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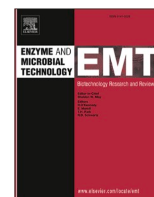

Effect of pH on pulsed light inactivation of polyphenol oxidase

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ABSTRACT

The inactivation of diverse food enzymes by pulsed light (PL) has been described before, including the inactivation of polyphenol oxidase (PPO) (at pH 6.5). Since the pH affects the conformation of enzymes, it may influence the inactivation of enzymes by PL. The aim of this work was to evaluate the effect of pH on the kinetics of the PL-inactivation and associated structural changes of a case enzyme. To this, PPO was treated by PL at different pHs (4.0–6.5) and its inactivation kinetics and changes in its structure were evaluated by spectrophotometric and spectrofluorometric methods. The inactivation proceeded faster at low pH and was highly correlated with the decrease in peak intrinsic fluorescence intensity. Phase diagrams and parameter A evolution indicated the absence of intermediate unfolded states during the course of the inactivation. No protein aggregation was detected by turbidimetry. Results indicate that although a low pH favors the PL-inactivation of PPO, the mechanism of inactivation is pH-independent. Beyond the specific outcome for PPO, the results are evidence of a general pH-independence in the mechanism of enzyme inactivation by PL in the pH range 4.0–6.5 and acidification can be a strategy to decrease treatment times during PL processing.

1. Introduction

Pulsed light (PL) is a non-thermal technology that uses high-power pulses of wide-spectrum light including a significant UV-C portion [1]. The use of PL technology for the inactivation of food enzymes is an active research field that emerged in the decade of 2010 and extended the scope of application of PL beyond its traditional field of microbial inactivation. The first study focused on the inactivation of enzymes and associated structural changes was published by Manzocco et al. (2013) [2]. This application of pulsed light had been already discussed in the patents that pioneered the development of this technology in the Western hemisphere [3]. We and others have previously reported on the inactivation of different food enzymes by PL and its effect on enzyme structure [4–9].

Different factors can affect the inactivation of enzymes by PL. Besides those factors that affect the transmission of light, matrix composition can affect the efficacy of this technology by promoting or arresting the inactivation. It has been demonstrated that enzyme concentration affects the kinetics of inactivation of polyphenol oxidase (PPO). The inactivation gets slower with the increase of enzyme concentration. Results of HPLC-gel permeation analysis has shown that a low concentration of PPO gives place to a more abundant tetrameric structure upon PL inactivation, whereas the dimeric structure is predominant at high PPO concentration. The authors hypothesized that the finding could be a consequence of the crowding effect [2], which refers to the different behavior of the enzyme as response to inactivation factors depending on its concentration, or that of other components of the suspension, due to steric exclusion [10]. As far as we know, there is no other report about the effect of this or other factors on enzyme inactivation by PL and associated structural changes.

It has been shown that the pH affects the structure of different food enzymes [11–13] and its rate of inactivation by different physical methods such as ultrasound [14] and heat [15]. But the effect of pH on pulsed light enzyme inactivation is unknown to the best of our knowledge.

pH influences the ionization of aminoacids, which affects in turn their susceptibility to photoionization [16]. Moreover, enzymes occur with different levels of folding depending on pH [11], which may affect their susceptibility to different treatments. This can lead to a higher proneness to inactivation by further unfolding and different tendencies to cross-linking or internal rearrangements as consequence of the exposure of hydrophobic aminoacids originally hidden in the core of the native protein.

