal production of lactones (mg L^{-1}) or the maximal consumption concentration of oxygen (mg L^{-1} of air), respectively.

2.7. Statistical Analysis

All experiments have been carried out three independent times. The STDEVA function in Microsoft Excel was used for standard deviation calculation. The one-way analysis of variance (ANOVA) in Microsoft Excel was used to determine whether there are any statistically significant differences between variables.

3. Results

3.1. Growth of Y. lipolytica W29 Depends on the Oxygen Ratio

The growth of *Y. lipolytica* W29 in SSF was studied in different conditions of oxygen ratio between 20% and 50%, using luffa sponge as the solid support. The yeast growth was evaluated from the production of carbon dioxide (%) detected in the SSF bottles and from the number of yeast cells (cells/g of dried weight). The growth kinetics of *Y. lipolytica* in different conditions of oxygen ratio are shown in Figure 3. Under the condition of initial oxygen of 20%, the yeast growth reached its stationary phase at 40.5 h with a concentration of approximately 4.27×10^8 cells g^{-1} of dried weight. Under the condition of 30% initial

oxygen ratio, the stationary phase was reached faster and the cell concentration was higher at approximately 5.86 × 10^8 cells g^{-1} of dried weight at 27.5 h. For higher initial oxygen ratio conditions of 40 and 50%, the time to reach the stationary phase was increased to 40.5 h and 50 h, but the maximal cell concentration increased, reaching 7.02 × 10^8 and 7.12 × 10^8 cells g^{-1} of dried weight at the early stationary phases, respectively. The logistic differential Equation (1) for the growth study in different conditions was used to evaluate the specific growth rates and the maximum biomass concentrations of *Y. lipolytica* W29 (Table 1). The model parameters obtained were physically significant. The specific growth rate of *Y. lipolytica* W29 in the condition of 30% oxygen ratio reached the highest value (0.14 h^{-1}) compared to other conditions.

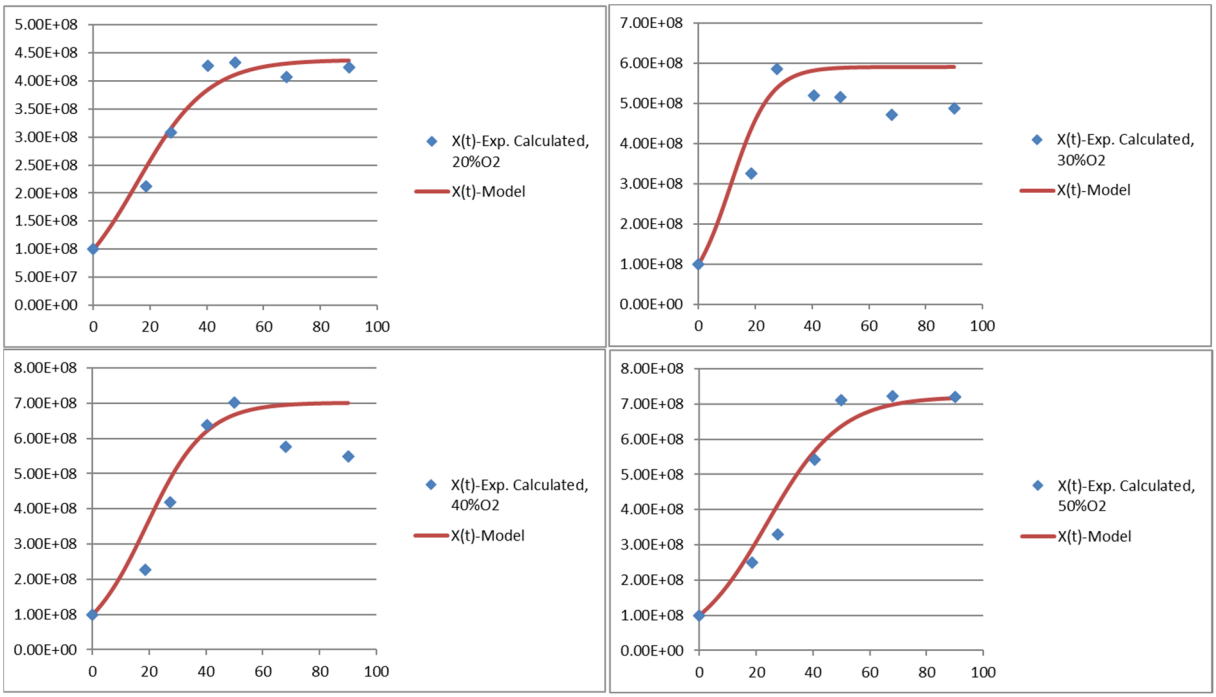

Figure 3. Kinetics of *Y. lipolytica* W29 growth in different conditions of initial oxygen-enriched air injection.

Table 1. Specific growth rates (μ_{max}) and maximum biomass concentrations (X_{max}) of *Y. lipolytica* W29 grow in SSF in different conditions of initial oxygen-enriched air.

