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# Optimization of pure linoleic acid 13-HPX production by enzymatic reaction pathway: unravelling oxygen transfer role

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## Highlights

- \_ Relevant determination of SBLOX1 kinetic parameters taking into account  $k_{La}$ .
- \_ Sufficient oxygen supply leads to pure LA 13-HPX production.
- \_ The use of  $k_{La}$  and Damköhler number is essential to optimize SBLOX-1 reactor.
- \_ The enzymatic reactor allows a green production of 13-HPX with high added-value.

## **Abstract**

This work is, to our knowledge, the first to have investigated the role of gas-liquid oxygen transfer on the determination of the kinetic parameters of an oxidase, and on oxidase reactor sizing for pure hydroperoxides production using soybean lipoxygenase-1. The addition of a small amount of ethanol (2% v/v) was found suitable for simple linoleic acid handling and probably a better dispersion of the latter in the reaction medium. Then, the kinetic parameters of the enzyme in a pH 9 buffered medium were determined and compared to the literature data. Following a simple methodology which accounts for gas-liquid oxygen transfer, the dissolved oxygen kinetics were reprocessed to correct the initial reaction rates.  $k_{La}$  was thus found essential to determine accurate reaction rates. The latter were found consistent with the hydroperoxide production and linoleic acid consumption rates, confirming that the data reprocessing method was necessary. This sheds new light on several literature results on lipoxygenase activity characterizations, where a discrepancy is often observed between the apparent oxygen kinetics and the hydroperoxide production rates. Based on these results and the Damköhler number ( $Da$ ) in relation to oxygen uptake and transfer, a reactor sizing procedure was proposed and led to the almost full conversion of linoleic acid into pure 13-hydroperoxide.

## **Keywords**

Oxygen transfer; Lipoxygenase; Linoleic acid; Oxidation; 13-Hydroperoxide; Reactor sizing.

## **Graphical abstract**

## 1. Introduction

Oxidation of poly-unsaturated fatty acids (PUFAs) has been widely studied for decades [1], particularly the first aerobic step of the oxidation reaction cascade, leading to the production of fatty acid hydroperoxides (HPX). These molecules have an important contribution in flavor formation and taste modification of food [2]. They are commonly used as analytical standards for lipid characterization and as reaction precursors for the lipid oxidation investigations, or for controlled flavor synthesis [3-5]. Fatty acid hydroperoxides are also used to investigate the cyto-toxicity and pathogenicity of lipid oxidation in mammals [6, 7].

Currently, commercial PUFA hydroperoxides are produced by chemical synthesis and are moderately pure. Linoleic acid (LA – *cis,cis*-9,12-octadecadienoic acid) is the most commonly used PUFA for lipid oxidation investigations in the literature [4, 8]. Its chemical oxidation in the presence of oxygen and metal ions (such as  $Fe^{2+}$ ) leads to a mixture of 13- and 9-linoleic acid hydroperoxides, with *cis-trans* and *trans-trans* conjugated dienes for the first one and *trans-cis* and *trans-trans* conjugated dienes for the second one [9, 10]. Consequently, most of the commercialized fatty acid hydroperoxide standards are composed of a mixture of these isomers. In order to obtain a high purity isomer, complementary purification steps are required to separate the isomers and the resulting product is thus extremely expensive.

To simplify the production of a pure hydroperoxide isomer and reduce its cost, an enzymatic production by the widespread enzymes lipoxygenases (LOX - EC 1.13.11.12; iron as co-factor) can be considered, given the high region-selectivity and enantio-selectivity of the lipoxygenase. After enzymatic oxidation of linoleic acid, a mixture of 9 and 13 – hydroperoxides is obtained. However, according to the isoform activity and the ionization of the considered PUFA, some of these enzymes can produce a specific isomer[11].

In the case of linoleic acid oxidation by soybean lipoxygenase-1 (SBLOX-1, the most used LOX for HPX formation investigation), it is well established that pH has an important effect on the isomeric

26 composition of the hydroperoxides produced. As a matter of fact, the pKa for linoleic acid was  
27 experimentally determined at 7.9 by Bild *et al.* [12]. At pH higher than this pKa value, most of the  
28 linoleic acid is in its linoleate form and SBLOX-1 is known to favor the formation of the 13-hydroperoxy-  
29 *cis-9-trans-11* form [11, 13, 14] (Figure 1). At pH 9, the most commonly used pH in SBLOX-1 reaction  
30 investigations, this leads to 92.6% of the linoleate form. Moreover, most of literature data show that  
31 the 13-*cis-trans*-hydroperoxide is formed at pH 9 with SBLOX-1 at a purity higher than 90% [13, 15, 16].  
32 Additionally, this reaction also requires non-limiting amounts of dissolved oxygen in the reaction  
33 medium to occur. The lack of dissolved oxygen switches the reaction pathway to an anaerobic (non-  
34 enzymatic) pathway, generating dimers, oxodienes, hydroxyacids and *n*-pentane (see Figure S.1 in the  
35 Supplementary Information for reaction pathways). The hydroperoxides present in the medium are  
36 then used as substrates for anaerobic reactions [1]. Therefore, if an enzymatic oxidation of linoleic  
37 acid to produce high purity hydroperoxides is considered, a sufficient supply of oxygen must be  
38 provided. However, several published studies show enzymatic oxidation kinetics with different  
39 lipoxygenases for which the reactions are only monitored through (i) either the consumption of PUFA  
40 (by HPLC analyses) [17]; (ii) or hydroperoxides synthesis (by UV absorbance measurements) [16, 18-  
41 20], regardless oxygen availability.

42 Oxidation kinetics are seldom monitored through the dissolved oxygen (DO) evolution [6]. However,  
43 the change in DO is a balance between enzymatic oxidation (Oxygen Uptake Rate, OUR) and dissolution  
44 of oxygen from the atmosphere into the liquid medium (Oxygen Transfer Rate, OTR). In the above-  
45 mentioned investigations, the oxygen transfer from atmosphere into liquid phase was not taken into  
46 account during the oxidation reaction. Consequently, the OUR may have been underestimated. The  
47 consideration of this phenomenon is particularly important when initial LA amount and/or the studied  
48 enzymatic activity are high. Likewise, Aziz *et al.* [5] demonstrated that increasing the stirring speed  
49 leads to an improvement of the reaction velocity. The authors attributed this result to a better  
50 homogenization of LA substrate. It would be interesting to check if this is not rather the result of a  
51 better oxygen transfer with the increase of stirring speed.

52 Consequently, most of the available investigations lead to an apparent affinity constant value of the  
53 enzyme for PUFAs and rarely investigate its affinity for oxygen: only Glikmann and Klinman [6] and  
54 Knapp [11] experimentally determined the affinity constant of SBLOX-1 towards oxygen. Under the  
55 same experimental conditions (i.e. in 0.1 M borate buffer pH 9), they found a value between 33.3  $\mu\text{M}$   
56 and 40.5  $\mu\text{M}$  and a value of 11  $\mu\text{M}$  respectively (values recalculated from the data in the article). Both  
57 studies performed the dissolved oxygen monitoring with a Clark electrode, but neither considered the  
58 impact of oxygen transfer from ambient air during stirring on their recorded curves. The obtained  
59 oxygen uptake rates are then underestimated in both cases. This not only makes it difficult to extract  
60 the real kinetic parameters of the used enzymes but also complicates the comparison between the  
61 different results available in the literature, as the oxygen transfer conditions always differ from one  
62 experimental device to another.

63 Generally, this leads to an unwise sizing of the enzymatic production of the fatty acid hydroperoxides.  
64 Gardner *et al.* [21] reported this issue from the work of Glikman and Klinman [6] without explanation.  
65 Indeed, Glikman and Klinman [6] compared the reaction constants of linoleic acid oxidation by SBLOX-  
66 1, measured spectrophotometrically (HPX production) and with a Clark electrode (oxygen  
67 consumption). They noticed that both values did not match (they differed by an order of magnitude of  
68 3). They attributed this difference to the stirring, which leads to an underestimation of the reaction  
69 kinetics measured by the Clark electrode compared to those measured by UV-spectra. Authors did not  
70 give a robust explanation. Anyhow, they did not attribute it to oxygen transfer at the gas/liquid  
71 interface, but suggested that premicellar fatty acid aggregates are probably the preferred substrate  
72 for LOX and are disrupted by the force of stirring. A similar observation was reported by Pourplanche  
73 *et al.* [15] without suitable explanation. Although this issue is valid for all oxidases, only Van Hecke *et*  
74 *al.* [22] have appropriately considered oxygen transfer kinetics to study enzymatic oxidation of  
75 saccharides.

76 In the present work, SBLOX-1 is used to oxidize linoleic acid to the 13-hydroperoxide isomer in sodium  
77 tetra borate buffer (pH 9) at 5 °C. The choice of low temperature is justified by a better solubility of

78 oxygen, especially since Elshof *et al.* [16] have demonstrated that the oxidation of LA by LOX is not  
79 negatively affected by the decrease of temperature. They even demonstrated that, over a temperature  
80 range of 5 °C to 40 °C, the highest oxidation rate is obtained at 5 °C. Additionally, several investigations  
81 have shown that SBLOX-1 remains active in certain organic solvent media[23], of which ethanol is the  
82 least toxic. It is well known that the presence of ethanol in an aqueous medium significantly reduces  
83 the surface tension [24] and thus improves the transfer of oxygen from the gas to the aqueous  
84 media[25]. Moreover, LA is easier to handle when dissolved in ethanol. Therefore, in the present work,  
85 the effect of ethanol on the SBLOX-1 activity is also studied and a suitable amount is used in the  
86 reaction medium. After the characterization of SBLOX-1 kinetics towards LA, the main aim of this work  
87 is to propose a simple and economical methodology to monitor the enzymatic production reaction of  
88 pure LA 13-hydroperoxide based on the systematic characterization of both oxygen transfer and  
89 uptake kinetics.

## 90 **2. Material and Methods**

### 91 **2.1. Chemicals**

92 All chemicals used were listed in Table 1. All solutions were prepared in ultrapure water. The soybean  
93 lipoyxygenase-1 solution was prepared in sodium tetraborate buffer 0.1 M pH 9 at 4 mg.mL<sup>-1</sup> and stored  
94 at -20 °C as 1 mL aliquots until use. The enzyme used contains mainly SBLOX isoform 1 (SBLOX-1)  
95 representing 75% of the total activity, and isoform 2 (SBLOX-2) representing about 25% of the total  
96 activity. At pH 9, SBLOX-1 expresses 100% of its activity while SBLOX-2 expresses only around 1% [26].  
97 Moreover, according to the supplier, the enzyme preparation is free of other enzyme activities such as  
98 lipase and peroxidase. Consequently, all reactions presented in the present work and occurring at pH 9  
99 can be attributed to SBLOX-1 isoform.

