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PII:	S0896-8446(18)30274-2
DOI:	https://doi.org/10.1016/j.supflu.2018.07.026
Reference:	SUPFLU 4340
To appear in:	*J. of Supercritical Fluids*
Received date:	26-4-2018
Revised date:	13-6-2018
Accepted date:	25-7-2018

Please cite this article as: Benito-Román Ó, Sanz MT, Melgosa R, de Paz E, Escudero I, Beltrán S, Studies of Polyphenol Oxidase inactivation by means of High Pressure Carbon Dioxide (HPCD), *The Journal of Supercritical Fluids* (2018), https://doi.org/10.1016/j.supflu.2018.07.026

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Studies of Polyphenol Oxidase inacvaon by means of High Pressure Carbon Dioxide (HPCD)

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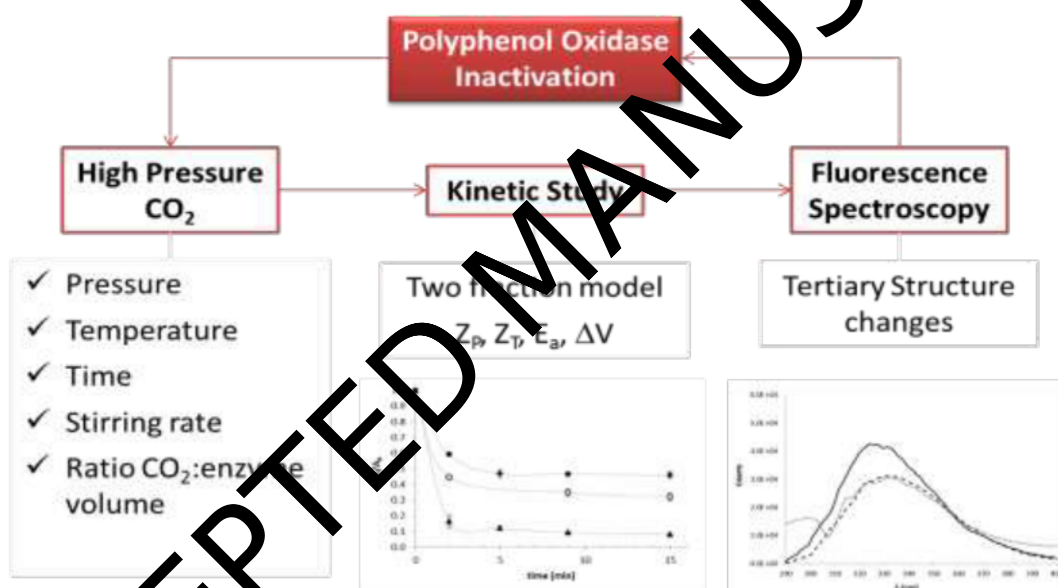
Graphical Abstract



Highlights

- Polyphenol oxidase (PPO) was successfully inacvated by means of supercrical CO_2
- The rao CO_2:volume of enzyme dissoluon was found to be a crical parameter
- Pressures of 20 MPa led to the complete inacvaon of PPO in mes <10minutes
- A complete inacvaon kinec study was done using a two fracon model
- Dramac changes in the enzyme terary structure were observed

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Abstract

Tyrosinase from mushroom was used as a model polyphenol oxidase (PPO) enzyme to perform a systemac inacvaon study using high pressure carbon dioxide (HPCD). The ratio CO_2/volume of enzyme (g/mL) loaded in the reactor was found to be crical. Above a crical rao, pressure, temperature and me did not control the inacvaon performance. Exposure me (2 to 15 minutes), temperature (25 to 45 °C) and pressure (5 to 20 MPa) allowed to show a characterisc inacvaon paern for PPO: a sudden decrease in acvity (at least 75% of the total acvity loss was observed within the rst 2 minutes) was followed by a slowed decay. The experimental data were ed into a two fracon kinec model and the main kinec parameters (Z_P, Z_T acvaon volume and acvaon energy) were calculated. The uorescence spectroscopy analysis of the samples treated with HPCD revealed signicant changes in the terary structure of the enzyme.

Keywords: polyphenol oxidase, HPCD, enzyme inacvaon, supercrical carbon dioxide

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1. Introducon

Polyphenol oxidase enzymes (PPO; EC 1.14.18.1) are extensively distributed in plants, fruits or sea food. These enzymes catalyze the oxidaon of phenolic compounds to quinones, which are responsible for the producon of the brownish pigments [1] that appear when the fruit is exposed to air or when the shrimps are taken out of the water. This browning process is irreversible and induces costumers to perceive the foodstu as spoiled and not to buy it. Therefore, the consequences of the browning formaon pigments induced by PPO are a signicant problem with huge economic impact for the food industry. This problem demands the development of new food preservaon strategies (based on non-thermal treatments in order to preserve the organolepc properes of the food) to prevent the acvity of PPO enzymes in juices or sea food.

Among the new treatments arising HPCD (High Pressure Carbon Dioxide) is a promising alternave. This technique uses CO_2 in condions of pressure and temperature usually above the crical point (7.4 MPa and 30.9 °C). Typical operang values are pressures up to 30 MPa and temperatures not higher than 50 °C [2] whereas the exposure mes to the CO_2 are variable and can last from a few minutes to one hour. In all cases the temperatures are much lower than those of the convenonal thermal treatments which helps to preserve the organolepc properes of the treated food [3]. HPCD has been widely used to inacvate PPO in juices from many dierent fruits and vegetables: apple [4][5][6][7], carrot and peach [8], orange [9], red beet [10], beet root [11] watermelon [3] or carrot and celery juices [12] among others. Most of these papers present a similar working procedure: dierent combinaons of pressure, temperature and me are tried, then the extent of the enzyme inacvaon is measured and in the last stage the experimental data are ed to dierent kinec models. Table 1 summarizes the experimental condions and experimental results published in the literature, regarding the PPO inacvaon by HPCD. As can be seen there are a wide variety of working procedures which makes it dicult to compare the results among authors: on one hand the source of the enzyme determines the properes the enzyme has and on the other hand experiments were performed under very dierent experimental condions (dierent pressures, temperatures, volumes of enzyme soluon or reactor conguraons). Moreover enzymes directly isolated from foods are not pure, but they are linked to other compounds and the inacvaon process is more complex [7].

In order to evaluate the eect of the above menoned process parameters in the inacvaon of PPO and to get useful informaon about the inacvaon mechanism it is necessary to carry out a set of experiments using a soluon of pure PPO in order to avoid the interferences of other species present in juices. In the literature there are only a few studies that treat pure polyphenol oxidases with HPCD: Manzocco et al. (2016) [2] and Manzocco et al. (2014) [13] studied the inacvaon of mushroom tyrosinase by HPCD, ng the inacvaon results to a rst order kinec model. Hu et al. [14], treated tyrosinase from mushroom with pressures up to 15 MPa at 55 °C, no srring was applied and poor inacvaon eciencies were reached. PPO from mushroom is a type-3 metalloprotein containing a di-nuclear copper acve site bound to the protein matrix (four α-helix bundles that surround the acve site) by six hisdine residues [15]. The mechanism why enzymes are inacvated under HPCD has been hypothesized in dierent works. In general pressure and temperature can alter directly the

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structure of the enzyme or change the properes of the CO_2 (hence determining the interacons between solvent and enzyme) [1]. Under certain pressure and temperature condions a change in the protein structure can happen with an increase of the β-sheet structure that leads to the burial of the catalyc center [16]. According to Li et al. [17], the inacvaon of a PPO enzyme is related to the way that carbon dioxide interferes with the hydrophobic acve site. CO_2 is a very non polar molecule whose physical properes change when changing pressure and temperature (especially above the crical point) being able to stay in gas, liquid and supercrical state. These changes in the properes will aect the way it interacts with the enzyme and will have to be studied.

In the literature an engineering approach for the enzyme inacvaon by HPCD is oen missed, since no systemac studies had been carried out to idenfy the crical process parameters. Besides pressure and temperature other parameters such as the srring rate, the holding me and the rao CO_2 versus the volume of enzyme soluon loaded into the reactor must be considered. This factor plays a signicant role in the inacvaon performance and to aect the economic feasibility of the HPCD process. Therefore, insights about the way it aects the enzyme inacvaon are required.

All in all, the aim of the present work is on one hand to obtain experimental data useful for the up-scaling of the HPCD enzyme inacvaon process and on the other to gain knowledge about the PPO inacvaon mechanism under dierent condions of CO_2, below and above the crical point, using a commercial tyrosinase from mushroom as a model PPO enzyme. This approach requires a mulstage work: in a rst stage the idencaon and evaluaon of the main process parameters that aect the enzyme inacvaon (pressure, temperature, me, srring rate and the volume of enzyme soluon loaded in the reactor) must be tackled. In a second stage a kinec study of the inacvaon data has to be performed considering the insights obtained in the rst stage of the work, trying three dierent temperatures (25, 35, 45 °C) and three dierent pressures (5, 10 and 20 MPa). The dierent combinaons of pressure and temperature led to condions in which CO_2 was present in three dierent states (gas, liquid and supercrical) and were used to explain how the physical state of the CO_2 aects its physical properes and how the interacon between CO_2 and the enzyme is aected. The obtained experimental inacvaon rates (or residual acvity of the enzyme) were ed to dierent kinec models, being used the kinec constants to calculate kinec parameters such as the decimal reducon me, the decimal reducon parameters (Z_P and Z_T), and the energy and volume of acvaon, all of them dened and explained in secon 2.5. Finally, the inacvaon results were compared to the changes induced in the structure of the enzyme determined by uorescence spectroscopy.