Parameters	Experimental Conditions of Oxygen-Enriched Air *			
	20%	30%	40%	50%
μ_{max} (h^{-1})	0.08 ^{a}	0.14 ^{b}	0.09 ^{a}	0.08 ^{a}
X_{max} (10^8 cells g^{-1} of dried weight)	4.38 ^{a}	5.50 ^{b}	6.70 ^{c}	6.91 ^{c}

* Mean values followed by the same letter are not significantly different according to Fisher's test ($p > 0.05$).

3.2. Oxygen Consumption of Y. lipolytica W29 Depends on the Oxygen Ratio

The kinetics of oxygen availability during the growths studied in Section 3.1 are shown in Figure 4. Oxygen availability under the condition of 20% initial oxygen ratio decreased significantly from 20% until a low concentration of approximately 0.3% was reached in the SSF bottles at 40.5 h. For the conditions of 30, 40 and 50% initial oxygen ratios, the concentrations decreased to 1.4%, 1.8% and 2.7% in SSF bottles at 27.5 h, 40.5 h and 50 h, respectively. When using the logistic differential equation (2) for the oxygen consumption study in different conditions, the model fitted with the experimental data is shown in Figure 5. Furthermore, the specific oxygen consumption rates and the maximal

consumption concentrations parameters represented physically acceptable values (Table 2 and Figure 5).

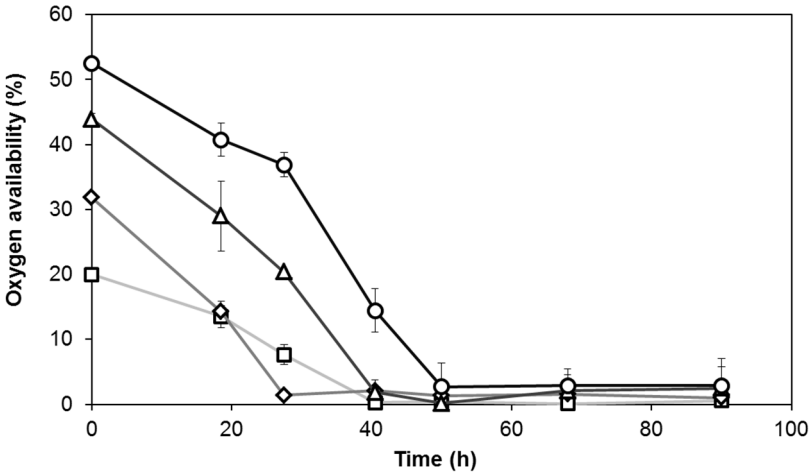

Figure 4. Kinetics of oxygen availability in SSF using *Y. lipolytica* W29 in different conditions of initial oxygen-enriched air injection. Initial oxygen ratios set to 20% (Squares), 30% (Diamonds), 40% (Triangles) and 50% (Circles).

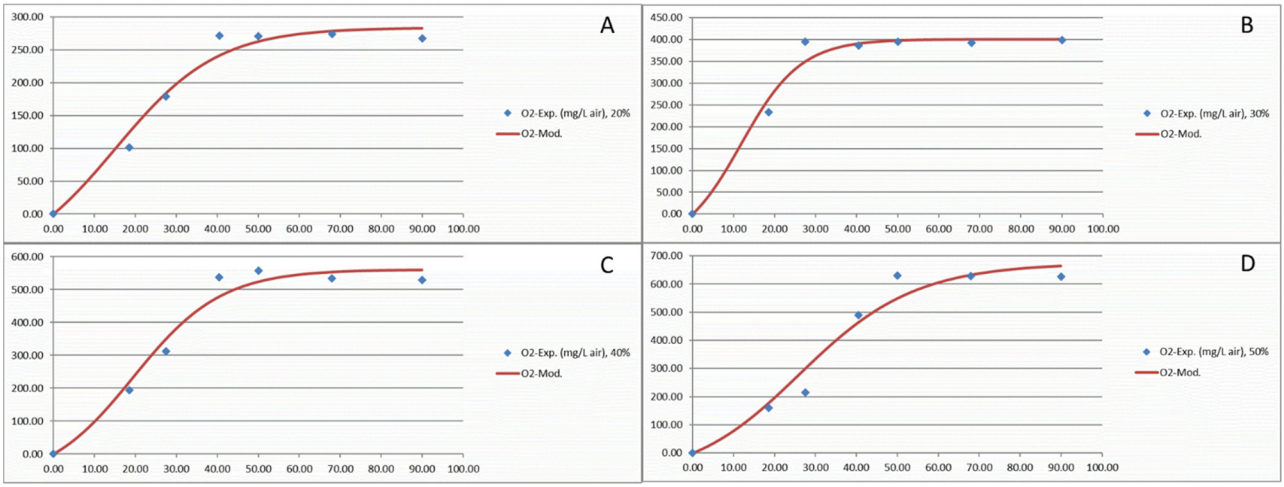

Figure 5. Data of Table 2 adjusted to logistic model for the conditions of initial O2, (**A**): 20%, (**B**): 30%, (**C**): 40% and (**D**): 50%, respectively.