100 To facilitate the accurate addition of the substrate to the reaction medium, a concentrated ethanoic  
101 solution of LA was prepared at 500 mM and stored at -20 °C. This high concentration was useful to  
102 minimize the change in the amount of ethanol in the reaction medium upon addition of the substrate.

103 Indeed, for LA concentrations from 20  $\mu\text{M}$  to 100  $\mu\text{M}$  in the reaction medium, the volumes of ethanol  
104 added were of 10  $\mu\text{L}$  to 100  $\mu\text{L}$ , in a total reaction volume of 500 mL. This leads to a percentage of  
105 ethanol of 0.002% to 0.02% (v/v) in the reaction medium, which can be neglected for its impact on the  
106 volumetric transfer coefficient of oxygen at the gas-liquid interface ( $k_{\text{La}}$ ).

## 107 **2.2. Enzymatic oxidation set up and monitoring**

108 Enzymatic reactions were performed in a 500 mL jacketed reactor with magnetic stirring, equipped  
109 with a Clark electrode (Heito BOT2, adapted for temperature effect correction) connected to an OSD23  
110 oximeter for measuring dissolved oxygen (DO). DO data were recorded with the Data Studio 1.9.8r6  
111 software.

112 The reaction medium was a 0.1 M sodium tetraborate pH 9 buffer containing different amounts of  
113 ethanol to improve substrate solubility and facilitate LA addition. All enzymatic reactions were  
114 monitored at 5 °C. Soybean lipoxygenase-1 solution was used at 0.1% v/v in the reaction medium,  
115 unless otherwise stated.

116 Based on the pKa value of LA of 7.9 determined experimentally by Bild *et al.* [12], 92.5% of linoleic acid  
117 is in its linoleate form (sodium linoleate) in sodium tetraborate buffer pH 9. The use of air bubbling to  
118 deliver oxygen to the reaction medium is therefore risky because of foam formation leading to a  
119 decrease in the substrate availability. To avoid this, a special and simple procedure based on exclusive  
120 stirring without bubbling (oxygen transfer through the upper surface of the liquid medium) was applied  
121 and presented in section 2.5.

## 122 **2.3. Quantification of oxidation products and verification of the** 123 **purity**

124 The kinetics of hydroperoxide formation were monitored by UV absorbance at 234 nm (UVIKON xs  
125 Secoman, software UVxLite). HPX concentrations were calculated after calibration of the UV  
126 absorbance with pure LA hydroperoxides. The extinction coefficient of HPX was determined to be  
127 24.000  $\text{M}^{-1}\cdot\text{cm}^{-1}$ .

128 For verification of product purity, HPX and LA were analyzed with RP-HPLC. The HPLC analysis were  
129 performed on a Dionex Ultimate-3000 HPLC system equipped with a DAD detector and a ThermoFisher  
130 C30 Accucore column (2.6  $\mu\text{m}$ , 150 mm x 2.1 mm) was used. The mobile phase was water (A)-  
131 acetonitrile (B), with 0.1% formic acid and 5% THF added to each solvent. Gradient elution was adopted  
132 and the detailed program was as follows: initial 5% B linearly increased to 63% B in 3.0 min, kept at  
133 63% B from 3.0 to 10.0 min, then linearly increased to 85% from 10.0 to 11.0 min and maintained at  
134 85% B from 11.0 to 15.0 min, and returned to 5% in 1.0 min and maintained 5.0 min. The flow rate  
135 used was 0.25 mL.min<sup>-1</sup>. All the separations were performed at room temperature. LA was identified  
136 from its retention time (17.5 min) with UV absorbance at 205 nm. 13-HPX showed a retention time of  
137 12.5 min and was detected at 234 nm. 9-HPX eluted slightly later than 13-HPX, with a retention time  
138 of 13 min and was detected at the same wavelength of 234 nm. The main oxodiene peak co-eluted  
139 with 13-HPX but showed no significant absorbance at 234 nm and was detected at 278 nm.

#### 140 **2.4. Effect of ethanol content on enzymatic activity**

141 The enzymatic reaction procedure described above was tested with a fixed initial LA concentration of  
142 50  $\mu\text{M}$  and a varying ethanol concentration between 0% (v/v) and 10% (v/v) to study the effect of  
143 ethanol on the enzymatic activity. The buffer medium with the enzyme at each ethanol content was  
144 incubated for different durations (0, 24 and 72 h) at 5 °C before addition of LA and monitoring the  
145 reaction kinetics through HPX formation.

#### 146 **2.5. Determination of gas-liquid oxygen transfer coefficients**

147 To take into account the oxygen transfer, the oxygen mass balance during the LOX reaction is written  
148 according to equation (1):

$$149 \quad \frac{d[O_2]}{dt} = OTR - OUR \quad (1)$$

150 where *OTR* is commonly expressed as equation (2):

$$151 \quad OTR = k_L a \times ([O_2]^* - [O_2]) \quad (2)$$

152 with  $k_{La}$  the volumetric transfer coefficient of oxygen at the gas-liquid interface and  $[O_2]^*$  the  
153 solubility of oxygen in the reaction medium at the fixed temperature.

154 The volumetric oxygen transfer coefficient between gas and liquid phases ( $k_{La}$ ) was determined for  
155 each oxidation reaction experiment in this way:

156 Before the additions of enzyme and LA, the reaction medium was deaerated with nitrogen. LA was  
157 then added as a concentrated ethanoic solution and the reaction medium was let under controlled  
158 stirring to reaerate without air bubbling. The  $k_{La}$  was determined from the slope of the curve  
159  $\ln([O_2]^* - [O_2]) = f(t)$ .

160 Once SBLOX-1 has been added to the reaction medium, the decrease of dissolved oxygen  
161 concentration corresponds to the balance between oxygen uptake by lipoxygenase and oxygen  
162 transfer from atmosphere, as described in the equation (1).

## 163 **2.6. Determination of the kinetic parameters of the enzyme**

164 In a first set of experiments, initial dissolved oxygen was fixed at saturation (theoretically 407  $\mu\text{M}$  at  
165 5 °C in pure water) to ensure its supply in large excess. The initial concentration of LA was varied  
166 between 10 and 100  $\mu\text{M}$ , considering a Michaelian model (equation (3)) to determine the kinetic  
167 constant of SBLOX-1 towards LA:

$$168 \quad v_i = \frac{V_{max} \times [LA]}{K_{M(LA)} + [LA]} \quad (3)$$

169 where  $K_M(LA)$  is the affinity constant and  $V_{max}$  the maximum reaction rate.

170 In a second set of experiments, the initial concentration of LA was set to 20  $\mu\text{M}$  or 90  $\mu\text{M}$  and the initial  
171 dissolved oxygen was varied between 24  $\mu\text{M}$  and 407  $\mu\text{M}$  to check its possible effect on the initial  
172 reaction rate. A small reaeration was technically unavoidable before the start of the reaction due to  
173 the incompressible experimental delay between the end of deaeration step and the addition of the  
174 enzyme.

175 The concentrations of HPX and DO were monitored to evaluate the kinetics of the reaction. The  
176 recorded DO values were analyzed as mentioned in 2.5 to take into account the oxygen transfer  
177 kinetics (*OTR*) and determine the oxygen uptake values (*OUR*).

### 178 **3. Results and discussions**

#### 179 **3.1. Effect of ethanol content on enzymatic activity**

180 The effect of ethanol on SBLOX-1 activity was investigated. Enzyme was incubated (0 to 72 h) with  
181 different ethanol contents ranging from 0% (v/v) to 10% (v/v), then 50  $\mu\text{M}$  LA were added to measure  
182 SBLOX-1 activity. Figure 2 shows the evolution of the initial reaction rates of LOX towards LA as a  
183 function of the ethanol concentration.

184 The initial reaction rates are calculated from the HPX production rates, which correspond to the  
185 increase in absorbance at 234 nm. Given the low initial concentration of LA, dissolved oxygen is in large  
186 excess in all the experiments shown in Figure 2. This figure shows that between 0% and 3% ethanol,  
187 there is no negative effect on the initial reaction rates with a plateau reached between 2% and 3%.  
188 There is even a positive effect, which could be due to an improvement of dispersibility or solubility of  
189 LA due to the presence of ethanol, leading to a better availability of substrate for LOX. It also could be  
190 due to a better dispersion of the enzyme in the aqueous medium with ethanol. Figure 2 also shows  
191 that the inactivation effect is enhanced for longer contact times with ethanol. Above an ethanol  
192 content of 3%, a decrease in the initial reaction rate is observed and may be due to a partial inactivation  
193 of the enzyme.

194 Therefore, an ethanol content of 2% seems to be a very good compromise between a high dispersion  
195 of LA in the reaction medium, the amount of organic solvent added, and the inactivation of SBLOX-1,  
196 especially if the reaction time is limited to a few hours. This percentage of ethanol was chosen for the  
197 following experiments.

#### 198 **3.2. SBLOX-1 kinetic parameters towards LA**

199 For the determination of enzyme kinetic parameters towards LA, experiments were performed with  
200 2% (v/v) ethanol, at 5 °C by fixing initial dissolved oxygen at saturation (407  $\mu\text{M}$ ) and by varying initial

201 LA concentration between 10  $\mu\text{M}$  and 2500  $\mu\text{M}$ . The kinetics of the reaction can then be modeled as a  
202 one-substrate (LA) reaction kinetics because the initial DO concentration is in large excess compared  
203 to  $K_M(\text{O}_2)$  reported in literature[6, 11]. The results are given in Figure 3 as Michaelis-Menten (a) and  
204 Lineweaver-Burk representations (b).