Polyphenol oxidase (PPO) (E.C. 1.14.18.1) was selected as case

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enzyme because it is one of the most important enzymes related to food quality. It catalyzes the oxidation of phenols leading to browning. Its activity is generally undesirable because it is associated with the loss of quality of fruits, vegetables, mushrooms and crustaceans. The native enzyme is a tetramer with a molecular mass of 120 kDa composed by two kind of subunits, one of 43 kDa and ~392 residues called subunit H and another of 14 kDa and ~150 residues called subunit L. The active site is located in the H subunit and it is made of a bundle of four helices with two copper ions in which three histidine residues coordinate each copper ion [17]; which are His^{61}, His^{85} and His^{94} of the subunit A and His^{259}, His^{296} and His^{263} of the subunit B [11]. It is known that pH affects both, the secondary and tertiary structure of PPO from *Agaricus bisporus* [15]. The proportion of α-helix decreases from 38 to 28 % and the amount of β-sheet structure increases from 17 to 21 % when the pH changes from 6.8 to 3.0. Likewise, the intensity of the intrinsic fluorescence decreases when the pH varies from 6.8 to 4.0, then increases at pH 3.0 [15]. Previously, similar effects have been reported when PPO was exposed to malic acid [14]. Furthermore, a model of the fine structure of PPO has revealed the unfolding of the native enzyme at acidic pH causing exposure of its hydrophobic residues [11].

Among the different analytical techniques available to study the structure and unfolding of proteins, tryptophan fluorescence spectroscopy is one of the most interesting. Besides the analysis of the spectra itself (position, shape and intensity), a big amount of information can be derived from simple fluorometric measurements by using diverse analysis techniques, mainly used in biomedical sciences, such as spectral shifts, phase diagrams [18], parameter A [19] and spectral center of mass [20]. Some of them are sometimes redundant and useful to confirm trends.

Therefore, the aim of this work was to provide insight on the effect of pH on the inactivation by pulsed light of a case enzyme, namely PPO, using spectrophotometric and spectrofluorometric methods.

2. Materials and methods

2.1. Chemicals and reagents

Mushroom polyphenol oxidase from *Agaricus bisporus* (T3824, Sigma-Aldrich) was employed in this study. The rest of analytical reagents were purchased from Sigma. The enzyme solution was prepared at a concentration of 0.24 mg/mL in 0.1 M phosphate buffer pH 6.5. The samples were stored at − 20 °C to preserve the enzymatic activity throughout the different determinations and to minimize potential batch-to-batch differences given the existence of enzyme concentration effects on the inactivation by PL [2].

2.2. Pulsed light system

A bench-top PL system (XeMaticA-Basic-1 L system, Steribeam, Germany) was used in all experiments. The system was worked at 2.5 kV, which generates a characteristic emission spectrum reported before [21]. The fluence impinging on the sample surface was 2.14 J/cm^2 and was determined by analysis of in-built photodiode readings using a PC-Lab 2000 L T PC oscilloscope (Velleman Instruments, Gavere, Belgium), and manufacturer performance charts. Different fluences were generated by increasing the number of pulses.

2.3. Experimental procedure

Before each experiment, 6 mL of enzyme solution were suspended in 14 mL acetate buffer (pH 3.5 and 5.0) or phosphate buffer (pH 6.5) and allowed to interact at room temperature for 30 min, yielding final pH's of 4.0, 5.3 and 6.5 respectively. After this period of time, the Petri dish containing 20 mL of PPO solution was put on a stirrer just below the center of the lamp, which was only activated between pulses. This volume (4 mm height) was selected as the minimum required to fully covering the plate bottom. The distance between the lamp and the sample surface was 6.7 cm.

2.4. Enzymatic activity

PPO activity was measured at 400 nm in a standard reaction mixture containing: 700 μL of buffer 0.1 M (pHs 3.5, 5.0 or 6.5), 200 μL of the enzyme solution and 100 μL of 4-tertbutylcatechol (TBC) 2.5 mM [22]. The enzymatic activity was measured at 0, 5, 10, 15, 20, 30, 40, 50 and 60 light pulses. PPO activity was calculated from the slope of the inactivation curve and described according to a first-order kinetics as indicated in Eq. 1:

$$\ln A = - k F + \ln A_0 \tag{1}$$

where A is the PPO activity at fluence F (J/cm^2), k is the first order inactivation constant (cm^2/J) and A_0 is the PPO activity of the native enzyme.