Table 2. Specific oxygen consumption rates (q_{O2max}) and maximal concentration consumed (P_{O2max}) by *Y. lipolytica* W29 during the production of lactones in SSF in different conditions of initial oxygen-enriched air.

Parameters	Unite	Experimental Conditions of Oxygen-Enriched Air **			
		20%	30%	40%	50%
q_{O2max}	mg $(10^8$ cells$)^{-1}$ h^{-1} *	5.15	9.57	7.60	6.18
P_{O2max}	mg L^{-1}	275	401	561	676

* 1 mg dried weight cells is equal to 5.016×10^7 cells. ** Mean values obtained in all conditions are significantly different according to Fisher's test ($p \leq 0.05$).

3.3. Production of Lactones Depends on the Oxygen Ratio

Four γ-decalactones, γ-decalactone, dec-2-en-4-olide, dec-3-en-4-olide and 3-hydroxy-γ-decalactone, were detected in all the conditions of initial oxygen-enriched air concentrations tested. γ-Decalactone, under the conditions of a 30 and 40% oxygen ratio, reached the maximal concentrations of approximately 270 mg L^{-1} and 206 mg L^{-1}, respectively, at 68 h. Under the conditions of oxygen ratios of 20 and 50%, γ-decalactone reached its highest level at 90 h with the concentrations of approximately 143 mg L^{-1} and 216 mg L^{-1}, respectively (Figure 6A). 3-Hydroxy-γ-decalactone reached the highest concentrations of approximately 700 mg L^{-1}, 1190 mg L^{-1}, 1440 mg L^{-1} and 1650 mg L^{-1} in initial oxygen ratios of 20, 30, 40 and 50%, respectively. 3-Hydroxy-γ-decalactone produced from the conditions of initial oxygen ratio of 20 and 30% reached their maximum concentrations at 40.5 h and 27.5 h, respectively. For the conditions of initial oxygen ratios of 40 and 50%, lactone contents reached their maximum concentrations at the same time (68 h) (Figure 6B). The kinetics of the production of dec-2-en-4-olide and dec-3-en-4-olide in all conditions were similar to those of 3-hydroxy-γ-decalactone. Dec-2-en-4-olide reached its highest concentration of approximately 118 mg L^{-1}, 212 mg L^{-1}, 148 mg L^{-1} and 151 mg L^{-1}, under the conditions of initial oxygen ratios of 20, 30, 40 and 50%, respectively (Figure 6C). Dec-3-en-4-olide reached its highest concentration of approximately 50 mg L^{-1} under the initial oxygen ratios of 20, 30 and 50% and 60 mg L^{-1} for 40% (Figure 6D).