205 It appears from Figure 3b that the Lineweaver-Burk curve is linear only for low LA concentrations  
206 ( $[\text{LA}] \leq 100 \mu\text{M}$ ) and that increasing LA initial concentration above this order of magnitude (red  
207 symbols) did not lead to an improvement of the initial reaction rates. No results in literature  
208 demonstrate an inhibition by excess substrate (LA). As an example, Berry *et al.* hypothesized such  
209 inhibition [27] but we emphasize that this is a lack of oxygen or a poor substrate accessibility (micellar  
210 form). The evidence of our statement is that a simple addition of oxygen or an increase of its solubility  
211 (by adding pure oxygen instead of compressed air) is sufficient to overcome this limitation and an  
212 improvement of the enzymatic activity is noticed, revealing that there was no inhibition but a simple  
213 lack of oxygen as substrate. This is in full agreement with the results of Berry *et al.* [27]. Consequently,  
214 it can be considered that in the present case, amounts of substrate above the observed limit of  
215 100  $\mu\text{M}$  range are not accessible to the enzyme and the most likely explanation is the micellar form of  
216 the LA substrate at these high concentrations. Thus, from data at low LA concentrations (blue symbols,  
217  $[\text{LA}] \leq 100 \mu\text{M}$ ),  $V_{\text{max}}$  and  $K_M(\text{LA})$  values are calculated with the Michaelis-Menten model (dotted line  
218 in Figure 3a) and the corresponding values were obtained:  $V_{\text{max}} = 11.4 \mu\text{M} \cdot \text{min}^{-1}$  and  $K_M(\text{LA}) = 45 \mu\text{M}$ .  
219 This  $K_M(\text{LA})$  value is of the same order of magnitude as the values reported in literature (Table 2).  
220 However, the operating conditions differ between the literature and the present work, with respect to  
221 two parameters: a lower temperature and the presence of ethanol. If only data from experiments with  
222 borate buffer are considered for comparison, the  $K_M(\text{LA})$  value obtained in the present work is higher.  
223 This may be explained by (i) the negative effect of a lower temperature on the access of LA to the  
224 internal active site of the enzyme due to lower mobility and (ii) the likely negative effect of ethanol on  
225 the conformation of the active site.

226 Above  $[LA] = 100 \mu\text{M}$ , initial reaction rates stop fitting the Michaelis-Menten model. It is well known  
 227 that the solubility of LA in aqueous media is very low. Micellar structures are often present even at low  
 228 concentrations between  $20 \mu\text{M}$  and  $200 \mu\text{M}$  [28-32], depending on the ionic composition and pH of  
 229 the medium, but also on the analytical method used to determine the solubility or the critical micelle  
 230 concentration (CMC). In the case of the presence of micelles, special kinetic models are used, to  
 231 describe enzymatic reactions towards micellar PUFA, but these models assume that the CMC values  
 232 are known. Firstly, the literature does not provide consistent data on CMC values of LA in aqueous  
 233 media [30-32] and secondly, data on media containing ethanol are really scarce [32].

234 Therefore, for the following sections, the experiments are performed with initial LA concentrations  
 235 within the range fitting with Michaelis-Menten model ( $< 100 \mu\text{M}$ ), in order to guarantee the LA  
 236 solubility condition and to simplify the present kinetic investigation, with the aim of studying the  
 237 oxygen transfer.

### 238 **3.3. Effect of dissolved oxygen concentration on enzyme kinetics**

239 To investigate the effect of dissolved oxygen on SBLOX-1 activity, experiments were performed at two  
 240 LA concentrations lower than  $100 \mu\text{M}$ , with 2% ethanol and varying the initial dissolved oxygen  
 241 concentration.

242 Mass balances must be written on dissolved oxygen by taking into account transfer and uptake rates,  
 243 according to the equation system (4):

$$244 \left\{ \begin{array}{l} \frac{d[O_2]}{dt} = OTR - OUR \\ \frac{d[LA]}{dt} = -\frac{V_{max}[LA]}{K_{M(LA)} + [LA]} = -OUR \\ \frac{d[HPX]}{dt} = OUR \\ OTR = k_L a \times ([O_2]^* - [O_2]) \end{array} \right. \quad (4)$$

245 The assumption made to facilitate the interpretation of oxygen kinetics is that the  $k_L a$  value remains  
 246 constant during conversion of LA to HPX. The applied experimental methodology is presented in  
 247 Figure 4.

248 The buffer medium containing 2% ethanol was poured in the jacketed reactor and its temperature was  
249 regulated at 5 °C. It was partially deaerated by nitrogen bubbling before the addition of LA (step not  
250 shown in Figure 4). After the addition of LA at two concentrations lower than 100 µM, the reaction  
251 medium was let under controlled stirring to reaerate. This step is mentioned as “zone 1” in Figure 4  
252 and is used to calculate  $k_{L,a}$  in the initial reaction medium. Under these conditions, the maximum  $k_{L,a}$   
253 value is determined to be 10 h<sup>-1</sup>.

254 When the desired DO concentration was reached, LOX was added and the resulting decrease in  
255 dissolved oxygen is used to calculate the enzymatic reaction rate, taking into account the previously  
256 calculated transfer rate (OTR). This is referred to as “zone 2” in Figure 4 where the oxygen mass balance  
257 is the result of both oxygen uptake rate (OUR) and oxygen transfer rate (OTR).

258 At the end of the conversion step in the mentioned “zone 3” of Figure 4, it can be noticed that the  
259 amount of dissolved oxygen increased again. During this new reaeration phase,  $k_{L,a}$  can be evaluated  
260 and compared to the value obtained in “zone 1”. The former (zone 1) corresponds to a medium loaded  
261 with LA while the latter (zone 3) corresponds to a medium loaded with HPX. This comparison validates  
262 the modeling parameters used for “zone 2” and the above-mentioned assumption of a constant  $k_{L,a}$   
263 value.

264 A series of experiments was performed with an initial LA concentration of 20 µM and the initial  
265 dissolved oxygen concentration was adjusted from 10% to 80% of saturation (i.e. 40 µM to 325 µM) to  
266 check if it influences the reaction kinetics and/or the product purity.  $k_{L,a}$  measurements in “zone 1”  
267 range between 0,99 and 1,5 h<sup>-1</sup> for the different experiments and show a variation of less than 10%  
268 between “zone 1” and “zone 3” for each experiment, which confirms the hypothesis of a rather  
269 constant value along each experiment.

270 Figure 5 shows the variation of dissolved oxygen from the initial aeration step to the end of the  
271 enzymatic reaction, after enzyme addition (zones 1 to 3). Based on the  $k_{L,a}$  values in “zone 1” and the  
272 DO change in “zone 2”, the initial oxygen consumption rates are calculated and lead to a common

273 mean value of  $3.9 \pm 0.2 \mu\text{M}\cdot\text{min}^{-1}$ . Even for the lowest initial dissolved oxygen concentration, no  
274 significant decrease in the initial reaction rate is found, indicating that dissolved oxygen is in large  
275 excess. The  $v_i$  value found is consistent with that presented in section 3.2, Figure 3, for  $20 \mu\text{M}$  LA  
276 ( $3.5 \mu\text{M}\cdot\text{min}^{-1}$ ).

277 Furthermore, since the stoichiometry of enzymatic oxidation is one mole of  $\text{O}_2$  for one mole of LA, the  
278 initial rate of HPX production should equal the initial oxygen uptake rate. Indeed, Figure 6 shows that  
279 the initial HPX production rate is the same regardless of the initial dissolved oxygen concentration and  
280 its value is  $3.92 \pm 0.04 \mu\text{M}\cdot\text{min}^{-1}$ . Likewise, Figure 7 shows that for an initial LA concentration of  $90 \mu\text{M}$ ,  
281 regardless of the initial DO amount (25% to 95% of the saturation, i.e.  $101 \mu\text{M}$  to  $387 \mu\text{M}$ ), the initial  
282 HPX production rate is constant and equal to  $8.6 \pm 0.5 \mu\text{M}\cdot\text{min}^{-1}$ , which corresponds to the value  
283 obtained at oxygen saturation, presented in section 3.2. This excellent correspondence between  
284 oxygen consumption rates and HPX production rates validates the  $k_{\text{L}}a$  estimates and confirms that an  
285 accurate measurement of oxygen transfer leads to a valid determination of the enzyme kinetic  
286 parameters.

287 The relatively constant oxygen uptake rate for different initial concentrations results from a very low  
288 affinity constant  $K_{\text{M}}(\text{O}_2)$  of the enzyme for oxygen, which would be lower than the minimum amount  
289 used ( $24 \mu\text{M}$ ). The few available literature data on  $K_{\text{M}}(\text{O}_2)$  ( $33\text{-}45 \mu\text{M}$  and  $11 \mu\text{M}$ ) [6, 11] are consistent  
290 with this observation although they may be overestimated because of the oxygen transfer not taken  
291 into account (the authors only followed the change in dissolved oxygen concentration and assumed it  
292 to be equal to the reaction rate). Also, due to the accuracy limitations of Clark electrode, it is not  
293 possible to determine a precise  $K_{\text{M}}(\text{O}_2)$  value if it is very low. In any case, this observation confirms that  
294 for Figure 3, it was relevant to model the kinetics by considering LOX as a single-substrate enzyme,  
295 since the concentration of dissolved oxygen was significantly above the  $K_{\text{M}}(\text{O}_2)$ . Therefore, the values  
296 of  $V_{\text{max}}$  and  $K_{\text{M}}(\text{LA})$  estimated in section 3.2 are also valid and represent the true kinetic constants and  
297 not just the apparent ones.

298 Thus, when the SBLOX-1 kinetic parameters and the oxygen transfer constant are taken into account,  
299 the zones 1, 2 and 3 previously defined in Figure 4 can be modeled into curves fitting the experimental  
300 data very well, as shown in the example in Figure 8. Similarly, the evolution of DO, LA and HPX  
301 concentrations is also well modeled, as shown in Figure 9.

302 However, it is obvious that the phenomena described above and the corresponding kinetic model are  
303 only relevant when dissolved oxygen is available. Figure 10 shows a typical case where dissolved  
304 oxygen was rapidly consumed and its renewal slower than consumption. This experiment was  
305 performed in a 1 L jacketed reactor, with a  $k_{La}$  value of  $1.1 \text{ h}^{-1}$ , an initial LA concentration of 2 mM and  
306 3 mL of the SBLOX-1 solution added after  $k_{La}$  measurement.