2. Material and Methods

2.1. Enzyme and chemicals

Mushroom tyrosinase (polyphenol oxidase -PPO- EC 1.14.18.1) (T3824, 25KU, obtained from mushroom *Agaricus bisporus*) was purchased from Sigma Aldrich (St. Louis, MO). The contents of the enzyme vial were dissolved in 10 mL of phosphate buer soluon (50mM, pH 6.5), which was divided in several aliquots that were frozen at -20 °C. The PPO soluons used in the experiments were prepared from diluon of this soluon, in order to have a 25 U/mL

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concentraon.

Carbon dioxide (99.9%) was supplied by Air Liquide S.A. (Spain). The physical properes of CO_2 were taken from NIST Database and dielectric constant was calculated according to the equaon presented by Eltringham et al.[20].

All other chemicals used in this work to prepare the phosphate buer soluon or catechol soluon were analycal grade purchased from Sigma Aldrich.

2.2. Experimental Set-up

All the experiments were carried out in a stainless steel high pressure batch srred reactor with an internal volume of, approximately, 80 mL which was submerged in a thermostac water bath. The experimental set up consists of a CO_2 reservoir, a high pressure syringe pump (260D Teledyne ISCO) and it was a slight modicaon of Melgosa et al. [21]. A schemac representaon of the experimental set up is shown in Figure 1.

In a typical experiment, the desired volume of preheated tyrosinase soluon was loaded into the reactor which was ghtly closed. Subsequently it was placed in the water bath previously set at the desired temperature. Aer two minutes (me enough to reach the working temperature) CO_2 was bubbled directly into the enzyme soluon through a sintered stainless steel micro-lter (pore size of 10 μm) in a sucient amount to reach the desired working pressure (pressurizaon rate 10 MPa/min) and temperature were held for the desired operang me (from 2 to 15 minutes). Experiments were done in a temperature and pressure range commonly used in non-thermal inacvaon HPCD systems: temperature from 25 to 45 °C and pressure from 5 to 20 MPa. Aer dierent inacvaon mes, the reactor was depressurized and the enzyme soluon was taken out. It was le at room temperature for ten minutes and shaken in order to release the CO_2 that was sll dissolved. All the samples were frozen at -20 °C unl the moment of being analyzed in order to evaluate the residual acvity of the enzyme aer a treatment.

2.3. Experimental Work

The experimental work was divided in three dierent stages:

2.3.1.Screening experiments

Inially several screening experiments were carried out in order to idenfy the experimental parameters that had the greatest inuence on the tyrosinase inacvaon. In a HPCD batch process, temperature, pressure, holding me, stirring rate and the volume of enzyme soluon loaded into the reactor were idenfy as the parameters that may aect the inacvaon performance. In this work, it was carried out a fraconal factorial design of those ve process parameters at two levels (low and high) each: volume of enzyme soluon used (25 and 40 mL), pressure (10 and 20 MPa), me (5 and 15 minutes), temperature (35 and 45 °C) and srring rate (250 and 600 rpm). In total, 8 dierent treatments were tried (it was a 2^{5-2} experimental design, with resoluon III) and the ANOVA study of the experimental results was used to idenfy the parameters that played the most important role on the enzyme inacvaon.

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2.3.2. Volume of enzyme soluon used

In a second stage of the experimental work the volume of enzyme soluon loaded into the reactor was changed. In all the experimental results presented in the literature, a constant volume of enzyme soluon was loaded in the reactor, being the remaining volume lled up with CO_2. Then, dierent combinaons of pressure and temperature were applied. Since the physical properes of CO_2 (such as density, which relates pressure and temperature) change dramacally when changing pressure and temperature, the total mass of CO_2 loaded in the reactor signicantly changes depending on the working condions, leading to experiments done at dierent pressures, temperatures and masses of CO_2 to inacvate the enzyme. Since the mechanism why PPO is inacvated is not completely clear, the amount of CO_2 used may play a signicant role on the inacvaon performance. From an upscaling and economic outlook, the amount of CO_2 used per gram of soluon is crical, since an excess of CO_2 might not increase the inacvaon rate but it would increase the operaonal costs of the HPCD process. Therefore, the opmal rao must be determined in order to avoid excessive use of CO_2.

In order to evaluate the inuence that the rao CO_2:enzyme soluon has on the PPO inacvaon performance, some experiments loading dierent amounts of enzyme soluon were carried out. Temperature was set at 35 and 45 °C, and the rao grams of CO_2: volume of enzyme soluon was varied from 0.7 g/mL (more soluon than CO_2 was loaded in the reactor) to 7 g/mL (seven more mes of CO_2 than enzyme soluon). The CO_2 density was obtained from (NIST Chemistry Webbook 2017), the volume of enzyme soluon was varied to fulll the rao desired and the volume of enzyme soluon needed to reach the CO_2:soluon rao was calculated.

Srring rate was set at 250 rpm, and all the experiments lasted 9 minutes.

2.3.3. HPCD treatment: kinec study

25 mL of the enzyme soluon were placed in the reactor. Three dierent temperatures below the convenonal thermal treatment were selected: 25, 35 and 45 °C and three pressures: 5, 10 and 20 MPa. Inacvaon kinec experiments lasted 2, 5, 9 and 15 minutes. In total 36 dierent condions were tested. Once the inacvaon rate was measured the experimental data were ed into dierent kinec models, as described in secon 2.5.

Some control experiments were done at atmospheric condions in order to compare the HPCD treatment performance: 25 mL of tyrosinase soluon were placed in a water bath at 25, 35 and 45 °C at atmospheric pressure. Samples were taken periodically up to 180 minutes and kept frozen unl further analysis.

2.4. Analycal procedures

2.4.1. Enzymac Acvity Evaluaon

The enzyme acvity was assayed spectrophotometrically (Jasco V-750, Japan) using catechol as substrate and following a slightly modied protocol from [16]: 2 mL of phosphate buer soluon were added to 0.3 mL of the enzyme soluon; aer the addion of

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0.3 mL of catechol soluon (20 mM, prepared in a phosphate buer soluon 50 mM and pH 6.5) the reacon was started. The changes in absorbance at 420 nm and 25 °C were recorded every second for 150 seconds. The enzyme acvity was calculated from the slope of the inial secon of the absorbance versus me. One unit of PPO acvity was dened as the change in absorbance of 0.001 units per minute at 420 nm.

The residual acvity (RA) was calculated as the rao of the measured acvity aer a treatment (A) and the acvity before treatment (A_0), according to the equaon 1:

(1)

2.4.2. Fluorescence Spectroscopy

The terary structure of the PPO was determined by uorescence spectroscopy using the FLS980 photoluminescence spectrometer (Edinburgh Instruments, Livingstone, UK). The sample was excited at a λ_{em}=280 nm and the emission spectra were recorded in the range 290 to 400 nm. A 1 cm path length cell was used and the emission and excitaon slits were set at 4 and 2 nm, respecvely.

2.5. Kinec models of PPO inacvaon

Four dierent kinec models were used to correlate the PPO inacvaon data: rst order kinec model, two fracon model, fraconal conversion model and the Weibull model. For each model, the decimal reducon me (D), dened as the treatment me needed for a 90% inacvaon of the inial enzyme acvity at a given condion was calculated.

The pressure and temperature increase needed for a 90% reducon of the D value, dened as Z_P (MPa) and Z_T (°C), respecvely (equaons 2 and 3) was also calculated. The values of Z_P and Z_T are obtained as the negave reciprocal slope of the regression line that represents the log D versus pressure and temperature relaonships, respecvely.

$$\frac{D_1}{D} \quad \frac{2 \quad 1}{Z} \qquad (2)$$

$$\frac{D_1}{D} \quad \frac{2 \quad 1}{Z} \qquad (3)$$

Finally two more parameters were calculated: in order to evaluate the kinec constant dependence on pressure the acvaon volume (V_a, cm³/mol) was rst calculated using the Eyring equaon (eq. 4). Secondly, in order to evaluate the kinec constant dependence on temperature the acvaon energy (E_a, kJ/mol) was calculated by means of the Arrhenius equaon (eq. 5). Both V_a and E_a were calculated from linear regression of *ln k* versus *P* or *(1/T)*, respecvely.

$$\frac{k_1}{k} \quad \frac{V_A}{}[P \quad P \qquad (4)$$

$$\frac{k_1}{k} \quad \frac{A}{} \frac{}{T} \quad - \qquad (5)$$

2.5.1. First order kinec model

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The rst order kinec model is commonly used to describe the inacvaon of enzymes. This model assumes that the logarithm of the residual acvity decreases linearly with me. Equaon 6 describes mathemacally this model:

(6)

A is the acvity of the enzyme aer treatment, A_0 is the inial acvity of the enzyme, *k* is the inacvaon rate constant (kinec constant) at given pressure and temperature condions and *t* is the me the enzyme is exposed to the pressure and temperature condions. From the kinec constant, it is possible to calculate the decimal reducon me (D).