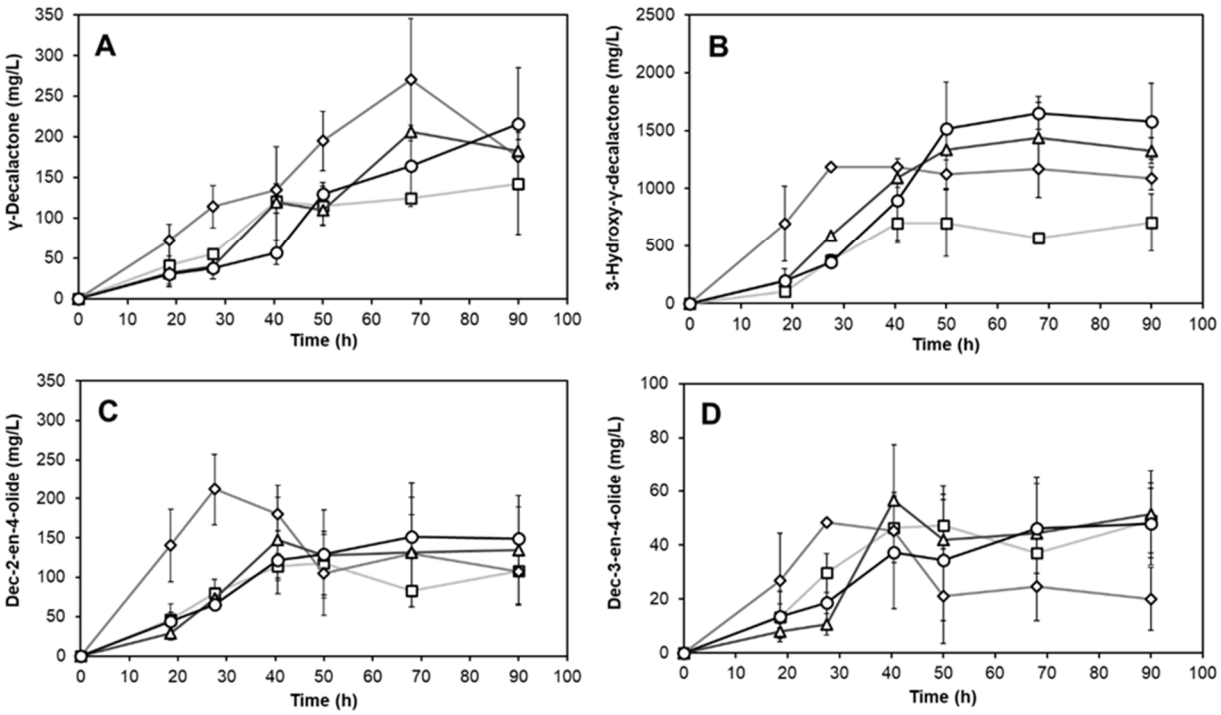

Figure 6. Kinetics of production of lactones by *Y. lipolytica* W29 in SSF using Luffa sponge as inert support, in different conditions of initial oxygen-enriched air injection. Initial oxygen ratios set to 20% (Squares), 30% (Diamonds), 40% (Triangles) and 50% (Circles). (**A**) γ-decalactone; (**B**) 3-hydroxy-γ-decalactone; (**C**) dec-2-en-4-olide; (**D**) dec-3-en-4-olide.

The logistic differential equation (2) was used as a mathematical model to study the lactone production kinetics. The results obtained from the parameters fitting using experimental data under different conditions of oxygen ratio are presented in Table 3. The model used described the kinetics as shown in Figure 7 and the model parameters represented the physical significance values. The specific production rates of dec-2-en-4-olide ($q_{2DECmax}$) and 3-hydroxy-γ-decalactone (q_{3OHmax}) under the condition of 30% oxygen ratio reached higher values compared to other conditions (20%, 40% and 50% oxygen ratios). However, the specific production rates of γ-decalactone decreased with the increase

in oxygen concentration from 20 to 50%. To study the effect of oxygen concentration on the production of different lactones in SSF, the specific molar production rates of lactones, related to the consumption of oxygen by yeast, were determined ($n_{Lactone}$) (Table 3 and Figure 7) by dividing the specific molar production rates of lactones by the specific molar consumption rates of oxygen by *Y. lipolytica* W29. The total molar production of lactones in the condition of 30% oxygen reached a higher value compared to the other conditions tested (0.73 mole mole^{-1} oxygen consumed).

Table 3. Specific production rates (q_{max}) and maximal production concentrations (P_{max}) of lactones in SSF in different conditions of initial oxygen-enriched air.

Parameters	Unite	Experimental Conditions of Oxygen-Enriched Air **			
		20%	**30%**	**40%**	**50%**
q_{GDLmax}	mg (10^8 cells)$^{-1}$ h^{-1} *	1.68 ^{ab}	1.93 ^{a}	1.03 ^{bc}	0.74 ^{c}
$q_{2DECmax}$		2.07 ^{a}	6.45	1.73 ^{a}	1.61 ^{a}
$q_{3DECmax}$		0.83 ^{a}	1.06 ^{a}	0.45 ^{a}	0.45 ^{a}
q_{3OHmax}		10.32 ^{a}	29.91	12.52 ^{a}	11.07 ^{a}
P_{GDLmax}	mg L^{-1}	147	289 ^{a}	227 ^{b}	253 ^{ab}
$P_{2DECmax}$		121 ^{a}	198	149 ^{a}	159 ^{a}
$P_{3DECmax}$		48 ^{a}	47 ^{a}	53 ^{a}	50 ^{a}
P_{3OHmax}		714	1197 ^{a}	1497 ^{ab}	1740 ^{b}
n_{GDL}	mole lactone per mole oxygen consumed	0.061	0.038	0.024 ^{a}	0.023 ^{a}
n_{2DEC}		0.077	0.128	0.043 ^{a}	0.050 ^{a}
n_{3DEC}		0.031 ^{a}	0.021 ^{ab}	0.011 ^{b}	0.014 ^{b}
n_{3OH}		0.345 ^{a}	0.538	0.283 ^{a}	0.308 ^{a}
$n_{Total\ lactones}$		0.513 ^{a}	0.725	0.362 ^{b}	0.394 ^{ab}

n: specific molar rate of lactone production/specific molar rate of oxygen consumption. GDL: γ-decalactone. 2DEC: dec-2-en-4-olide. 3DEC: dec-3-en-4-olide. 3OH: 3-hydroxy-γ-decalactone. * 1 mg dried weight cells is equal to 5.016×10^7 cells. ** Mean values followed by the same letter are not significantly different according to Fisher's test ($p > 0.05$).