2.5. Steady-state tryptophan fluorescence

The changes in the tertiary structure were followed by the analysis of the intrinsic Trp fluorescence. Trp fluorescence spectra were measured with a spectrofluorimeter model RF-Shimadzu 5301 PC (Shimadzu, Japan), using a quartz cuvette of 1-cm optical path length at room temperature (25 °C). The samples were analyzed at 0, 5, 10, 15, 20, 30, 40, 50 and 60 light pulses. Determinations were carried out with a sampling interval of 1 nm and slits of 5 nm. The excitation wavelength was set to 293 nm, and the fluorescence emission spectra were scanned from 300 to 450 nm. Sample fluorescence was corrected for Raman scattering by subtracting solvent fluorescence. Fluorescence data were analyzed by maximum fluorescence intensity, phase diagram, spectral center of mass and parameter A.

Phase diagrams were elaborated according to Kuznetsova et al. (2004) [18] using Eq. 2, which reads as:

$$I(\lambda_1) = a + b\, I(\lambda_2) \tag{2}$$

where $I(\lambda_1)$ and $I(\lambda_2)$ are the spectral intensities at wavelengths λ_1 and λ_2 under different fluences and a and b are the intercept and the slope of the plots.

Fluorescence changes were also characterized in terms of parameter A [19] using Eq. 3, which reads as follows:

$$parameter\ A = \left(\frac{I_{320}}{I_{365}}\right)_{293} \tag{3}$$

where I_{320} and I_{365} are the fluorescence intensities at 320 and 365 nm respectively for an excitation wavelength set to 293 nm.

Furthermore, the spectral center of mass of the fluorescence spectra was determined according to Eq. 4:

$$\lambda_{av} = \sum \lambda\, F(\lambda) \Big/ \sum F(\lambda) \tag{4}$$

where λ_{av} is the center of mass (nm) and F(λ) is the fluorescence at wavelength λ. Only data from 305 to 400 nm was used in these calculations.

2.6. Spectrophotometric analysis

UV–vis spectra of samples were recorded in the UV-1700 spectrophotometer using a quartz cuvette with a 1-cm path length in the range from 200 to 600 nm. From the spectra, turbidity was determined as absorbance at 420 nm [23].

2.7. Statistical analysis

Linear regressions were carried out using Microsoft Excel 2016.

Statistical comparisons were performed by ANOVA with $p < 0.05$ using IBM Statgraphics 24. Each experiment was repeated three times.

3. Results and discussion

3.1. Inactivation kinetics

The inactivation of PPO by PL followed a first-order kinetics ($R^2 > 0.99$) at all pHs tested. Results showed a pronounced effect of pH on the enzyme inactivation (Fig. 1), with a faster inactivation at low pH. Compared with the inactivation rate at pH 6.5, the inactivation rate was 1.5 times faster at pH 5.3 and 2.4 times faster at pH 4.0 (Table 1). PPO inactivation rate changed linearly with pH ($R^2 = 0.99$) according to the following relationship:

$$\text{Inactivation rate (cm}^2\text{/J)} = 0.0544 - 0.0066\ \text{pH}$$

The inactivation of PPO by PL under the working conditions was exclusively a photochemical process. It is known that PL can generate heat as unwanted side effect, which can contribute to enzyme inactivation. However, we has previously shown that under identical conditions, the temperature of the samples increases < 3 °C [5] which allows ruling out any heat contribution to the inactivation.

While to the best of our knowledge this is the first time that the effect of pH on enzyme inactivation by PL is described, it has been reported that an acidic environment facilitates the inactivation of PPO by other technologies. For example, lower pHs decreased the pressure resistance of grape [24], avocado [25] and pineapple [26] PPO during high pressure processing. Morevoer, the inactivation of this enzyme by ultrasound was faster when malic acid was added [14], its heat inactivation was faster at low pH [15] and the UV-C light inactivation of PPO proceeded faster at pH 3.5 than at pH 6.8 [27]. From a practical perspective, the results obtained for the inactivation of PPO by PL indicates that it will be easier to inactivate in acidic products and that the use of acidulants would enhance the efficacy of this technology.