307 For this experiment, Figure 10 shows that the concentration of DO decreased in the first 15 minutes  
308 and remained null from this point until the end of recording (100 min). Also at the 15<sup>th</sup> minute, the  
309 rate of HPX accumulation decreased dramatically, confirming that the absence of dissolved oxygen had  
310 a direct effect on the enzymatic reaction. Given the amount of enzyme used ( $3 \text{ mL.L}^{-1}$ ), the initial  
311 reaction rate is expected to be about  $34 \mu\text{M.min}^{-1}$ . The results for HPX production and LA decrease  
312 shown in Figure 10 give initial rates of  $32.8 \mu\text{M.min}^{-1}$  and  $32 \mu\text{M.min}^{-1}$  respectively, which is consistent  
313 with this expected value. After the 15<sup>th</sup> minute, the HPX accumulation rate is equal to  $2.7 \mu\text{M.min}^{-1}$   
314 while the OTR is constant and equal to  $7.3 \mu\text{M.min}^{-1}$ . Since there was no oxygen accumulation, this  
315 means that all oxygen transferred to the liquid medium was directly consumed by the oxidation  
316 reactions ( $\text{OTR}=\text{OUR}$ ). Therefore, the difference between these two calculated rates may be due to the  
317 conversion of a significant part of the HPX produced into degradation products, with a net rate of  
318  $4.6 \mu\text{M.min}^{-1}$ . Simultaneously, the LA concentration decreased linearly in this second period. The  
319 calculated rate between 15 and 50 minutes is about  $15 \mu\text{M.min}^{-1}$ , which is twice the OTR value,  
320 confirming thus the presence of secondary anaerobic reactions.

321 The modeled curves presented in Figure 10 show that the modeling equations (4) cease to fit the  
322 experimental data from 15 min. This is due to the secondary reactions mentioned above, that are not  
323 taken into account in the modeling procedure and require specific investigations to be characterized.

324 To conclude, this study reveals the importance of the integration of gas-liquid oxygen transfer in  
325 SBLOX-1 kinetics studies, if a correct evaluation of oxygen consumption kinetics is aimed. It also reveals  
326 that even very low amounts of residual dissolved oxygen (a few percentages of saturation) are not  
327 limiting for the SBLOX-1 activity, which confirms that  $K_M(O_2)$  is very low. However, anaerobic conditions  
328 should be avoided to preserve the medium from secondary and uncontrolled reactions.

329 From the expression of oxygen transfer rate, it can be noticed that the maximum transfer rate is  
330 obtained with the lowest amount of residual DO in the reaction medium, according to equation (5):

$$331 \quad OTR_{max} = k_L a \times ([O_2]^* - 0) \quad (5)$$

332 The previous results show that this case is however not recommended. In fact, it is well known that in  
333 the absence of dissolved oxygen, SBLOX-1 goes through the anaerobic pathway. The anaerobic  
334 reaction starts, like the aerobic reaction, with the formation of radicals due to hydrogen abstraction  
335 from the LA. Without sufficient oxygen for the oxygenation step, this results in the formation of various  
336 carbonyl compounds: LA dimers, pentane, 13-oxotridecadienoic acid, AL epoxides, AL-epoxide dimers  
337 [33]. Some of these compounds are not detectable in UV and mass balances are therefore impossible  
338 to establish on the sole basis of the present analyses.

339 Therefore, it is necessary to identify or at least encompass the lowest permitted (or critical) DO  
340 amount, especially if significantly higher LA concentrations are used for large-scale HPX production.

341

### 342 **3.4. Enzymatic reactor set up for large scale HPX production and** 343 **product purity**

344 For a proper scaling of an enzymatic HPX production process, it is obvious from the previous section  
345 that gas-liquid oxygen transfer must be controlled. One relevant criterion for scaling up is the

346 Damköhler number[34],  $Da = \frac{\text{maximum reaction rate}}{\text{maximum transfer rate}}$ . Ideally,  $Da$  value should be less than 1 to  
347 ensure sufficient oxygen transfer, especially for long duration reactions. The maximum transfer rate  
348 can be calculated using a low residual oxygen concentration.

349 Therefore, two production experiments were performed in a 1 L jacketed reactor. Together with the  
350 previous experiment shown in Figure 10, this led to a set of three production experiments with a  $k_{La}$   
351 between  $1.1 \text{ h}^{-1}$  and  $5.3 \text{ h}^{-1}$ , and an initial LA concentration of 2 mM. 3 mL of the SBLOX-1 solution were  
352 added after  $k_{La}$  determination. The evolution of the DO concentration over time for three different  $k_{La}$   
353 values is shown in Figure 11. If the lowest DO reached concentration during reaction, used in section  
354 3.3, is considered, the  $Da$  numbers obtained are 4.5, 1.3 and 0.9 for  $k_{La}$  values of 1.1, 3.7 and  $5.3 \text{ h}^{-1}$   
355 respectively. For  $k_{La}$  values of 1.1 and  $5.3 \text{ h}^{-1}$ , the  $Da$  values confirm the obtained results in terms of  
356 lack and availability of oxygen, respectively. For the intermediate  $k_{La}$  value of  $3.7 \text{ h}^{-1}$ , there was no  
357 observed lack of oxygen for the only reason that the duration of the reaction was short enough. For a  
358 higher initial LA concentration, thus for a longer duration reaction, a lack of oxygen would have been  
359 noticed around the 20<sup>th</sup> minute as predicted by the  $Da$  number value.

360 For  $k_{La} = 3.7 \text{ h}^{-1}$  and  $5.3 \text{ h}^{-1}$ , the DO amounts remain above  $20 \mu\text{M}$  and  $170 \mu\text{M}$  respectively. Even for  
361 that very low residual DO concentration, pure HPX was obtained (Figure 11). The purity of HPX (234  
362 nm and retention time 12.5 min in the chromatograms) and mainly the absence of oxodienes, is  
363 verified by the absence of peaks on the chromatograms recorded at 278 nm. Additionally, in the  
364 chromatograms at 234 nm, no peaks are identified at 13 min attesting to the absence of 9-  
365 hydroperoxide. Even with a very low residual DO ( $20 \mu\text{M}$ ), almost 100 % LA is converted ( $>99.9 \%$  after  
366 6h for  $k_{La} 3.7$  and  $5.3 \text{ h}^{-1}$ ). The level of HPX produced ( $\frac{\text{total HPX}}{\text{Initial LA}}$ ) reaches 95 %. Complementary results  
367 on the purity of the obtained product are given in [35].

368 We show that even extremely low DO concentrations ( $[\text{O}_2]_{\text{lim}} \sim 20 \mu\text{M}$ ) allow almost total conversion  
369 of LA ( $>95\%$ ) and pure 13-HPX production. However, it is difficult to determine an accurate  $[\text{O}_2]_{\text{lim}}$  value  
370 due to Clark electrode sensitivity.

371 In comparison, commercial hydroperoxides used as standards contain a significant amount of  
372 oxodienes (additional peaks in the chromatograms in Figure 12). This may be due to a lack of oxygen  
373 during the oxidation reactions (usually chemical oxidations) or to the presence of reactive pollutants  
374 interacting with the hydroperoxides produced.

375 In conclusion, it is possible to produce pure 13-HPX with very low residual amounts of DO. However,  
376 once the latter is null, the anaerobic SBLOX-1 pathway is activated, and both LA and HPX are used as  
377 SBLOX-1 substrates to produce secondary degradation products. To predict this effect, several  
378 numerical simulations are performed with different experimental conditions, based on the equation  
379 system (4). Some examples are provided in the Supplementary Material.

380 In summary, to practically control the process by Damköhler number, the following procedure is  
381 necessary:

382 a. The value of  $k_La$  can be precisely predicted by dimensional analysis, knowing the  
383 specification of the reactor and stirrers, as well as the physicochemical properties of  
384 the solution (viscosity and oxygen solubility  $[O_2]^*$ ); it can also be simply measured  
385 experimentally as described in the present work;

386 b. Once the value of  $k_La$  is known, the amount of enzyme to be added can (and should)  
387 be adjusted so that  $V_{max} < k_La[O_2]^*$  is obtained (one should have  $Da < 1$  so

388  $\frac{\text{maximum reaction rate}}{\text{maximum transfer rate}} < 1$ ).

#### 389 **4. Conclusions**

390 Currently, PUFA hydroperoxides are chemically synthesized and the commercial products available are  
391 of moderate purity, often containing significant amounts of oxodienes and other secondary reaction  
392 products. Moreover, they are very expensive, which limits their use as reaction substrates in lipid  
393 oxidation research. Soybean lipoxygenases are known to be highly regio-selective and are therefore

394 very attractive for the production of pure fatty acid hydroperoxides, especially pure isomer by  
395 controlling pH and dissolved oxygen concentration.

396 The present work has demonstrated the need to consider oxygen transfer for satisfactory reactor  
397 sizing, as lack of oxygen induces the production of secondary oxidation and scission products. Once  
398 the oxygen uptake kinetics are corrected with the transfer rates, simple, economical and accurate  
399 inline characterization of the kinetics is possible, with oxygen uptake rates that were consistent with  
400 the LA and HPX kinetics.

401 The use of an optimal amount of ethanol allows an easier handling of the substrate and improves the  
402 dispersion into the reaction medium. A precise study on solubility of LA, its CMC and micelle sizes in  
403 such a reaction medium is in progress and will be published later in order to consolidate and clarify the  
404 data available in the literature. The use of low temperature improves the oxygen solubility and stability  
405 of the enzyme in a hydro alcoholic medium.

406 The proposed strategy results in almost full conversion of LA and very high purity of 13-HPX, which is  
407 currently used as a standard in our laboratory, and as a pure precursor for the study of lipid oxidation  
408 in food matrices. When high purity 13-HPX is produced, without any degradation products in the  
409 reaction medium, no free radical reactions are possible during storage, and the resulting product can  
410 be thus stored longer.

#### 411 **Declaration of competing interest**

412 The authors declare that they have no known competing financial interests or personal relationships  
413 that could have appeared to influence the work reported in this paper.

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416 this work.

417

418 **Figure captions**

419 *Figure 1* : Linoleic acid and its two main hydroperoxide products obtained by SBLOX-1 oxygenation  
420 under aerobic conditions

421 *Table 1* : List of chemicals

422 *Figure 2* : Effect of ethanol content and incubation time on initial reaction rates of LOX towards LA  
423 (initial concentration 50  $\mu\text{M}$ ) calculated as HPX production rates

424 *Figure 3* : Michaelis-Menten (a) and Lineweaver-Burk (b) curves for SBLOX-1 conversion of LA into HPX  
425 in borate buffer pH 9 (2% (v/v) ethanol)

426 *Table 2* : SBLOX-1  $K_M(\text{LA})$  values reported in literature

427 *Figure 4* : Illustration of the different steps (« zones ») followed to determine  $k_{\text{La}}$  and calculate initial  
428 enzymatic reaction rate.

429 *Figure 5* : Modification of dissolved oxygen concentration during enzymatic reaction with 20  $\mu\text{M}$  of  
430 initial LA concentration and various initial DO amounts. Zones 1 to 3 are represented. The sudden slope  
431 change corresponds to the enzyme addition for each curve.