3.4. Production of Lactones Using Gas with an Oxygen Ratio of 30% Injected at Different Times

An oxygen ratio of 30% was chosen to study the effect of oxygen availability on the production of lactones at different times of injection. Two experimental conditions were used: (i) injection of 30% oxygen ratio at 0 h and 20 h and (ii) injection of 30% oxygen ratio at 0, 20, 35, 48 and 60 h. In condition (i), γ-decalactone reached a concentration of approximately 154 mg L^{-1} at 48 h and remained almost stable until 90 h. In contrast, in (ii), γ-decalactone reached 120 mg L^{-1} at 42 h, then decreased to a final concentration of approximately 54 mg L^{-1} at 90 h (Figure 8A). 3-Hydroxy-γ-decalactone reached, at 73 h, the maximum concentrations of 1620 mg L^{-1} and 4550 mg L^{-1} for conditions (i) and (ii), respectively (Figure 8B). For both conditions, the kinetics of dec-2-en-4-olide and dec-3-en-4-olide production were similar to those of 3-hydroxy-γ-decalactone. Dec-2-en-4-olide reached its maximum concentration of 83 mg L^{-1} at 35 h in condition (i) and of 263 mg L^{-1} at 73 h in condition (ii) (Figure 8C). Dec-3-en-4-olide reached its maximum production of 17 mg L^{-1} at 35 h in condition (i) and of 46 mg L^{-1} at 73 h in condition (ii) (Figure 8D).

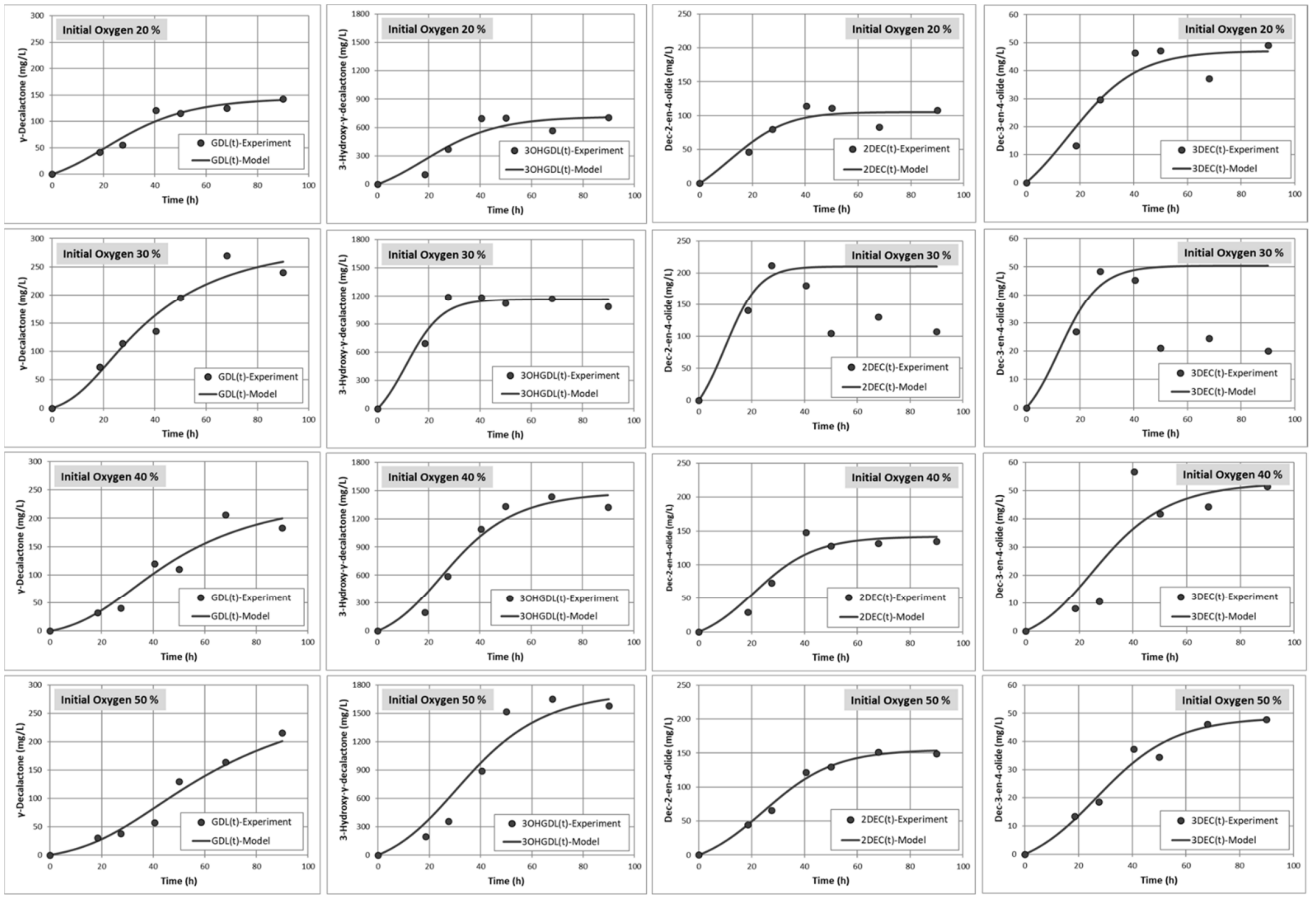

Figure 7. Experimental points and model curves corresponding to Table 3.