This model is the most commonly used to describe the inacvaon of enzymes (PPO or PME) in juices. Several examples are summarized in Table 1.

2.5.2.Two-fracon kinec model

This model takes into account the existence of several isoenzymes of PPO, grouped into two fracons, a labile (L) and a stable (S) fracon. Both enzymes were considered to be inacvated according to a rst-order kinecs, but independently of each other [6]:

xp(− t (xp(t (7)

where A_L and A_S (A_S=1-A_L) are the acvity of the labile and stable fracons respecvely and k_L and k_S (min^{-1}) the inacvaon rate constants of both the labile and stable fracons respecvely. In general under mild condions only the labile fracon is inacvated, whereas the stable fracon remains unaltered [23].

2.5.3.Fraconal-conversion model

A fracon-conversion model is a special case of a rst order kinec model that takes into account the non-zero residual acvity aer prolonged heang and/or pressure (A) treatment. Mathemacally is expressed according to the equaon (8).

(xp(kt (8)

2.5.4.Weibull model

This empirical model is described in equaon 9 and uses two dierent parameters: α, the scale parameter (characterisc me) and β, the so-called shape parameter [24]. It can be noted that when β equals 1 the model results is a linear reducon of the residual acvity versus me, similar to a rst order kinec model. The Weibull model is basically a stascal model of distribuon of inacvaon mes, contrary to the rst order approach. The rst order model assumes that all the enzymes present are equal and hence all of them have the same resistance to CO_2 and that the length of me that an enzyme is exposed to the treatment has no eect on the probability that it will be inacvated in the next unit of me [25]. However, if there are several isoenzymes with dierent sensivies to the changes in the environmental condions, a distribuon of inacvaon mes appears as a consequence.

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$$—(-$$ (9)

Similarly to the decimal reducon me (D) calculated using other models, for the Weibull model is possible to calculate the me required to reduce the enzyme acvity by 90%, using the equaon (10). t_D is valid when it refers to the treatment me starng at zero me [26].

(10)

The dependence of the scale parameter (α) with the temperature can be well modelled using an exponenal relaonship [24]:

(11)

From equaon [11] it can be seen that Z_T -1/b_1 by denion considers the non-linear part of the curve log(A/A_0) versus me, compared to the classic denion of , that is calculated from the linear part of the curve. A similar discussion was done for the dependence of the scale parameter with the pressure, and it was represented log(α) versus pressure. The reverse of the slope was considered the Z_P
linear model represents the pressure increase to reduce by 90% the t_D value. In order to validate this calculaons the pressure and temperature dependence of the shape parameter (β) was evaluated, since there must be no dependence in order to be valid these Z_P $_T$ calculaons [24].

2.6. Stascal analysis

All the stascal analysis was done using the soware Statgraphics X64. This soware was used to perform the ANOVA test of the screening experiments and the non-linear regression of the experimental data to obtain the kinec constants of the dierent models presented in secon 2.5.1.

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3. Results and Discussion

3.1. Screening experiments

Results of the screening experiments are presented in Table 2. From the ANOVA study (Table 3) it can be concluded that the volume of enzyme soluon, pressure and temperature are the stascally signicant factors. Time resulted not to be stascally signicant in the range from 5 to 15 minutes; it seems that the major part of the inacvaon occurs within the rst 5 minutes of the experiment when pressures and temperatures are in the range 10 to 20 MPa and 35 to 45 °C, respecvely. Therefore, in the kinec study experiments, inacvaon mes shorter than 5 minutes will be used.

The srring rate resulted not to be stascally signicant; apparently the srring in our experimental set up is ecient, probably because of the magnec srrer placed in the reactor and the fact that CO_2 is bubbled directly into the enzyme soluon, which already provides good mixing enhancing the diusion of CO_2 in the enzyme soluon. In another work [14] similar condions of pressure and temperature, but without srring, led to inacvaon eciencies signicantly lower (around 25%), aer 20 minutes at 45 °C and 8 MPa (15 mL of sample loaded in a 850 mL reactor) or around 30% aer 20 minutes at 35 °C and 15 MPa, compared to the inacvaons higher than 80% obtained in our system (experiments 1 and 4) using signicantly lower amounts of CO_2. In a non-srred system, since water and supercrical CO_2 are barely miscible, two phases are formed, which limits the eect of CO_2 on the enzyme. The volume of enzyme soluon loaded in the reactor is a crical parameter, since it determines the free volume of the reactor that can be lled up with CO_2. This free volume will lead to a dierent mass of CO_2 used in the experiment, as a funcon of pressure and temperature. The rao CO_2:volume of soluon studied is in the range 0.7 g/mL (experiments 2 and 6) to 1.9 g/mL (experiments 3 and 7). Signicant dierences between the two CO_2:volume of enzyme soluon raos were observed, especially at the lowest pressure, indicang the importance of this experimental parameter and the need of performing a deeper study, as will be presented in secon 3.2.

3.2. Rao CO_2:volume of enzyme soluon

Results regarding the dierent raos CO_2: soluon of enzyme (g/mL) are presented in Table 4 and Figure 2, where the trend can be clearly seen.

It can be clearly seen that the amount of CO_2 used in the experiment aects dramacally the inacvaon performance. , the rao CO_2 versus the volume of enzyme soluon has not been systemacally studied in any paper in the literature. Not all the papers report the volume of enzyme soluon loaded into the reactor. For instance Manzocco et al. (2016) [5] used 10 mL of enzyme soluon in a 150 mL reactor (which led to raos in the range 9.8-12.6 g/mL depending on the pressure and temperature selected); Liu et al. (2010), [10] used red beet as a raw material loading 5 mL of sample in a 10 mL vial, which was placed in the 300 mL reactor. Gui et al. (2006) [4] and Zhang et al. (2010) [8] and Zhang et al. (2011) [19] used similar experimental procedures: 3 mL of sample in a 10 mL vial placed in a 200 mL reactor; 1 mL of sample in a 15 mL vial and placed in a 850 mL reactor, and 2 mL of sample

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loaded in a 10 mL vial and placed into a 850 mL reactor, respecvely. The amount of CO_2 used was not reported in any of these works, but it can be intuited that raos CO_2:volume of soluon are very high.

The results presented in this work reveal that raos CO_2:volume of enzyme soluon higher than 3 g/mL do not produce an improvement of the inacvaon eciency regardless the experimental condions selected (temperatures between 35 and 45 °C and pressures in the range 10 to 20 MPa). Nevertheless, when raos are below 3 g/mL the selected condions of pressure and temperature play a signicant role in the inacvaon process. It seems that there is a crical amount of CO_2 which determines the inacvaon performance, under that value the inacvaon performance strongly depends on the pressure and temperature and above it the addion of further amounts of CO_2 does not improve the results, being a waste of CO_2 from the economic point of view.

The addion of CO_2 to an aqueous soluon modies the physical properes of the soluon, and as a consequence the stability of the enzyme. The solubility of supercrical CO_2 in water is very low, 5.27g/100g of water, slightly increasing with pressure and decreasing with temperature [27]. In general, and according to Tegetmeier et al. [28], the density of water is increased (up to 5%, the higher the pressure the higher the density increase) as well as the volume, being expanded up to 6% (with pressures up to 30 MPa and 40 °C). These authors observed that the higher the pressure the higher the expansion, having the temperature a very lile eect. The reason behind these changes in the properes of the water is aributed to solvaon eects of the CO_2. This eect is more stressed the higher the pressure and the lower the temperature. According to Song et al. [29], this eect is dependent on the amount of CO_2 used. Therefore, the use of high amounts of CO_2 per mL of enzyme soluon might induce an expansion of the liquid. This eect has to be added to the other eects that the presence of CO_2 in water has on the enzyme such as the pH decrease as a consequence of carbonic acid formaon. The solubility of CO_2 is also aected by pressure and temperature, being higher the higher the pressure and the lower the temperature. The presence of salts in the medium tends to reduce the solubility of CO_2 at given condions of pressure and temperature [30]. To the

2

induces in phosphate buer soluons, but as it has been seen, the amount of CO_2 used per mL of enzyme soluon is a crical parameter. Only Saraiva et al. [31], pointed out the presence of a phosphate buer aects the thermostability and the kinec paerns inducing a stabilizing eect of the enzymes; therefore the eects of CO_2 will be dampen by the salt. The control of the pH in these systems is crical and will be studied in future works.