432 *Figure 6* : HPX Production kinetics by SBLOX-1 (followed by UV absorbance measurements at 234 nm)  
433 in reaction media containing 20  $\mu\text{M}$  of initial LA and different initial DO amounts.

434 *Figure 7* : HPX Production kinetics by SBLOX-1 (followed by UV absorbance measurements at 234 nm)  
435 in reaction media containing 90  $\mu\text{M}$  of initial LA and different initial DO amounts.

436 *Figure 8*: Example of experimental and modeled DO curves for the successive steps of the proposed  
437 methodology (zones 1, 2 and 3) taking into account all transfer and reaction constants

438 *Figure 9* : Example of experimental and modeled data (equations 4). DO are monitored with Clark  
439 electrode, HPX and LA are quantified by HPLC-DAD.

440 *Figure 10* : DO, LA and HPX kinetics for  $k_{La}=1.1 \text{ h}^{-1}$

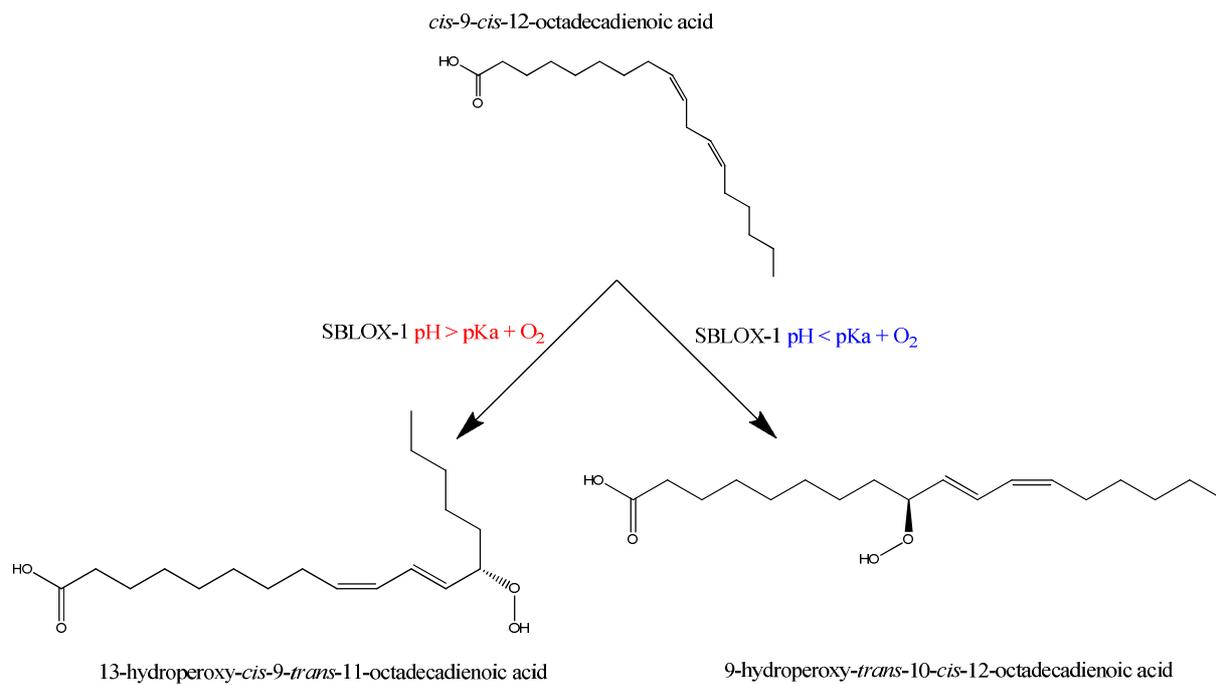
441 *Figure 11* : a) DO concentration curves for the production tests at three different  $k_{La}$  values. Only zones  
442 2 and 3 are present b) Chromatogram at 234 nm of the final product obtained with production test at  
443  $k_{La} 3.7 \text{ h}^{-1}$ . Included curve represents the UV spectrum of the HPX peak.

444 *Figure 12* : Chromatograms of fresh commercial linoleic acid 13-hydroperoxide at 234 nm and 278 nm.  
445 Included curve represents the UV spectrum of the main peak. 234 nm is the maximum absorbance  
446 wavelength for HPX and 278 nm the maximum absorbance wavelength for oxodienes.

447

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*Figure 1* : Linoleic acid and its two main hydroperoxide products obtained by SBLOX-1 oxygenation under aerobic conditions

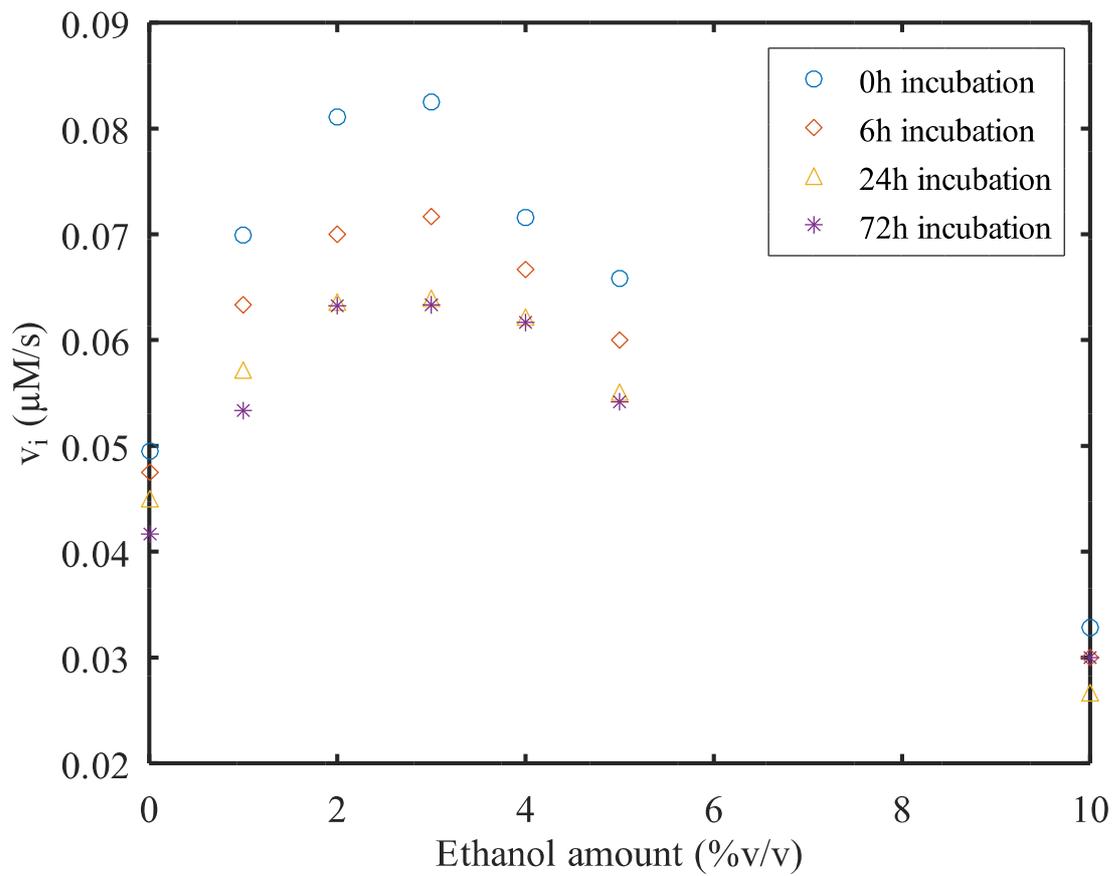


Figure 2 : Effect of ethanol content and incubation time on initial reaction rates of LOX towards LA (initial concentration 50  $\mu\text{M}$ ) calculated as HPX production rates

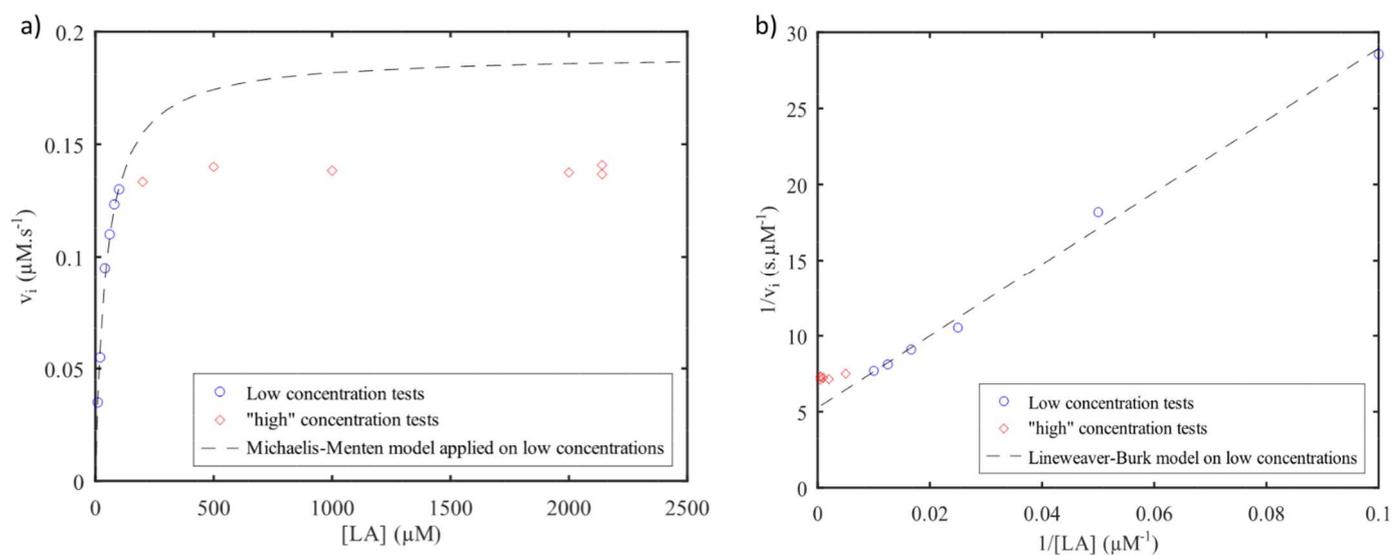


Figure 3 : Michaelis-Menten (a) and Lineweaver-Burk (b) curves for SBLOX-1 conversion of LA into HPX in borate buffer pH 9 (2% (v/v) ethanol)