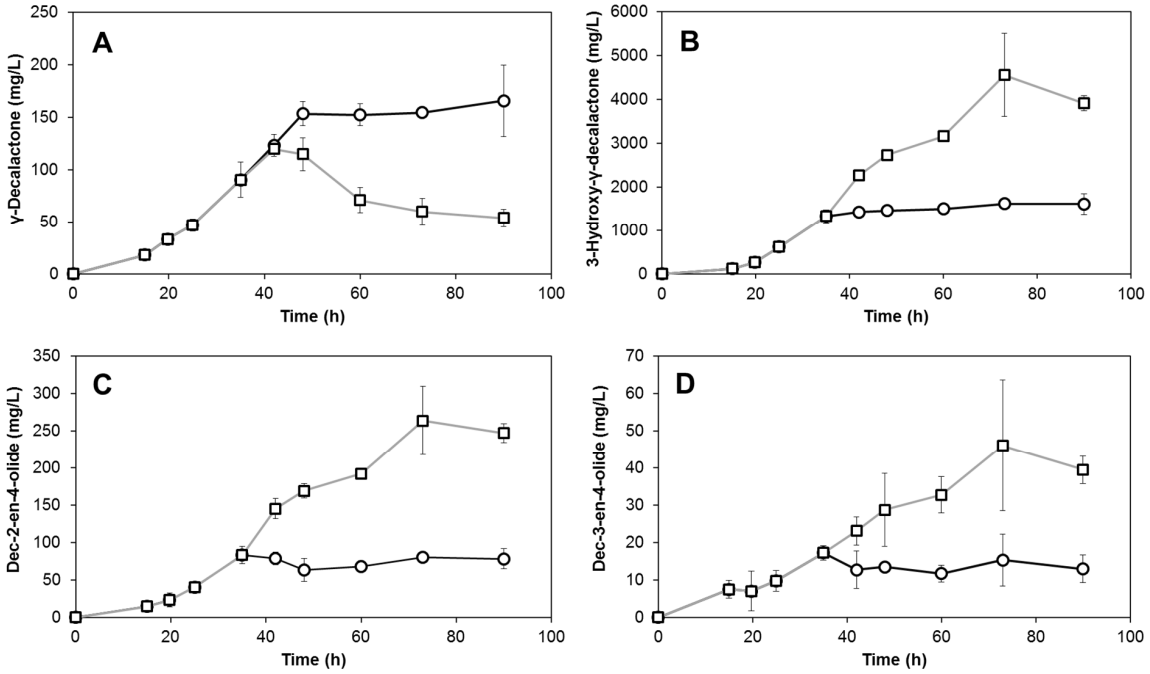

Figure 8. Kinetics of production of lactones by *Y. lipolytica* W29 in SSF using Luffa sponge as inert support using oxygen-enriched air (30% of oxygen ratio) injection at different times, (i) at 0 h and 20 h (Circles) and (ii) at 0 h, 20 h, 35 h, 48 h and 60 h (Squares). (**A**) γ-decalactone; (**B**) 3-hydroxy-γ-decalactone; (**C**) dec-2-en-4-olide; (**D**) dec-3-en-4-olide.

4. Discussion

Using oxygen-enriched air has been reported as an interesting practice currently used to support the aerobic growth of microorganisms in SmF bioreactors, to increase the cell density and to improve process productivity [22]. The present work emphasizes the effect of elevated oxygen concentration on the yeast *Y. lipolytica* for the study of growth and production of lactones in SSF, which is an efficient process to produce high-value compounds [23]. In bioprocesses, the transfer of oxygen from a gas bubble to a cell can be found in the scientific literature on basic/fundamental biotechnology and biochemical engineering [24–28]. In SSF, the transfer of oxygen from the gas phase to the cells can be represented by similar steps [26,27]. In addition, a gas bubble in SmF becomes gas phase (air oxygen) in SSF. Oxygen mass transfer pathways in our experimental conditions were assumed using the following steps: (i) transfer from the gas phase to the gas–liquid interface, (ii) movement across the gas–liquid interface, (iii) diffusion through the stagnant liquid film surrounding the liquid phase, (iv) transport through the liquid phase, (v) diffusion through the stagnant liquid film surrounding the solid particles/cells and (vi) movement across liquid–cell interface. The growth of *Y. lipolytica* was dependent on the availability of oxygen in the process (Figures 3 and 4). No yeast growth was detected when oxygen decreased to a value of approximately 1%. A condition using an initial oxygen ratio of 30% was favorable for the yeast growth, which reached a stationary phase earlier compared to the other conditions used (Figure 3, Table 1). The conditions using initial oxygen ratios of 20% and 40% reached a stationary phase later. This might be due to an insufficient level of oxygen occurring in the process under the condition of an oxygen ratio of 20%. On the other hand, a condition using an initial oxygen ratio of 40% or even 50% reached a stationary phase later compared to all the conditions tested. Oxygen is necessary for growth and production by aerobic microorganisms. However, oxygen can also be toxic for microorganisms. This can be explained by the fact that by-products of reactive oxygen species (ROS) such as superoxide ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2) are produced when cells are exposed to a high oxygen concentration [29–32]. The toxicity of concentrations of oxygen between 40 and 50% would delay the time to reach the stationary phase of yeast without inhibiting the growth and the cell count reached even a maximal value higher than for other conditions. In these conditions, the yeast concentration increased rapidly after the oxygen ratio in the system had reached a favorable concentration. This concentration was approximately 30% O_2, and it occurred after 20 h and 30 h under the conditions of initial oxygen ratio of 40% and 50%, respectively. The effect of oxygen toxicity has been reported to impact the exponential growth phase [22,33]. Furthermore, eukaryotic cells have been reported to be more sensitive than prokaryotic cells to atmospheric oxygen concentrations above 40% [34–36].