3.3. *Inacvaon kinecs*

In Table 5 it is possible to see the experimental results for the control experiments done at atmospheric pressure in the same temperature range as for the HPCD experiments. These experiments lasted up to 180 minutes. It is possible to see dierent trends: at 25 °C, an almost linear slow decay in the residual acvity was observed, but at higher temperatures a much faster decrease in the acvity was observed, tending to reach a minimum but constant residual acvity aer 180 minutes. The experimental results were ed to a two fracon model (data shown in table 6); the regression coecient was reasonably good at 25 °C (0.958) and better at

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35 and 45 °C (in both cases >0.991). The temperature sensivity parameter (Z_T=5.7±3.6 °C) and the acvaon energy (E_a, resulng 40.2±4.4 KJ/mol for the labile fracon and 296.3±48.9 KJ/mol for the stable fracon) were calculated for the inacvaon experiments done at atmospheric pressure.

Inacvaon data at atmospheric pressure were also ed to the Weibull Model: linear regression coecient was <0.98 in all cases, indicang a worse ng when using this model compared to the two fracon model. Weibull model led a Z_T value of 14.8±3.8 °C.

The addion of CO_2 signicantly changed the inacvaon paern of the PPO compared to the atmospheric pressure experiments. Inacvaon was much faster and, as can be seen in Figure 3, despite the temperature, all the inacvaon experiments had the same trend: an inial period with a very fast decay in the enzyme acvity (shorter than two minutes) followed by a slowed decrease period up to 15 minutes. At constant temperature higher inacvaon was observed the higher the pressure; while when increasing temperature, the inacvaon rate increased as well. All the experiments done at 20 MPa showed an almost complete inacvaon regardless the experimental temperature. Dierent models were used to study the kinec aspects of the experimental results obtained.

3.3.1. First Order kinec model

The obtained inacvaon results did not t into a rst order kinec model; the calculated R^2 were lower than 0.7 in all cases.

3.3.2. Two fracon model

The general appearance of the two fracon model is a sharp decrease in the enzyme acvity followed by a period of slower decay. This was observed in the experimental data presented in Figure 3. The experimental data ed well to the two fracon kinec model (Table 6). In general both fracons, labile and stable, were pressure and temperature sensive; higher pressures and temperatures led to much higher inacvaon constant rates, being the inacvaon constant of the stable fracon signicantly smaller than that of the labile fracon.
The inial acvity of the stable fracon (calculated as 1-A_L) decreased with pressure and temperature, probably due to the increased suscepbility of the enzyme to the HPCD treatment [6].

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When working at constant temperature, increases in pressure are translated into higher kinec rates. Pressure induces changes in the physical state of CO_2: at 25 °C density rises dramacally from 131.3 kg/m^3 at 5 MPa to 817.6 kg/m^3 at 10 MPa, changing the state from gas to liquid. Another small increase in the density up to 914.2 kg/m^3 is observed when increasing the pressure up to 20 MPa. At 45 °C density moves from 108.7 kg/m^3 at 5 MPa to 498.3 kg/m^3 at 10 MPa (from gas to supercrical state) and 812.7 kg/m^3 at 20 MPa. These increases in pressure also involve more CO_2 used per mL of enzyme soluon, which, as has been previously explained, has an important eect on the inacvaon of PPO. Nevertheless high pressure lead to higher density of the CO_2 and supercrical CO_2 is barely miscible with water. Regarding the dielectric constant, it slightly increases with pressure from 0.6 (at 5 MPa) to 1.6 (at 20 MPa), having the temperature a negligible eect. This increase in the dielectric constant might enhance its miscibility with water, whose dielectric constant decreases from a value of 78 at room temperature to approximately 72 at 45 °C. It is dicult to determine which of the above menoned processes happening prevails, considering the dampen eect of the phosphate salts used to prepare the enzyme soluon. Liu et al. (2010) [10], concluded that PPO and POD were less sensive to pressure changes under supercrical condions than under subcrical condions. The reported changes in the density can help to explain that behavior. An analysis of the pH at high pressures is necessary to understand the inacvaon dominant eect.

At constant pressure, kinetic constants increase when increasing temperature, which indicates thermal sensivity of the enzyme and lower solubility of CO_2. Increasing temperature decreases the density of the CO_2, which can lead to beer mixing with the enzyme soluon. On the other hand, the solubility of the CO_2 decreases with temperature; thus in this case the acidicaon of the media might not be the dominant mechanism considering the dampen eect of the phosphate buer used to dissolve the enzyme. At 20 MPa a similar behavior of both stable and labile fracon can be observed. Considering the experimental results and the errors associated to them, it seems that increasing temperature from 35 to 45 °C does not increase the inacvaon rate, however the change from 25 to 35 °C did increase the inacvaon rate. At 20 MPa when the working temperature is above the crical temperature of the CO_2 the inacvaon of tyrosinase is extremely fast; it is also observed that the inial acvity of the stable fracon ($1-A_L$) is very low which indicates the great suscepbility of the enzyme to pressure. The decimal reducon me for each temperature and pressure was calculated from equaon (7) and from those values the Z-parameters were calculated (Table 7). For these calculaons the simultaneous contribuon of both the labile and stable fracons to the total acvity was considered, as equaon 7 does.

The temperature sensivity parameter changed form 27.0±5.4 °C at 5 MPa to 40.7±0.1 °C at 20 MPa which indicates that the tyrosinase becomes less temperature sensive at higher pressures. At atmospheric pressure this value was only 5.7±3.6 °C, indicang that, at atmospheric condions, the inacvaon of the enzyme is very sensive to temperature, since only an increase in 5.7 °C would induce a reducon of 90% in the decimal reducon me, without the presence of CO_2. The values of Z_P changed slightly with temperature, from 6.9±0.6 to 7.9±1.1 MPa. Considering the experimental error it is not possible to stablish a clear trend; it seems that similar pressures are required to reduce the decimal reducon me regardless the temperature.

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3.3.2.1. Acvaon volume of tyrosinase soluons treated by HPCD

The pressure dependence of the kinec rate is represented by the Eyring equaon [equaon 4]. In general, and according to the transion state theory, the acvaon volume considers the dierence between the inial reactants and the acvated complex at the transion state.

The results of the acvaon volumes of both labile and stable fracons are shown in Table 8 and the logarithm of the kinec constants versus pressure in Figure 4.

It can be seen that acvaon volumes for the stable fracon are much lower than those for the labile fracon, proving the greater eect that pressure has on the stable fracon. At 25 and 35 °C (for both the labile and the stable fracon) acvaon volumes of the same order are obtained, considering the experimental error. By increasing temperature up to 45 °C the highest acvaon volume is obtained for the labile (-68.8±2.2 cm^3/mol) and the stable fracon (-131.3±64.2 cm^3/mol). At 45 °C the regression of the experimental data (lnK vs P) presents the poorest correlaon (R^2 around 0.81), probably due the fast inacvaon at the highest pressure. A similar trend was observed by Ma et al. [7] when using a commercial PPO and increasing temperature from 45 to 65 °C. A reacon that presents a posive acvaon volume tends to be slowed down with pressure [1]. More specically, when talking about enzymac reacons, high values of V_a (approaching to zero) indicate that the enzyme is less suscepble to CO_2 pressure [2]. This can be related to the calculated value of Z_P, which indicates that the higher the temperature the more pressure is required to decrease the decimal reducon me by 90%. The values of Z_P and V_a are in agreement and indicate that at

3.3.2.2. Acvaon energy (E_a) of tyrosinase soluons treated with HPCD

Figure 5 represents the dependence of the rate constant with temperature, according to the Arrhenius equaon. E_a from the slopes of Figure 5 have been evaluated revealing a good linear correlaon at 5 and 10 MPa; however, at 20 MPa, a poor regression was observed. At that pressure a very fast and complete inacvaon of the labile fracon happened, even regardless the temperature. It would have been useful to have done an experiment at me shorter than 2 minutes.

The E_a found for the labile fracon of the PPO thermally treated at atmospheric pressure was 40.2±4.4 KJ/mol, decreasing down to 13.4±0.8 KJ/mol at 10 MPa, a value that was similar to that obtained at 20 MPa (16.6±8.2 KJ/mol).

The values of E_a for the stable fracon are higher than those observed for the labile fracon: from 296 KJ/mol at atmospheric pressure to 33 KJ/mol at 10 MPa, this fact reveals that the stable fracon is less temperature sensive, as expected. At 20 MPa the acvaon energy of the stable fracon could not be calculated due to the fast inacvaon rate which leads to the presence of a very small amount of stable fraction or to the experimental error.

Based on the values of Z_T and E_a, it can be clearly seen how the addion of supercrical CO_2 tends to reduce the acvaon energy and increase Z_T parameter. This indicates that CO_2 accelerates the inacvaon of tyrosinase, decreasing its sensivity to temperature changes. These results are in agreement with those published by Liu et al. (2010)[10] for red beet and by Gui et al. [18] for apple juice.