Next to these results, fluorometric and spectrophotometric methods were used in order to explain the structure-function relationship underlying the effect of pH on PPO inactivation.

3.2. Inactivation pathway

Protein unfolding can be a process that changes the native folded protein to a completely unfolded state directly through several partially unfolded intermediates. The phase diagram is a data analysis technique that has been developed to allow a detailed description of unfolding pathways and fish up hidden intermediates [18]. A linear dependence of the fluorescence intensity at 320 nm versus that at 365 nm indicates an all-or-none transition between two conformational states. On the other hand, the non-linearity of this function points towards the sequential character of structural transformations, in which each linear portion will characterize an individual all-or-none transition [28]. Thus, the phase diagrams were built by monitoring the fluorescence intensity at 365 nm as a function of that at 320 nm during the progress of the inactivation at different pHs (Fig. 2). A linear dependence was obtained for each pH, which indicates that the PPO inactivation by PL is an all-or-none transition process at pHs between 4.0 and 6.5. A similar result has also been reported for the PL inactivation of PPO at pH 6.5 [5].

Table 1
First-order inactivation rates of PPO inactivated by PL at different pH's.

pH	Inactivation rate (cm^2/J)*	R^2
4.0	0.0286 ± 0.0005	0.9942
5.3	0.0184 ± 0.0009	0.9986
6.5	0.0121 ± 0.0007	0.9978

* Results are means ± standard deviation.

This conclusion does not mean that PPO reaches a completely unfolded state but just that has arrived to an inactive state that can be completely unfolded or not. Furthermore, these results are not ineludibly indicative that the inactivation proceeds by the same mechanism for all pHs under study since it can occur by different mechanisms, each one an all-or-none process.

The all-or-none feature of the inactivation of PPO by PL is further supported by the analysis of the change of parameter A as the PL inactivation progressed (Fig. 3), which gives no indication of the possible presence of intermediaries. This parameter characterizes the shape and position of the spectrum [19]. The intermediate states are revealed by the parameter A as changes in the shape of the curve as function of the denaturating agent [29], which are absent in Fig. 3.

It has been reported that the inactivation of PPO by other technologies follows other inactivation pathways. PPO thermal inactivation follows a multi-stage process related to oligomerization [30].

3.3. Changes in tertiary structure

Tryptophan fluorescence is considered a very suitable internal probe to monitor changes in the tertiary structure of proteins since it avoids potential conformational changes that external probes can induce and it is particularly sensitive to the microenvironment [11]. *A. bisporus* PPO has 22 tryptophan residues but only 10 are partially or completed exposed to the solvent [11]. The tryptophan fluorescence spectra of the native enzyme at different pHs are shown in Fig. 4. Comparing the initial fluorescence at different pHs, it can be observed that the initial fluorescence decreased at low pH. This finding is consistent with the report by Zhou et al. (2019) [15] although different from the result reported by Ioniţă et al. (2014a) [11], who observed a minimum peak fluorescence

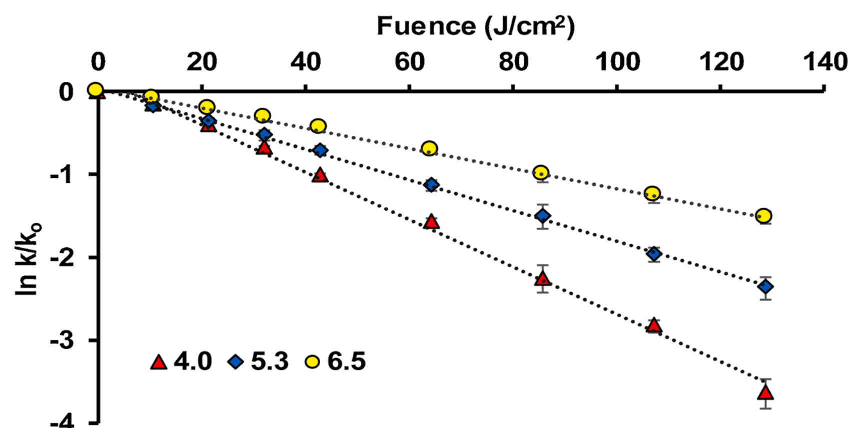

Fig. 1. Kinetics of PPO inactivation by pulsed light as affected by pH. Bars represent standard deviation.