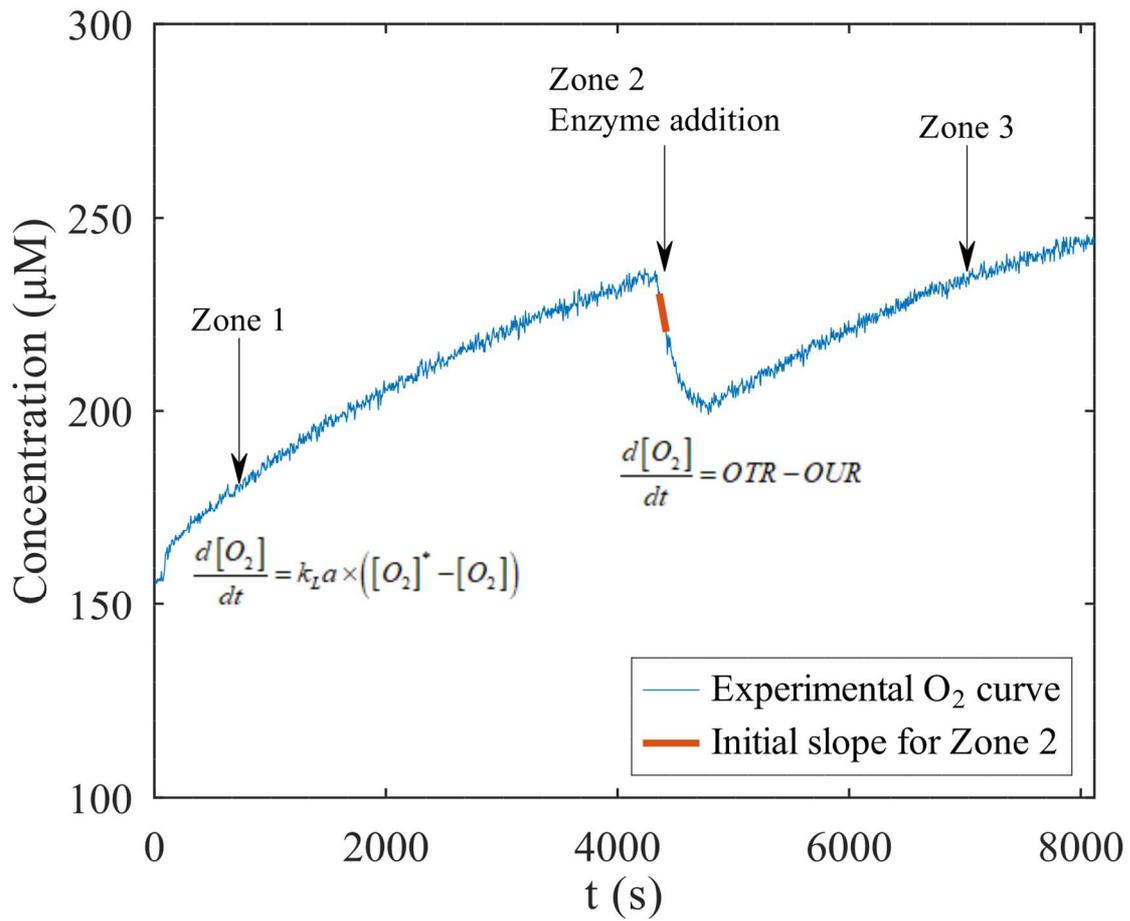


Figure 4 : Illustration of the different steps (« zones ») followed to determine  $k_L a$  and calculate initial enzymatic reaction rate.

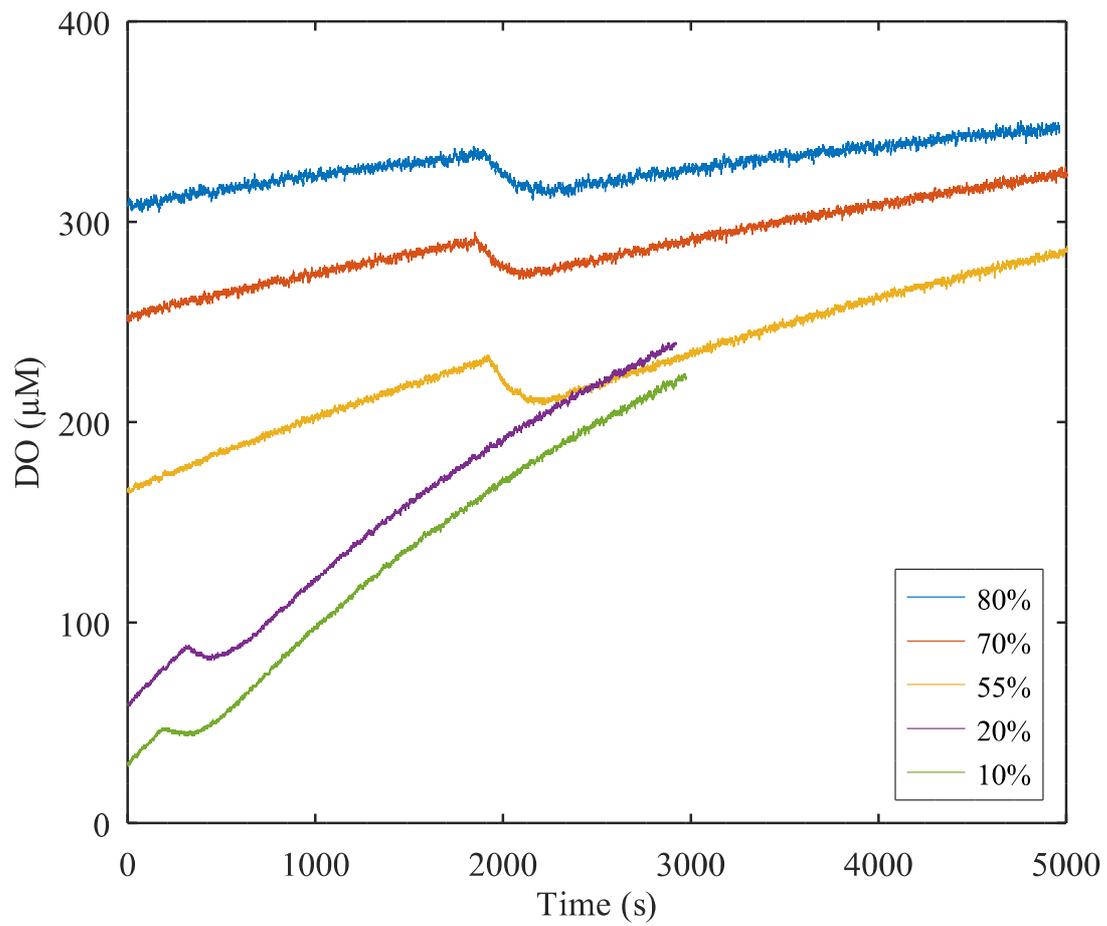


Figure 5 : Modification of dissolved oxygen concentration during enzymatic reaction with 20  $\mu\text{M}$  of initial LA concentration and various initial DO amounts. Zones 1 to 3 are represented. The sudden slope change corresponds to the enzyme addition for each curve.

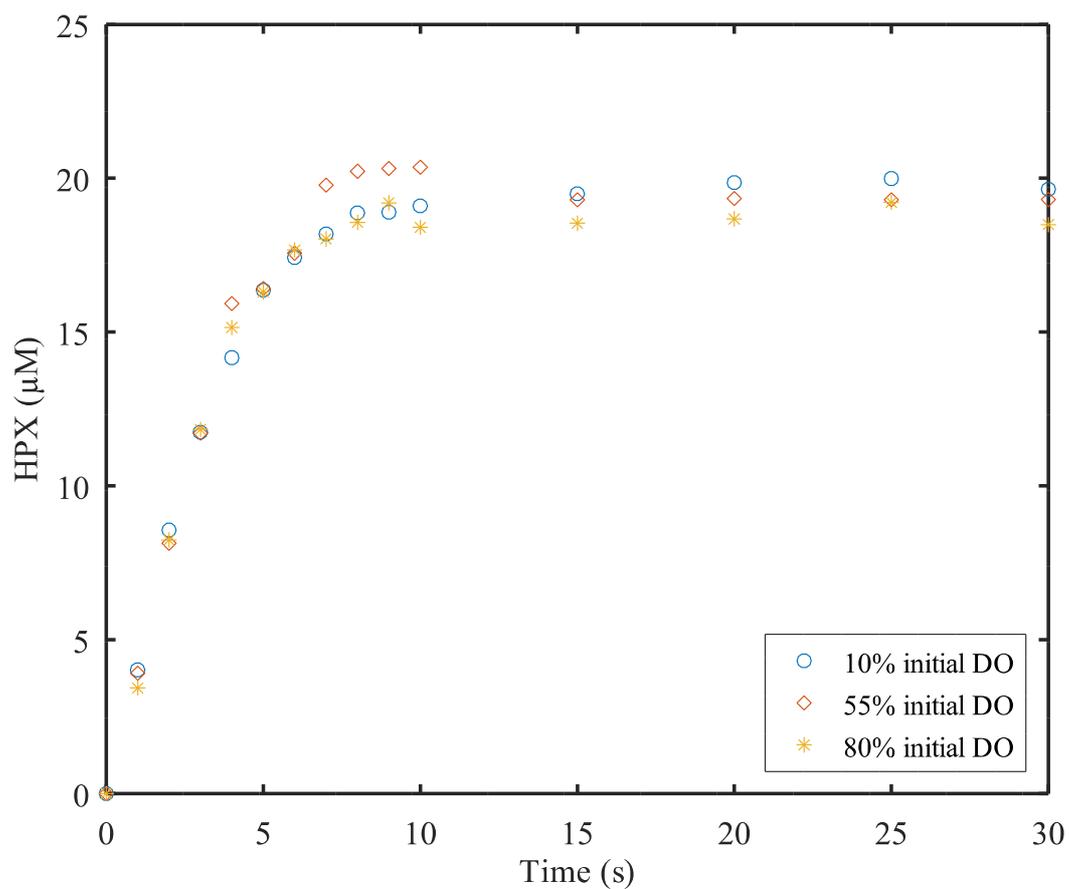


Figure 6 : HPX Production kinetics by SBLOX-1 (followed by UV absorbance measurements at 234 nm) in reaction media containing 20 μM of initial LA and different initial DO amounts.