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3.3.3. Fraconal Conversion Model

Although this model tted the experimental results quite well it predicted a fast decrease in the acvity of the enzyme to reach a minimum value that remains constant with the me; which was not actually seen experimentally. Therefore the fraconal conversion model was not discussed.

3.3.4. Weibull Model

The Weibull model together with the two fracon model ed best the experimental results obtained using HPCD. From the coecients of the model, the me needed to reduce the acvity of the enzyme by 90% as well as the pseudo decimal reducon parameters (Z_T ... $_P$ were calculated and presented in Table 9 and Table 10. Prior to these calculacons it was studied the dependence of the β parameter with both temperature and pressure. It was observed that the β parameter was not aected by pressure or temperature (when tested at the 95% signicance level for a linear relaonship).

The results from the experiments done at atmospheric pressure (Table 5) were also ed to the Weibull Model. The data did not t as well as they did to the two fracon model (linear regression coecient was <0.98 in all the cases). At atmospheric condions Z_T had a value of 14.8±3.4 °C calculated using the Weibull model, whereas the two fracon model had led to a Z_T value of 5.7±3.6 °C.

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It can be seen that the calculated parameters were in the same order of magnitude than those calculated for the two fracon model, but the trends are opposite: according to the Weibull model, higher temperatures make the enzyme more sensive to pressure changes, since lower pressures are required to reduce the Z_P $_T$ parameter, the higher the pressure the lower the Z_T parameter, which indicates that the enzyme is more sensive to the temperature. Right the opposite was obtained when using the Two Fracon model. Therefore it can be seen that the kinec parameters strongly depend on the model used to calculate them, being dicult to compare the results presented by other authors [2]. The model used to describe the experimental results is crical, since dierences in the D or $_D$ values can lead to underprocessing or overprocessing of the sample. However, it is possible to compare trends: Marszalek et al.[7], obtained similar trends for both parameters, but the absolute values of the parameters calculated resulted to be signicantly higher, probably because they used much broader pressure (10-60 MPa) and temperature (35-65 °C) ranges: Z_P at 35 °C was 268.5 MPa and 214.3 MPa at 45 °C and Z_T was 94.2 °C at 10 MPa; Manzocco et al. (2016) [2], did not calculate the Z_T parameter, but Z_P resulted 5.4 and 5.5 MPa at 20 and 35 °C, respecvely, using a rst order kinec model. Other authors who calculated these parameters for the nave PPO present in fruits obtained quite similar results: Gui et al. [18], used apple juice and obtained a Z_T value of 108 °C at 30 MPa, compared to the 27 °C obtained at atmospheric pressure (the experimental temperatures were in the range 35-55 °C); Manzocco et al. (2016) [5], when using apple as raw material reported values of Z_P decreasing with the temperature, just the opposite other authors have done. Zhang et al. (2011) [19], reported a value of 49 MPa for the Z_P parameters, when running the experiments at 37 °C and in the range 4-15 MPa. All the authors menoned in the last paragraph used rst order models to describe the experimental results obtained for polyphenol oxidase inacvaon. Liu et al. (2013) [3], calculated the Z-parameters using a two fracon model for the inacvaon of PPO from watermelon juice: Z_T was 44.5 °C for the labile fracon (at 30 MPa) and Z_P was 22.8 MPa for the labile fracon (at 50 °C). The observed variability in the results probes that the inacvaon kinecs of PPO strongly depends on the process parameters (temperature, pressure and rao CO_2 to volume of enzyme soluon, among others) but also on the source of the enzyme (either commercial or nave, meaning present in fruits and the way it is linked to other molecules) and on the kinec model used to represent the experimental data.

It is important to note that, according to Van Boekel et al. [24] it is not possible to calculate neither the acvaon energy nor the volume of acvaon from the pseudo-kinec parameters obtained from the Weibull model, since the calculated values do not reect the acvaon energy of a single reacon.

3.4. Conformaonal changes in the PPO enzyme

Fluorescence spectroscopy was used to characterize the terary structure of the tyrosinase used in this work and the changes induced in the structure as a consequence of the HPCD. Fluorescence spectroscopy uses the changes in the intrinsic uorescence of some residues (tryptophan TRP-, tyrosine TYR- and phenylalanine PHE-) to evaluate changes in the protein structure [32]. Tryptophan is part of the hydrophobic core of the enzyme and its emission

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wavelength is very sensive to the polarity of its surrounding environment: if the environment is non-polar the maximum tends to be around 305 nm, red shiing up to 350 nm when it is exposed to very polar media [33]. When the TRP residue is exposed to a polar solvent, part of the absorbed energy is transferred to the solvent (water, if it is in aqueous soluon), resulng in a higher emission wave length (340-350 nm) and lower intensity of the emission signal (quenching eect of water); these two phenomena are indicang changes in the structure of the protein [32]. The changes in the emission intensity can be explained by the fact that the TRP residues suer a reallocaon in the enzyme structure [34].

In Figures 6 the uorescence spectra of the nave tyrosinase (PPO) and high pressure carbon dioxide treated PPO are shown. Signicant changes in the emission spectra, compared to the nave enzyme can be observed.

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Figure 6-a shows the emission spectra for samples treated with CO_2 in gas state (5 MPa) at 25 and 45 °C, for 15 minutes (these experiments resulted in residual activities around 60 and 35%, respectively). Regarding the maximum wave length of emission, it can be observed that a red shift displacement up to 331 nm happened in both cases (from 324 nm in the native enzyme), together with significant decreases in the emission intensity. Under those conditions of pressure and temperature, CO_2 in gas state was bubbled into the samples; CO_2 rapidly diffused into the enzyme solution probably contributing to a relatively rapid decrease in pH (presence of carbonic acid), which can open the core on the enzyme exposing the TRP residues to the polar environment [17] that receives part of the energy of the TRP residues (water exert a quenching effect), resulting in a decrease of the emission intensity.

In Figure 6-b, the emission spectra for samples treated at 10 MPa revealed emission intensities quite similar to those of the native enzyme, and much higher than those observed in the samples treated at 5 MPa. At 10 MPa and 25 °C, CO_2 is in liquid state (density is 817.6 kg/m^3) and at 45 °C is in supercritical state (density 498.3 kg/m^3). At 45 °C, CO_2 is less dense and has better transport properties (diffusion), and a clear red shift of the maximum intensity is observed (up to 333.5 nm), higher than that registered at 25 °C (329.5 nm). In these experiments, higher amounts of CO_2 were used, therefore less energy from the TRP residues was transferred to the water which resulted in a mitigated quenching effect.

Finally Figure 6-c shows the emission spectra when CO_2 at 20 MPa was used to treat the tyrosinase. Residual activity after the 25 °C treatment was 7.2% and it was 3.7% when temperature was 45 °C. In both cases it is possible to talk about a complete inactivation of the enzyme. CO_2 under both conditions exhibits a similar density: 914.2kg/m^3 at 25 °C and 812.7kg/m^3 at 45 °C, but the state is different, being liquid in the former and supercritical in the latter conditions. In both cases the intensity registered is greater than that of the native enzyme; which indicates that the structure of the enzyme has changed and the quenching effects observed at 5 MPa have disappeared. These are the experiments in which more CO_2 was used (around 44 g per experiment versus 6 grams used at 5 MPa); the maximum intensity is now 322.5 nm (at 25 °C) and 322 nm at 45 °C, lower than the native enzyme, which indicates that TRP is oriented to a more non-polar environment, and hence water quenching effect is lower. Another effect must be considered, it is the effect that CO_2 exerts on the water: at high pressures and temperatures the density of the water exposed to CO_2 tends to increase by 20% and the volume is expanded by 5% due to solvation effects of the CO_2 molecule [28], being this changes proportional to the amount of CO_2 used [29]. The expansion that CO_2 produces in water affects the way the enzyme interacts with the media, leading to a much faster inactivation. According to the equation presented by Eltringham et al. [20], the dielectric constant of the CO_2 molecule slightly changes from 0.6 (5 MPa, 25 °C) to 1.6 (20 MPa, 45 °C), whereas the dielectric constant of water decreases from 78.6 (5 MPa, 25 °C) to 72.2 (20 MPa, 45 °C). CO_2 tends to be slightly more polar when increasing pressure and temperature, whereas water behaves in the opposite way. This fact can alter the way TRP residues interact with the surrounding environment, their allocation in the structure, affecting the activity of the enzyme. It would be very useful to have information about the dielectric constant of the

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water-CO_2 mixture under dierent condions; this informaon for sure would shed light on the TRP residues and enzyme structure.

In the results presented in Figure 6-c, a second peak appeared at around 300 nm, which is primarily assigned to the tyrosine residues [32]. In the nave state of the PPO there is not emission of the tyrosine when excited at 275 nm, probably due to an ecient energy transference from the tyrosine residues to the tryptophan residues. If a peak is observed aer the enzyme being treated with CO_2, this is indicang that the mechanism by which the energy was transferred for TYR to TRP might be broken. In any case the presence of this peak at around 300 nm is probing that the PPO structure is changed aer the CO_2 treatment, which is related to the dramac reducon in the PPO acvity observed.