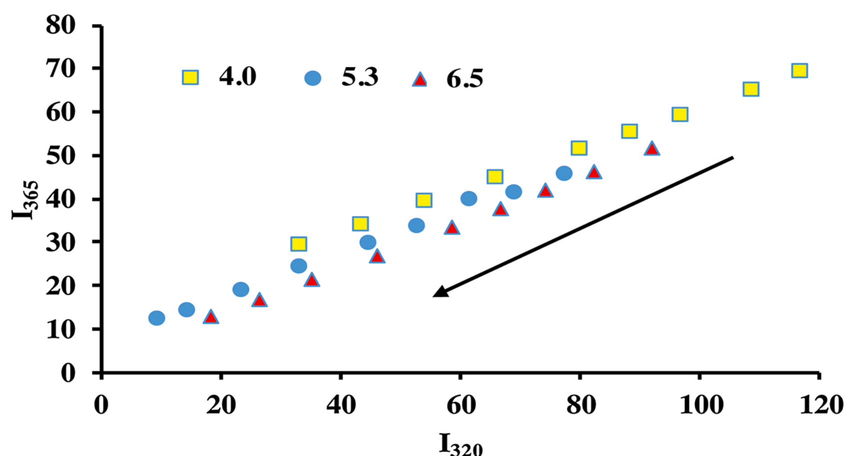

Fig. 2. Phase diagrams of the inactivation of PPO by PL at different pHs. The arrow indicates the progress of the treatment.

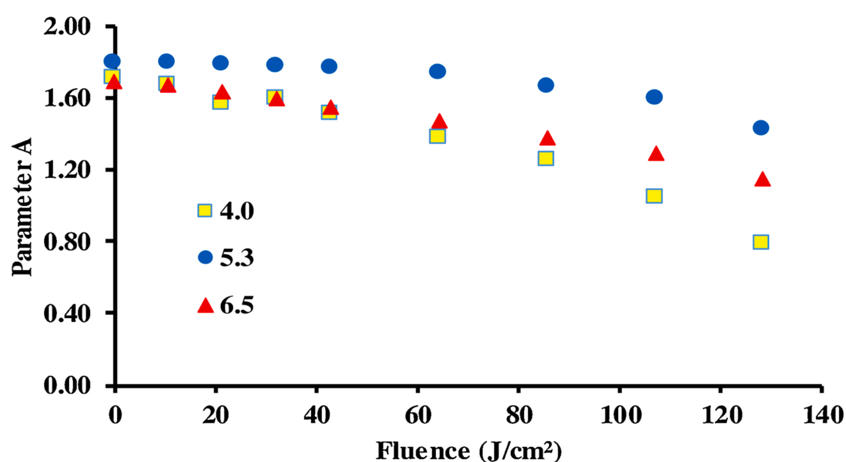

Fig. 3. Evolution of parameter A during the course of PL treatment at different pHs.