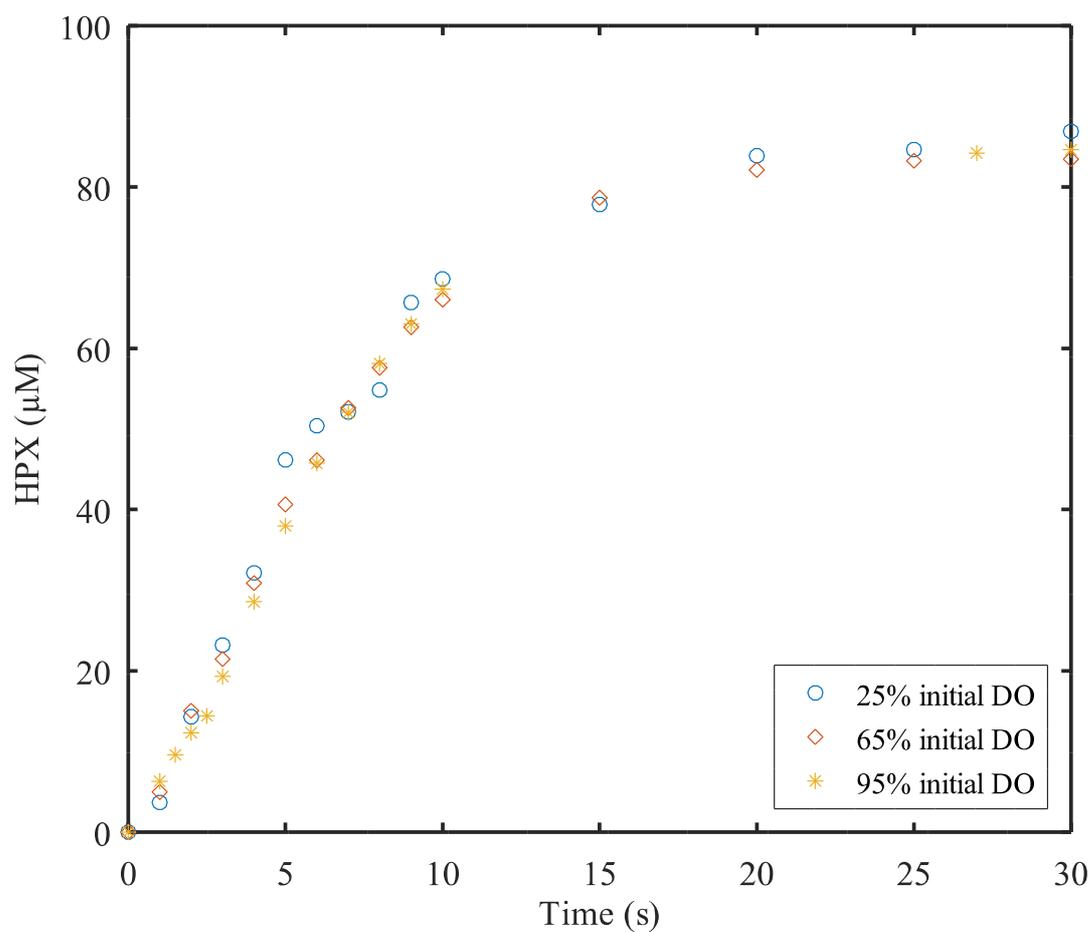


Figure 7 : HPX Production kinetics by SBLOX-1 (followed by UV absorbance measurements at 234 nm) in reaction media containing 90 μM of initial LA and different initial DO amounts.

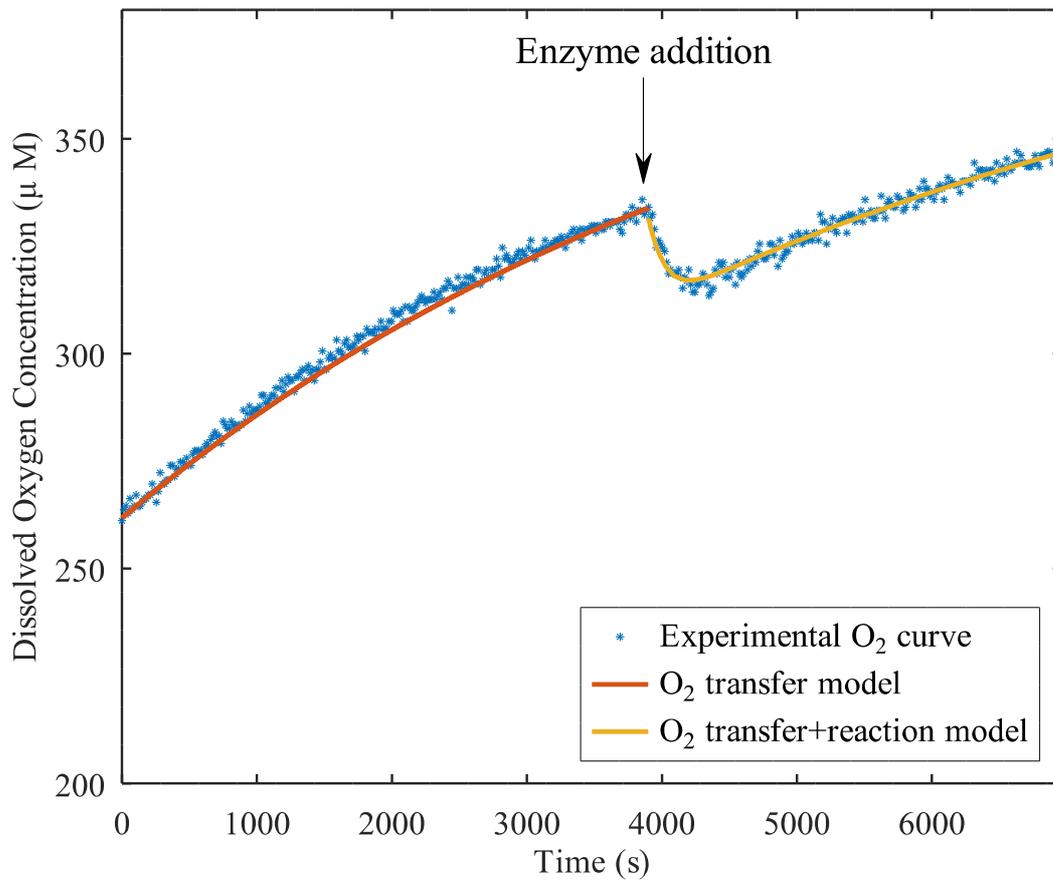


Figure 8: Example of experimental and modeled DO curves for the successive steps of the proposed methodology (zones 1, 2 and 3) taking into account all transfer and reaction constants

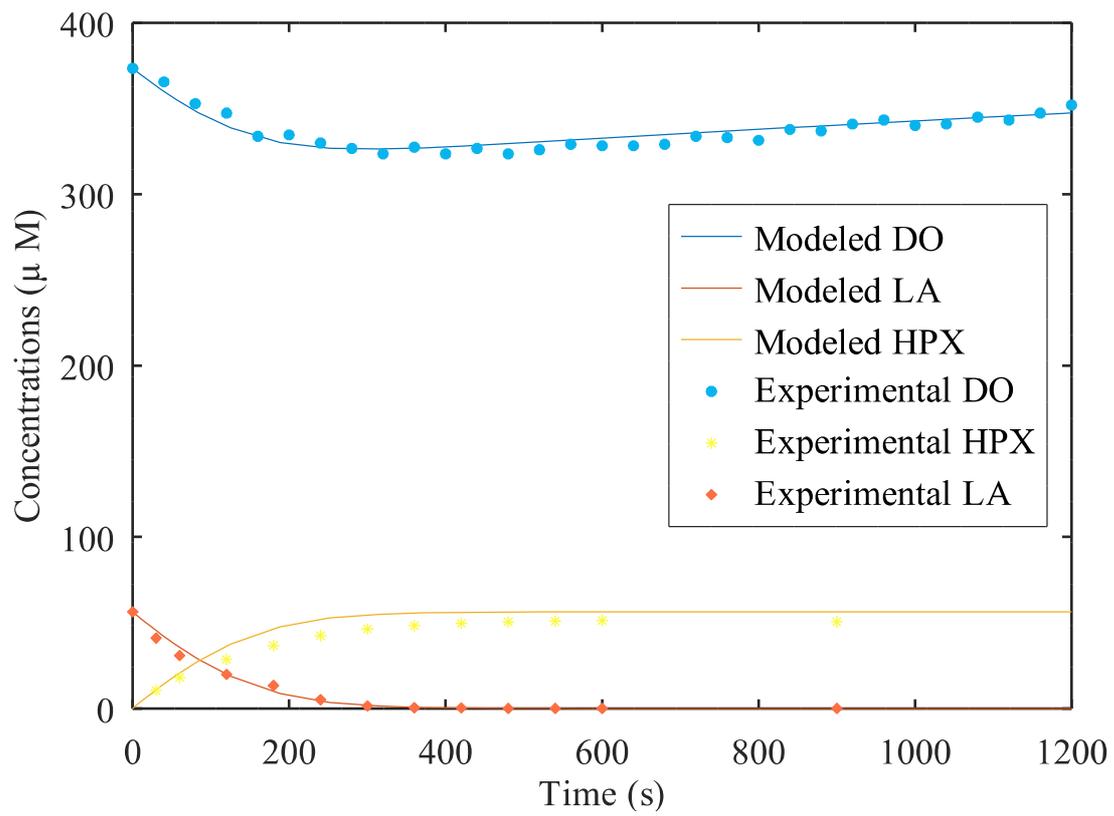


Figure 9 : Example of experimental and modeled data (equations 4). DO are monitored with Clark electrode, HPX and LA are quantified by HPLC-DAD.