4. Conclusions

CO_2 was successfully used to inacvate tyrosinase isolated from mushroom; almost complete inacvaons were achieved when the enzyme soluon was subjected to pressures as high as 20 MPa for less than 9 minutes with independence of the temperature used (25-45 °C). The amount of CO_2 used per unit of enzyme soluon resulted to be crical in the inacvaon performance. The experimental results were well described by the Two Fracon model and the Weibull model. The reason is to be found in the nature of those models: they gather an inial fast acvity decay followed by a slowed decrease in the residual acvity, which is the trend experimentally observed. The fraconal conversion model did not describe the experimental results as well as the other aforemenoned models because it assumed a constant residual acvity aer an inial period of fast decay.

CO_2 induced signicant changes in the structure of the enzyme, as probed the espectrouorimetry analysis: the extent of the change in the structure was dependent on the temperature and pressure used (which determined the state of the CO_2, either gas, liquid or supercrical). All in all HPCD resulted to be a useful technique to inacvate enzymes and this work contributed to shed light on the structural changes that HPCD treatment induced on the structure of the enzyme, as well as to demonstrate that the amount of CO_2 used in the inacvaon process is crical and must be carefully selected in order to avoid excessive CO_2 consumpon, what would aect the economic performance of the HPCD process.

5. Acknowledgements

To the Spanish Government (MINECO) and the European Regional Development Fund (ERDF) for nancial support of project CTQ2015-64396-R (MINECO/FEDER, UE). To Junta de Caslla y León and ERDF for O.Benito -doctoral contract funded by project BU055U16. To MINECO Cierva Contract (FJCI-2014-19850).

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Figure capons

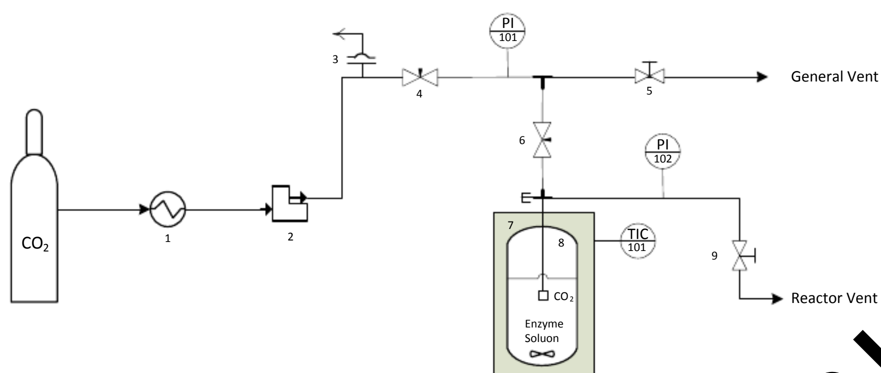

Figure 1. Experimental set-up for HPCD enzyme treatment. 1: CO_2 cryostat; 2: Syringe pump; 3: bursng disk; 4: general process valve; 5: general vent valve; 6: individual inlet valve; 7: water bath; 8: high pressure batch reactor with magnec srring; 9: depressurizaon valve. PI-101: general pressure gauge; PI-102: reactor pressure gauge; TIC-101 temperature controller of water bath

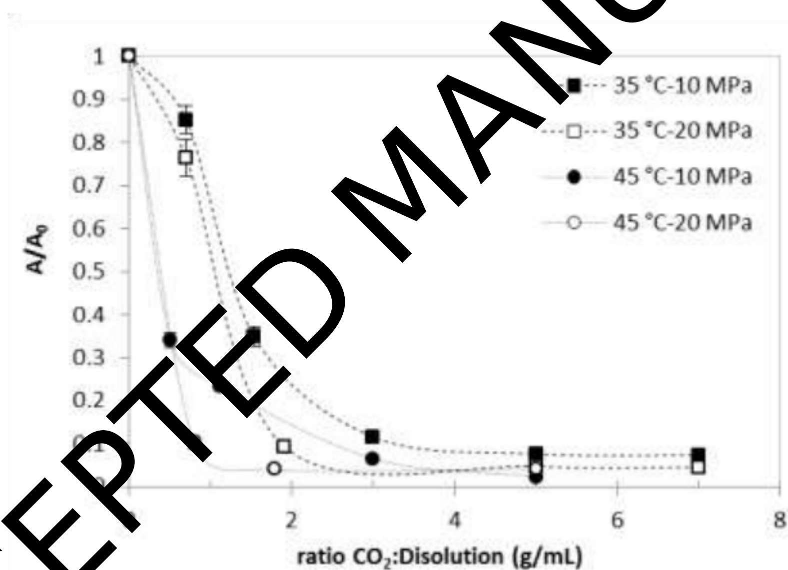

Figure 2. Residual acvity of PPO treated for 9 minutes at 35 and 45 °C and 10 and 20 MPa using dierent raos CO_2:volume of enzyme soluon.

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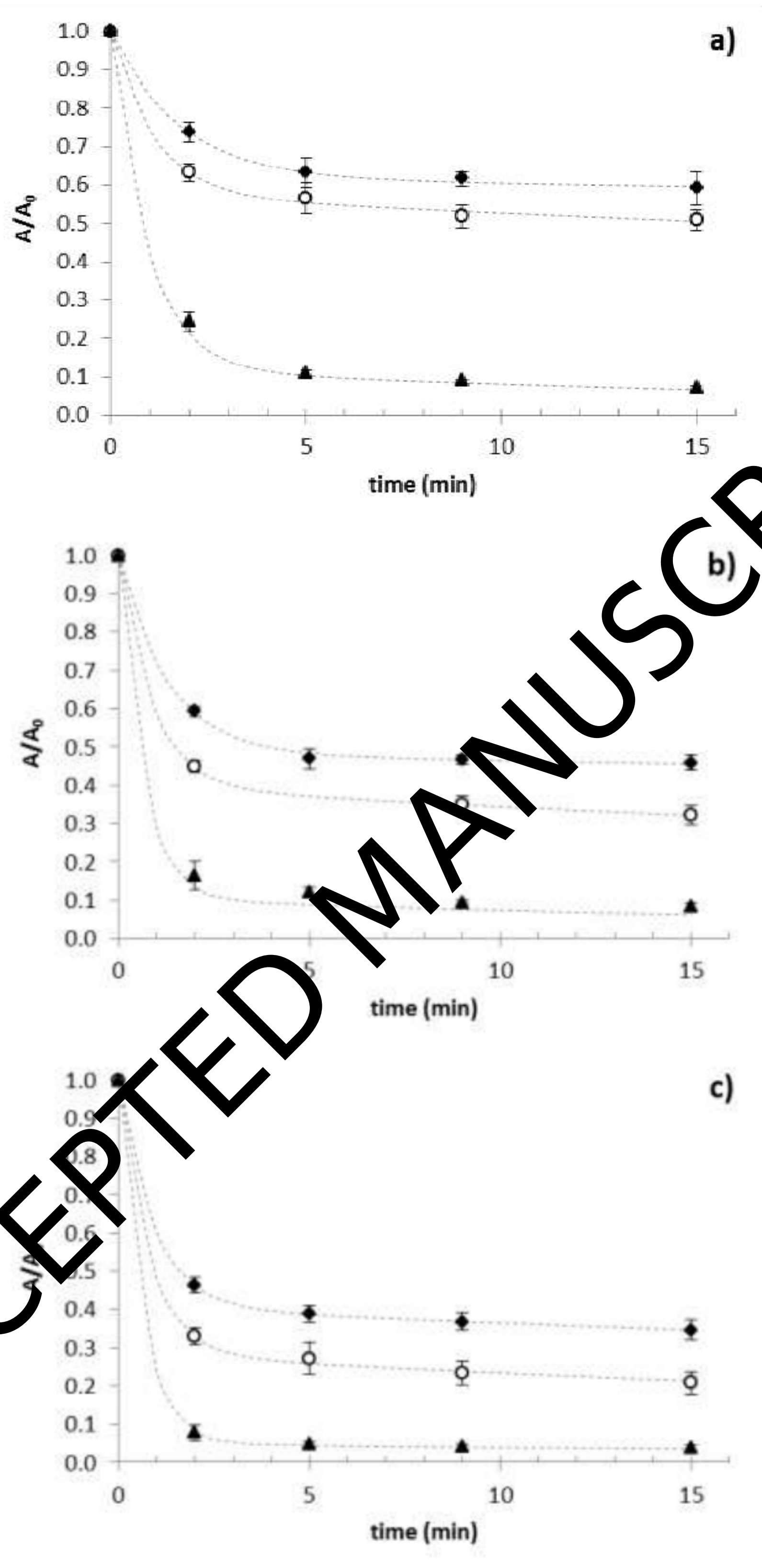

Figure 3. Polyphenoloxidase residual acvity as a funcon of the exposure me to HPCD at three dierent pressures: 5 MPa ( ), 10 MPa ( ) and 20 MPa ( ). In Figure a) experiments were done at 25 °C; in b) at 35 °C and in c) at 45 °C