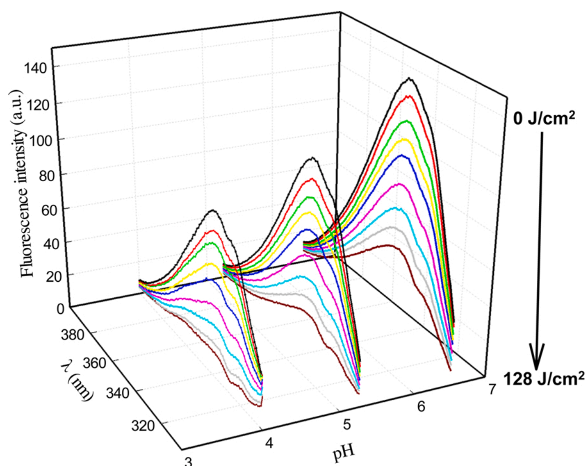

Fig. 4. Intrinsic fluorescence of PPO during the course of PL treatment at different pHs. The arrow indicates the progress of the treatment.

at pH 5.0. The change of initial fluorescence can be a consequence of a partial unfolding caused by the protonation of free carboxylic groups of the enzyme leading to electrostatic repulsive forces, as suggested by McCord and Kilara (1983) [31]. The reduction of fluorescence is due the exposure of tryptophan residues initially buried in the hydrophobic core of the protein, where they have a high quantum yield (emitted photons/exited photons); to the solvent, in which environment they have a low quantum yield [13]. In polymeric proteins such as PPO, it is also possible that the decrease in fluorescence be caused by dissociation, which would also expose tryptophan residues to the solvent. However, this possibility can be ruled out in the current research because no tryptophan residues are located at the subunit interfaces of the native PPO [11].

The tryptophan fluorescence decreased during PL inactivation (Fig. 4), which is indicative of enzyme unfolding. The peak fluorescence decreased by 65, 77 and 86 % during the PL inactivation for pHs 6.5, 5.3 and 4.0 respectively. The change was faster at low pH, which correlates with the observed inactivation pattern as a function of pH. Indeed, the reduction of residual PPO activity was correlated with the change of fluorescence intensity at the peak fluorescence (λ =335 nm) at different pHs, with $R^2 = 0.954$, 0.979 and 0.997 for pHs 4.0, 5.3 and 6.5 respectively (Fig. 5), suggesting that the degradation of the tertiary structure of the enzyme plays a direct role in the pH-dependent inactivation rate. Likewise, the spectral center of mass, which is sensitive method to detect spectral shifts, showed small red shifts in the fluorescence spectra (2−6 nm) at the different pHs. Unfolding of a protein almost always leads to a red shift in the emission from a λ_{max} range of 308−350 nm of the native protein to a λ_{max} of around 345−355 nm [32]. Spectral shifts are determined primarily by the electrical potential difference across the long axis of the tryptophan indole ring, which changes by interaction with solvent molecules [33].

While this kind of fluorescence change has been reported during the inactivation of PPO by different methods such as heating [15] and

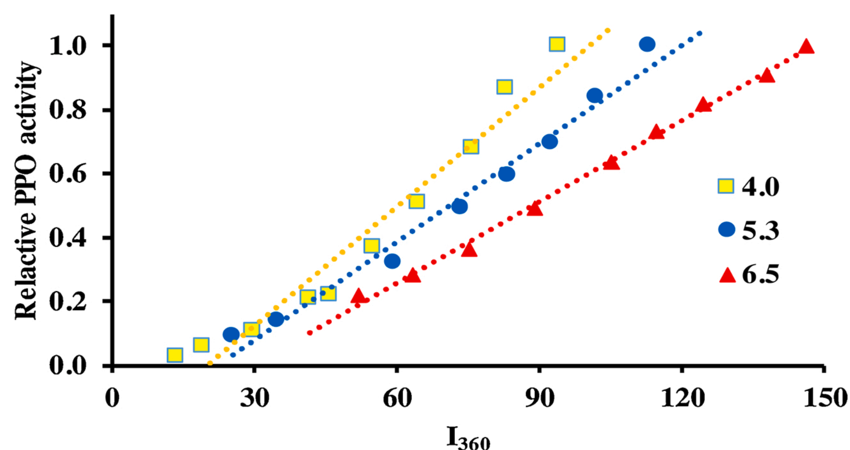

Fig. 5. Relationship between the evolution of peak intrinsic fluorescence intensity of PPO and residual activity during the course of PL treatment at different pHs.

conventional UV light [27], some technologies can promote the opposite effect. For example, fluorescence intensity increased during the inactivation of PPO from quince (*Cydonia oblonga* Miller) by ultrasonication, indicating an inward movement of tryptophan residues [34].