In all conditions, 3-hydroxy-γ-decalactone and both decenolides (dec-2 and dec-3-en-4-olide) were not produced after the yeast growth has reached its stationary phase (Figures 3 and 6). However, the γ-decalactone concentration increased until the end of fermentation (90 h). This suggests that the correlation of the yeast growth and the production of lactones can be divided into two periods. Firstly, the production of lactone corresponds exactly to the yeast growth phase (3-hydroxy-γ-decalactone, dec-2-en-4-olide and dec-3-en-4-olid). Then, the production of lactone still continues in the stationary growth phase (γ-decalactone). As previously described, no yeast growth was observed when the oxygen availability in the system reached a threshold value of approximately 1%. This shows that oxygen is the limiting factor for yeast growth and for the production of some lactones in our study conditions. A low oxygen availability in the system allows the production of γ-decalactone to continue, but not that of 3-hydroxy-γ-decalactone (Figure 9). This may result from the fact that oxygen, when present at a very low concentration, would be directed firstly to long-chain catabolism. In this case, the production of γ-decalactone results from β-oxidation at the C12 level whereas the production of 3-hydroxy-γ-decalactone comes from the C10 β-oxidation cycle (Figure 9). To gain a better understanding, the β-oxidation of the ricinoleic-hydroxylated fatty acid to shorter carbon chain intermediate compounds is repre-

sented in Figure 9 [2,5,6,9,37–40]. β-Oxidation is a complex metabolic pathway. The main peroxisomal β-oxidation enzymes are involved in the yeast *Y. lipolytica* for the production of lactones such as acyl-CoA oxidase, 2-enoyl-CoA hydratase, 3-hydroxy-acyl-CoA dehydrogenase and 3-keto-acyl-CoA thiolase (Figure 1) [2,6,9]. A low activity of acyl-CoA oxidase generally results from the accumulation of γ-decalactone. Similarly, a low 3-hydroxy-acyl-CoA dehydrogenase activity results in the accumulation of 3-hydroxy-γ-decalactone. When oxygen-enriched air was injected during the process, 3-hydroxy-γ-decalactone reached a very high concentration compared to the one obtained for the two-shot oxygen-enriched air injection (Figures 6B and 8B). This suggests that oxygen availability would increase the activity of acyl-CoA oxidase and decrease the activity of 3-hydroxy-acyl-CoA dehydrogenase, resulting in a high yield of 3-hydroxy-γ-decalactone. Only in the case of oxygen-enriched air injected many times into the system was γ-decalactone degradation was observed (Figure 8A). Yet, the degradation pathway of γ-decalactone has not been defined. However, two possible degradation pathways of γ-decalactone (lactonase/lactone-hydrolase and ω-oxidation) have been proposed by Endrizzi-Joran [41], and this was then discussed in detail in the work of Waché et al. [6], who emphasized the mutants of *Y. lipolytica* W29. When oxygen availability in the system is sufficient, the degradation pathways may take place (Figure 9). In addition, γ-decalactone may be hydrolyzed to 4-hydroxy-decanoic acid, a precursor of 3,4-dihydroxy decanoic acid, which can be lactonized to 3-hydroxy-γ-decalactone [5]. Another possible means of γ-decalactone degradation which has been suggested was that via ω-oxidation followed by further β-oxidation [41]. The results of the present study suggest that the degradation of γ-decalactone can be avoided in the case of low oxygen availability in the system in the stationary growth phase.