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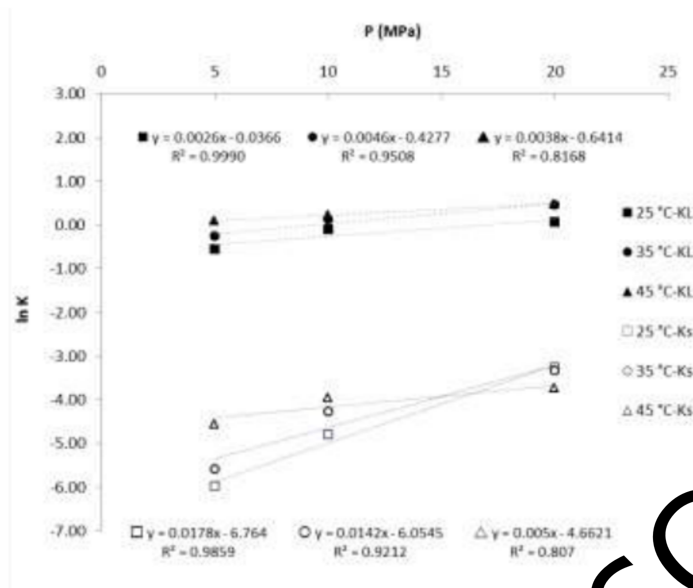

Figure 4. Eect of the pressure on the kinec constant for both labile (K_L) and stable (K_S) fracon of the tyrosinase enzyme treated with HPCD

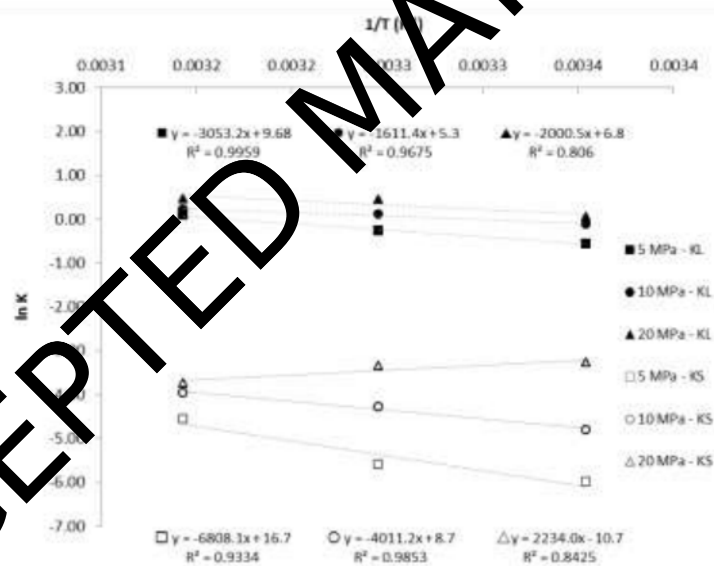

Figure 5. Eect of the temperature on the kinec constant for both labile and stable fracon of the tyrosinase enzyme treated with HPCD

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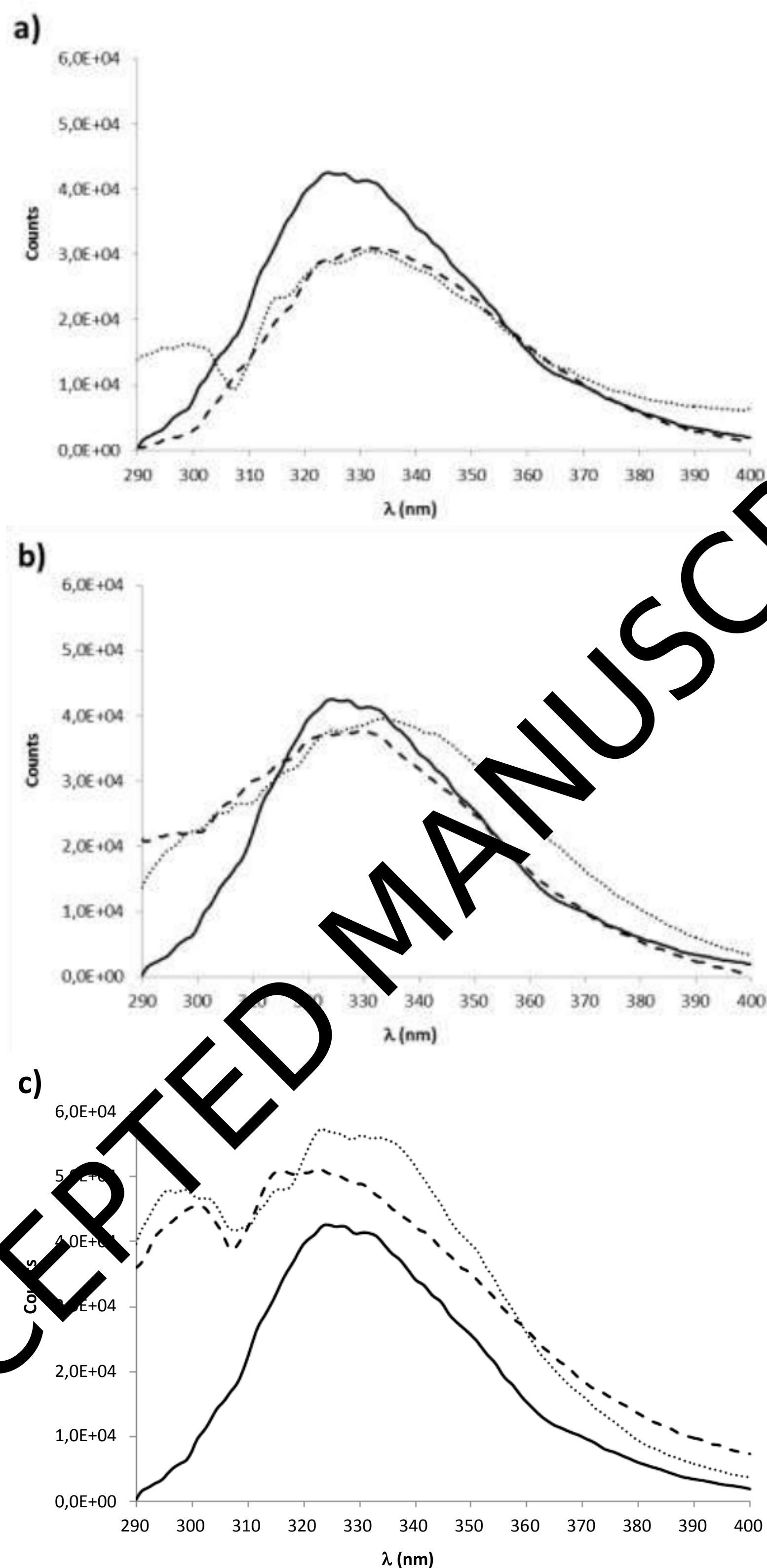

Figure 6. Emission spectra for nave tyrosinase (solid line), treated at 25 °C (dashed line - - -) and at 45 °C (doed line · · ·) at three dierent pressures: a) 5 MPa; b) 10 MPa; c) 20 MPa

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Table 1. Experimental condions and kinec parameters obtained by oth er researchers using HPCD inacvate PPO from dierent substrates

	Experimental conditions					Kinec Parameters					
Authors	rao CO_2:dis	Raw material	P (MPa)	T (°C)	t (min)	Model	D (min)	Z_P (MPa)	Z_T (°C)	V_a (cm^3/mol)	E_a (kJ/mol)
Manzocco et al. 2016 [5]	10 mL in a 150 mL reactor	Tyrosinase (EC 1.14.18.1)	6-18	20	Up to 15min	1st order	18.2-0.6	5.4	-	-1034.1	-
			6-18	35	Up to 15min	1st order	3.7-<0.2	5.5	-	-1081.1	-
Liu et al. 2010 [10]	5 mL in a 10 mL tubes. Placed in a 300 mL reactor	Red Beet juice	37.5	35-55	60	1st order	138.9-28.8	-	36.1	-	53.6
			1.5-7.5	55	60	1st order	75.8-50.5	34.1	-	-184.3	-
			15-37.5	55	60	1st order	41.5-38.8	125.0	-	-50.2	-
Gui et al. 2007 [18]	3 mL in a 10 mL tubes. Placed in a 200 mL reactor	Apple juice (*fuji*)	30	35-45	60	1st order	222.2- 166.7	-	108.0	-	18.0
			8-30	55	60	1st order	312.5-144.9	66.7	-	-94.3	-
Zhang et al. 2010 [8]	1 mL in a 15 mL tube. Placed in a 850 mL reactor	Peach juice	8	35-45	120	1st order	151.4 -19.8	-	22.6	-	82.9
			5-15	35	120	1st order	330.0 -183.5	20.2	-	292.3	-
		Carrot juice	8	25-45	120	Fraconal Conversion	1.4-0.4	-	55.3	-	34.1
			5-15	35	120	Fraconal Conversion	2.4-0.4	12.2	-	-485.0	-