The slight unfolding of PPO at low pH before treatment may explain the faster inactivation of this enzyme by PL at acidic environment because a partly unfolded protein is less stable against unfolding-promoting processes and a less compact protein has more surface exposed to photons that can induce photochemical reactions leading to enzyme inactivation.

3.4. Enzyme aggregation

The spectrophotometric readings were used to estimate the aggregation of PPO during the PL inactivation by measuring turbidity. Turbidity did not change significantly ($p > 0.05$) at any pH, (Fig. 6) showing no evidence of PPO aggregation. The result is in line with a previous report about the inactivation of PPO by PL at pH 6.5 [5]. The lack of oligomerization is further supported by fluorescence spectra data (Fig. 4); since aggregation occurs by interaction between hydrophobic residues that get exposed to the environment during the inactivation and/or by formation of disulfide bridges from *de novo* free sulfhydryls. Such interaction would hide tryptophan residues from the polar solvent again, which would cause a reverse of the evolution of the fluorescence signals to higher values. The absence of aggregation makes the mechanism of PL inactivation of PPO different from those of other technologies, since aggregation phenomena have been observed during the inactivation of PPO by heat [30], ultrasound [34] and high-pressure carbon dioxide [35].

4. Conclusions

The findings of this research show that the rate of inactivation of PPO by pulsed light is pH dependent while the inactivation mechanism seems to be pH independent. The inactivation proceeds faster at acidic pH in the pH range (4.0–6.5). The loss of enzymatic activity was highly correlated to protein unfolding, a process that coursed without formation of intermediate species and without evidence of enzyme aggregation. From the basic research point of view, these results indicate that the mechanism of enzyme inactivation by PL is independent of pH. From the practical point of view, it is possible to reduce treatment times for inactivating enzymes by PL in foods by means of controlling the pH. Softer treatment conditions would decrease the harmful side-effects that this technology may have on product quality as long as acidification be compatible with the organoleptic characteristics of the concerning food.

CRediT authorship contribution statement

José A. Pellicer: Investigation, Visualization, Writing - review & editing. **José A. Gabaldón:** Writing - review & editing, Funding acquisition, Project administration. **Vicente M. Gómez-López:** Conceptualization, Methodology, Formal analysis, Resources, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

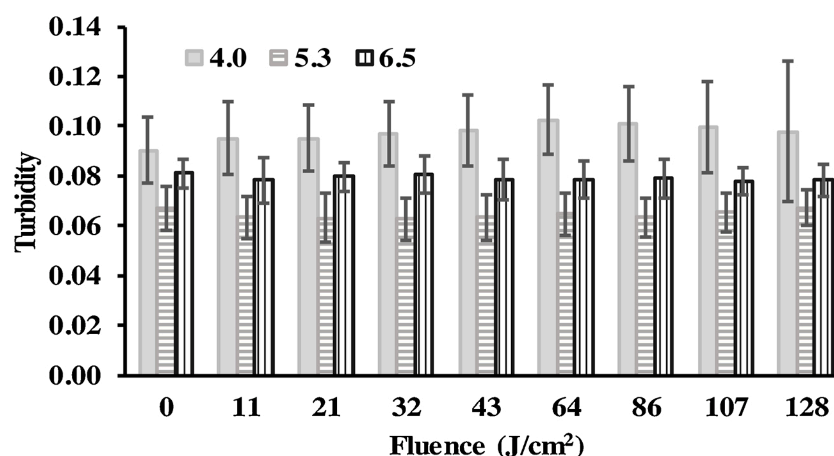

Fig. 6. Turbidity measurements of PPO inactivated by PL at different pHs. Bars represent standard deviation. Within a pH, means were not statistically different ($p > 0.05$).

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowlegment

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