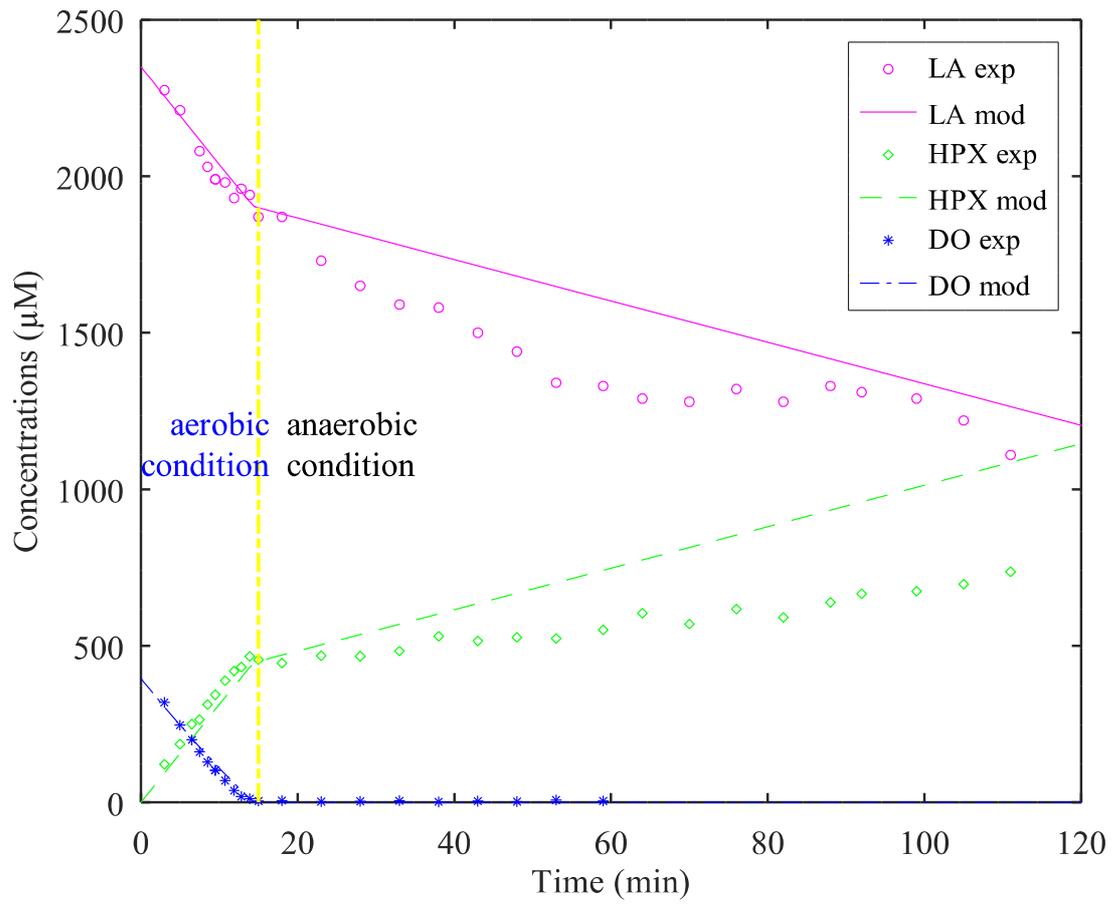


Figure 10 : DO, LA and HPX kinetics for  $k_{La}=1.1 \text{ h}^{-1}$

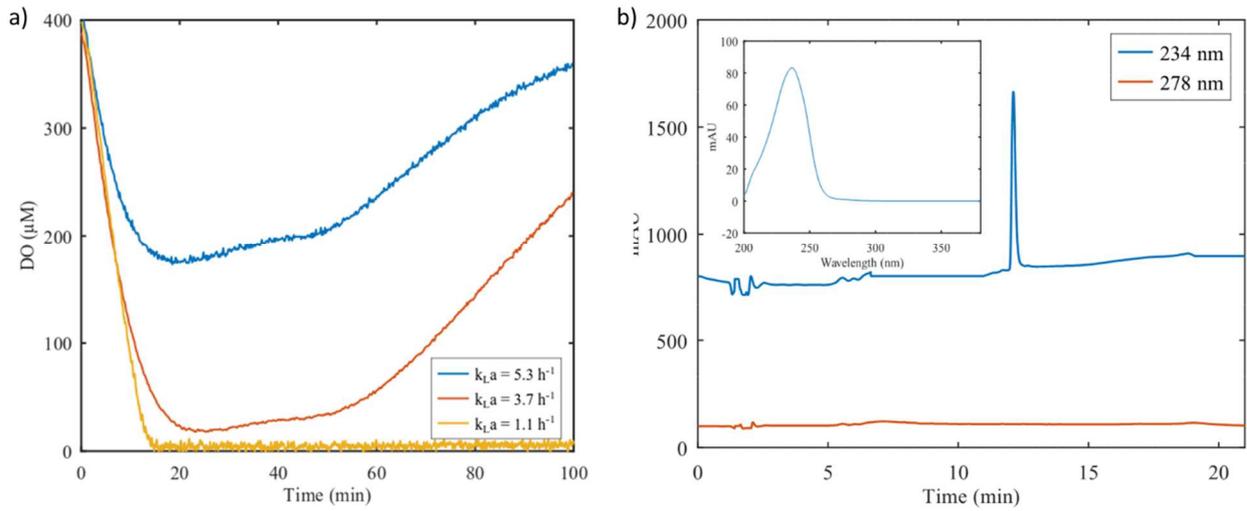
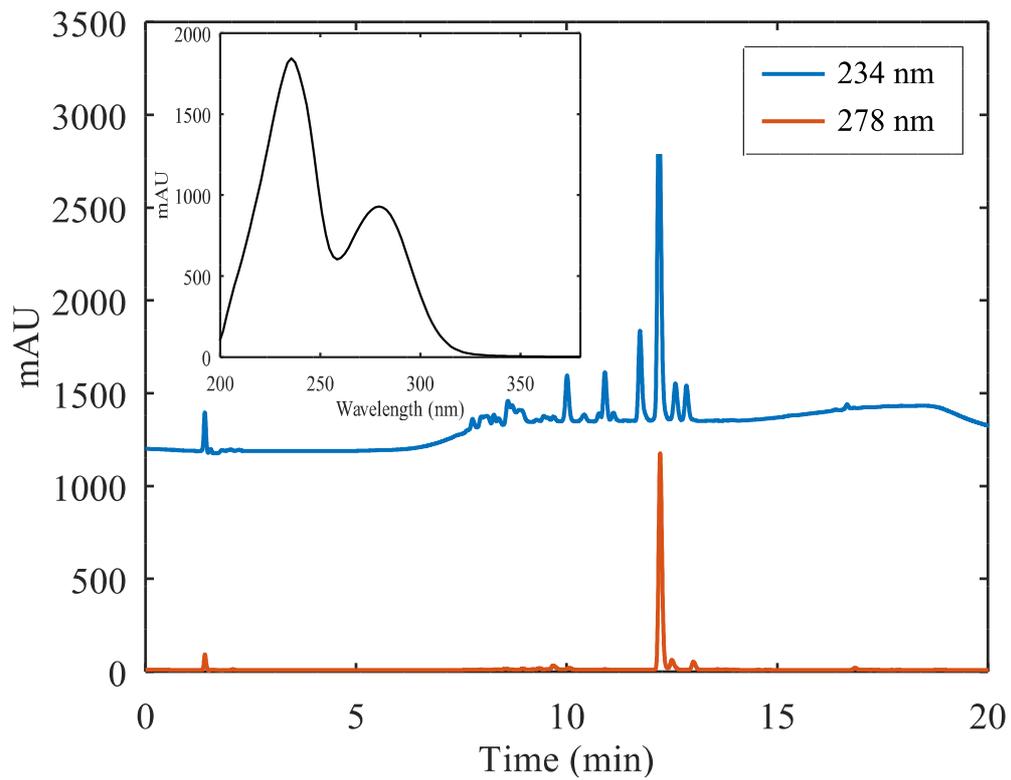


Figure 11 : a) DO concentration curves for the production tests at three different  $k_{La}$  values. Only zones 2 and 3 are present b) Chromatogram at 234 nm of the final product obtained with production test at  $k_{La} 3.7 h^{-1}$ . Included curve represents the UV spectrum of the HPX peak.



*Figure 12* : Chromatograms of fresh commercial linoleic acid 13-hydroperoxide at 234 nm and 278 nm.

Included curve represents the UV spectrum of the main peak. 234 nm is the maximum absorbance wavelength for HPX and 278 nm the maximum absorbance wavelength for oxidienes.

Table 1 : List of chemicals

Chemicals	Reference or CAS number	Quality/purity/activity	Supplier
NaOH	480507	98%	CARLO ERBA
Ethanol	1.11727.1000	96%	MERCK
Borax	1330-43-4	99%	SIGMA
Boric acid	B-6768	99%	SIGMA
Linoleic acid	10-1802-13	99%	LARODAN
13-HPX 9-HPX		laboratory produced + commercial*	
SBLOX-1	9029-60-1	7,9 U/mg	FLUKA
Acetonitrile	75-05-8	HPLC PLUS gradient grade	CARLO ERBA
Formic acid	64-18-6	98-100% for analysis	SIGMA

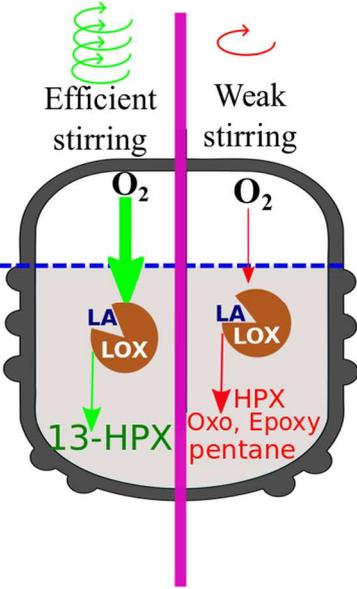
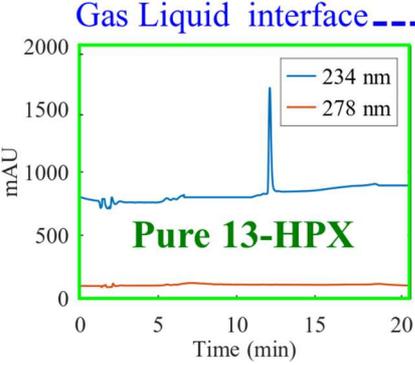
\* Supplier is not indicated as the product purity is criticized in the present work.

Table 2 : SBLOX-1  $K_M(LA)$  values reported in literature

Source	Reaction conditions	$K_M(LA)$ $\mu M$	Analytical method
Allen <i>et al.</i> [28]	Tris buffer pH 9 ; 25 °C	24	UV 234 nm
Berry <i>et al.</i> [27]	Phosphate buffer pH 9; 25 °C	62 $\pm$ 15	Dissolved oxygen
Galpin <i>et al.</i> [29]	0.1 M borate buffer pH 9 25°C	15	UV 234 nm
Glikmann <i>et al.</i> [7]	0.1 M borate buffer pH 9 25°C	22-27	UV 234 nm and dissolved oxygen
Karadag <i>et al.</i> [30]	0.1 M borate buffer pH 9 25°C	12.5	UV
Karout <i>et al.</i> [31]	0.05 M tris-HCl buffer pH 9 room temperature	67 $\pm$ 8	UV
Knapp <i>et al.</i> [12]	0.1 M borate buffer pH 9 20-21 °C	18 $\pm$ 3	Dissolved oxygen

Graphical abstract

**Damköhler < 1**



**Damköhler > 1**