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Zhang et al. 2011 [19]	2 mL in a 10 mL vial.	Shrimp (extract)	4-15	37	50	1st order	48.8 -29.0	49.0		-120.9	
			20-25	35	50	1st order	20.5 5.9	-			
			20	35	50	Two Fracon	D_L=0.94 D_S=29.4	-	-	-	-
			25				D_L=1.66 D_S=34.5	-	-	-	-
Manzocco et al. 2016 [5]	10 mL in a 150 mL reactor	Apple juice (*golden*)	0.1-18	20	30*	1st order	188.8 -22.1	20.3	-	-276.7	-
			0.1-18	35	30*	1st order	75.1 8.1	19.2	-	-307.9	-
			0.1-12	45	30*	1st order	21.5 2.4	14.8	-	-412.3	-
Liu et al. 2013 [3]	100 mL in a 850 mL reactor	Watermelon juice	30	30-50	30	Two Fracon	D_L=3.3-1.2 D_S=135.5-56.2	-	Z_{TL}: 44.5 Z_{TS}: 52.6	-	E_{aL}: 43.2 E_{aS}: 36.9
			8-30	50	30	Two Fracon	D_L=11.6-1.2 D_S=575.8-56.2	Z_{PL}: 22.8 Z_{PS}: 20.1	-	V_{aL}: -271.7 V_{aS}: -307.9	-
Marszałek et al. 2016 [12]	60 mL in a 500 mL reactor	Carrot and celery juice	10-60	31-55	10-30	1st order	Carrot: 1354 (at 47 °C-10 MPa) Celery: 767 (at 55 °C-60 MPa)	-	-	-	-
al. 2017 [11]	60 mL in a 500 mL reactor	Beetroot juice	10-60	31-55	10-30	1st order	281 (at 39 °C-10 MPa)	-	-	-	-

(for the 1st order model does not consider longest mes)

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Table 2. Experimental results for the inial screening experiments

run	A: Soluon Volume (mL)	B: Pressure (MPa)	C: me (min)	D: Temperature (°C)	E: Srring Rate (rpm)	A/A_0
1	25	10	15	45	250	0.21±0.03
2	40	10	15	35	600	0.57±0.05
3	25	20	15	35	250	0.08±0.01
4	40	20	15	45	600	0.10±0.01
5	25	10	5	45	600	0.27±0.04
6	40	10	5	35	250	0.85±0.03
7	25	20	5	35	600	0.12±0.01
8	40	20	5	45	250	0.17±0.02

Table 3. ANOVA table for the inial screening experiments

Source	SS	DF	Mean Square	F-Rao	p-Value
A: Soluon Volume	974.28	1	974.28	161.71	0.0061
B: Pressure	1546.28	1	1546.28	256.64	0.0039
C: me	4.93921	1	4.93921	0.82	0.4608
D: Temperature	733.602	1	733.602	121.76	0.0081
E: Srring Rate	54.0321	1	54.0321	8.97	0.0958
B*C	59.8761	1	59.8761	9.94	0.0876
B*E	86.6916	1	86.6916	14.39	0.063
Total error	12.05	2	6.025		
Total (corr.)	5463.[illegible]	9			

**p-Value stascal signicance*

Table 4. Experimental results for the rao CO_2: volume of soluon at dierent temperatures and pressures in experiments lasng 9 minutes

Pressure (MPa)	Temperature (°C)	Density CO_2 (kg/m^3)	rao CO_2: Enzyme Soluon (g/mL)	Enzyme Soluon (mL)	A/A_0
10	35	712.8	0.7	40.0	0.85±0.03
			1.5	25.0	0.35±0.02
			3.0	15.1	0.12±0.01
			7.0	7.3	0.07±0.01
20	35	865.7	0.7	44.2	0.76±0.04
			1.9	25.0	0.09±0.01
			5.0	11.8	0.05±0.01
			7.0	8.8	0.05±0.01
10	45	498.3	0.5	40.0	0.34±0.02
			1.1	25.0	0.24±0.01
			3.0	11.6	0.07±0.01
			5.0	7.4	0.02±0.00

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20	45	812.7	0.8	40.0	0.10±0.02
			1.8	25.0	0.04±0.01
			5.0	11.2	0.04±0.02

Table 5. Residual acvity for the control experiments, done at atmospheric pressure without CO_2

	Residual Acvity (A/A_0)		
T(°C) / t (min)	**25**	**35**	**45**
0	1	1	1
30	0.96±0.04	0.78±0.05	0.55±0.01
60	0.87±0.01	0.68±0.08	0.44±0.01
90	0.82±0.03	0.56±0.04	0.31±0.02
140	0.82±0.01	0.42±0.03	0.28±0.01
180	0.79±0.01	0.41±0.04	0.26±0.02

Table 6. Two fracon model kinec constants for commercial tyrosinase (PPO) treated with HPCD. Results for the experiments done at atmospheric pressure (without CO_2) are also presented in this table.

T (°C)	P (MPa)	A_L	K_L (min^{-1})	[illegible] (min^{-1})	R^2
25	**0.1**	0.247±0.139	0.011±0.[illegible]51	0.0±0.0	0.957
	5	0.381±0.021	[illegible]574±[illegible]063	0.002±0.003	0.999
	10	0.427±0.024	0.89[illegible]±0.147	0.008±0.004	0.998
	20	0.879±0.000	1.065±0.000	0.038±0.000	1.000
35	**0.1**	0.58[illegible]±0.207	0.013±0.016	0.001±0.001	0.991
	5	0.[illegible]18±0.0[illegible]3	0.772±0.045	0.004±0.003	0.999
	10	0.604±[illegible]000	1.128±0.000	0.014±0.000	1.000
	20	0.89[illegible]±0.019	1.582±0.169	0.036±0.016	1.000
4[illegible]	**[illegible]**	0.704±0.114	0.031±0.010	0.001±0.002	0.994
	5	0.595±0.001	1.093±0.007	0.011±0.000	1.000
	10	0.716±0.016	1.259±0.138	0.019±0.006	1.000
	20	0.951±0.002	1.618±0.029	0.024±0.003	1.000

Table [illegible]. Decimal reducon me and Z-values of commercial tyrosinase treated with HPCD and calculated using the two fracon model

	D (min)			
T(°C) / P(MPa)	25 °C	35 °C	45 °C	**Z_T (°C)**
5	733.1	418.6	133.0	27.0±5.4
10	211.9	98.7	54.5	33.9±2.4
20	5.5	3.1	1.8	40.7±0.1

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Z_P (MPa)	6.9±0.6	7.0±0.3	7.9±1.1	

45 °C the inacvaon of PPO is being less aected by pressure and more by temperature.

Table 8. Inacvaon kinec constants, acvaon volume and acvaon energy for tyrosinase treated with HPCD

	Labile fracon				Stable fracon			
	K_L values (min^{-1})				K_S values (min^{-1})			
P (MPa)	25 °C	35 °C	45 °C	E_a (KJ/mol)	25 °C	35 °C	45 °C	E_a (KJ/mol)
5	0.574	0.772	1.093	25.4±1.6	0.002	0.004	0.011	56.6±15.1
10	0.898	1.128	1.259	13.4±0.8	0.008	0.014	0.01[illegible]	33.4±3.8
20	1.065	1.582	1.618	16.6±8.2	0.038	0.036	0.0[illegible]4	----
V_a (cm^3/mol)	-93.8±44.4	-117.4±26.7	-68.8±2.2	-	-441.4±52.8	-364.0±1[illegible]6.5	-131.3±64.2	-

Table 9. Weibull model coecients calculated for commercial tyrosinase treated with HPCD

T (°C)	P (MPa)	β	α (min)	R^2
25	0.1	0.599±0.166	1[illegible]68.9±1438.0	0.918
	5	0.239±0.051	192.7±134.7	0.984
	10	0.2[illegible]±0.0[illegible]1	52.7±0.2	0.996
	20	0.27[illegible]±0.[illegible]54	0.4±0.2	0.984
35	0.1	0.740±0.087	195.0±13.6	0.982
	5	0.171±0.061	49.1±35.7	0.975
	10	0.173±0.001	7.0±0.1	1.000
	20	0.161±0.001	0.05±0.00	0.999
45	0.1	0.428±0.059	82.5±6.9	0.983
	5	0.152±0.020	9.2±0.9	0.997
	10	0.172±0.002	1.1±0.1	1.000
	20	0.122±0.020	0.0007±0.0001	0.997

Table 10 -values of commercial tyrosinase treated with HPCD and calculated with the Weibull model

	t_D (min)			
P (MPa) \ T(°C)	25 °C	35 °C	45 °C	Z_T (°C)
5	6295.0	6474.7	2236.8	15.2±0.9
10	1721.6	863.6	136.9	11.8±0.3
20	8.1	8.6	0.7	7.4±1.4
Z_P (MPa)	5.0±0.8	5.2±0.2	4.3±0.1	