In conclusion, this study provides an alternative model for studying the effect of oxygen using oxygen-enriched air for the production of active compounds in SSF. In this system leading to lower stripping, different concentrations of oxygen ratios were used and experiments also included different injection times of oxygen-enriched air (30% oxygen ratio) in the process. Logistic differential equations were used for modeling the growth, oxygen consumption and lactone production kinetics. All the model parameters obtained were physically significant. With a 20% oxygen ratio, an insufficient oxygenation was exhibited, resulting in a low production of lactones. When increasing the oxygen ratio to 30%, these conditions were the most favorable for lactones production among the experimental conditions used. The higher concentrations of oxygen ratio (40 and 50%) led to an incomplete inhibition of yeast growth, and resulted in a high concentration of yeast in the stationary phase and a slightly higher hydroxy-lactone accumulation. A high γ-decalactone concentration can be observed when the yeast growth reached a high specific growth rate. The availability of oxygen in the stationary growth phase appeared to be an important factor for the degradation of γ-decalactone. Furthermore, oxygen may be consumed preferentially for β-oxidation of long-chain fatty acids, rather than at the C10-β-oxidation level. Different strains exhibiting modified acyl-CoA activity will be used to gain a deeper understanding on this point, as well as in the β-oxidation metabolites fluxes in different conditions of oxygen ratio in the SSF process. Moreover, this SSF system can use a new continuous extraction system for decalactone harvesting [42].

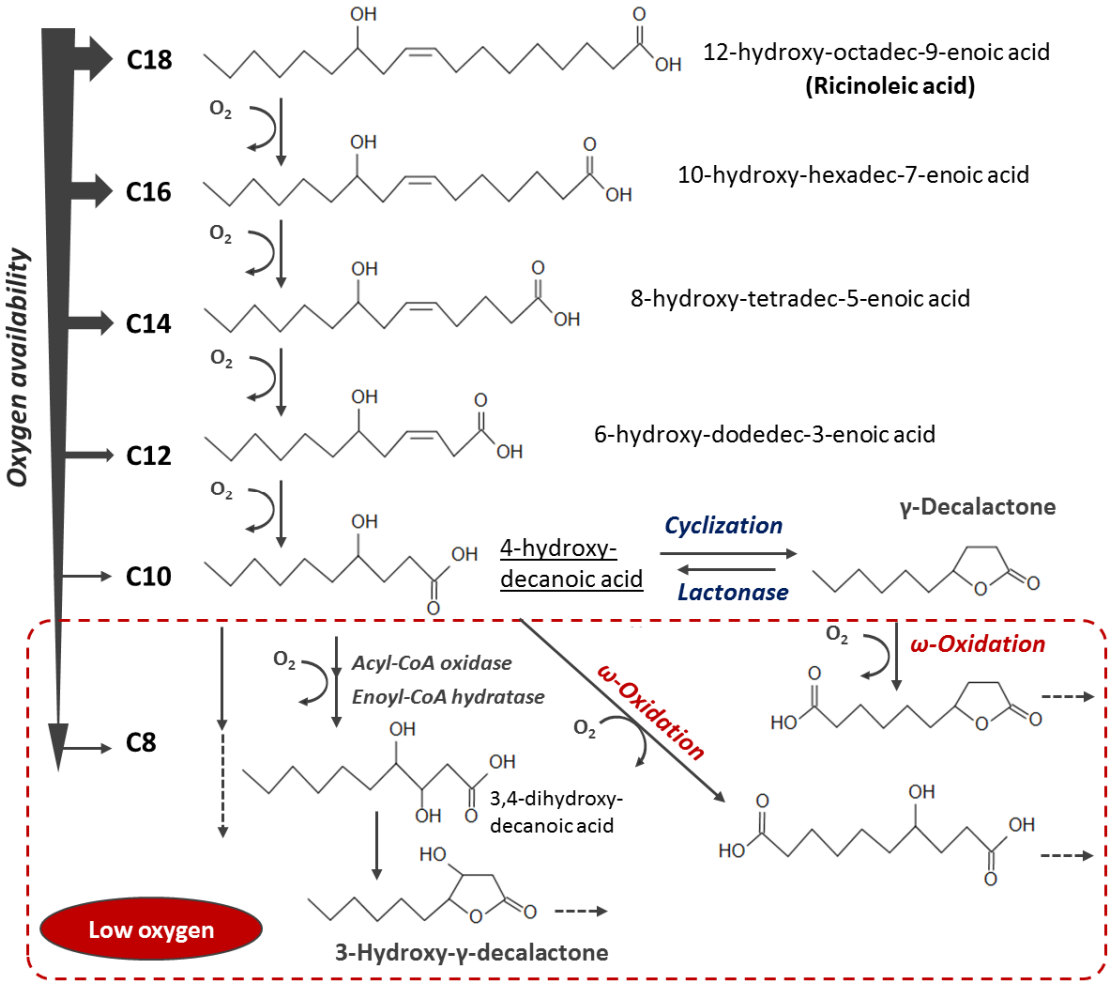

Figure 9. Bioconversion of ricinoleic acid (C18) to shorter-chain β-oxidation intermediates. C10 β-oxidation level and γ-decalactone degradation pathways are represented. Low oxygen concentration in the system would not make either the synthesis of 3,4-hydroxy-decanoic acid (precursor of 3-hydroxy-γ-decalactone) from 4-hydroxy-decanoic acid or the degradation of γ-decalactone possible.

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