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Research article

Laccase-based catalytic microreactor for BPA biotransformation

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ABSTRACT

A laccase-based catalytic reactor was developed into a polydimethylsiloxane (PDMS) microfluidic device, allowing the degradation of different concentrations of the emergent pollutant, Bisphenol-A (BPA), at a rate similar to free enzyme. Among the immobilizing agents used, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was capable of immobilizing a more significant amount of the laccase enzyme in comparison to glutaraldehyde (GA), and the passive method (2989, 1537, and 1905 U/mL, respectively). The immobilized enzyme inside the microfluidic device could degrade 55 ppm of BPA at a reaction rate of 0.5309 U/mL*min with a contaminant initial concentration of 100 ppm at room temperature. In conclusion, the design of a microfluidic device and the immobilization of the laccase enzyme successfully allowed a high capacity of BPA degradation.

1. Introduction

Bisphenol A (BPA) is part of the chemical compounds called emerging pollutants. The BPA manufactures pharmaceutical, cosmetic, plastic, additive, pesticide, and detergent products. Specifically, BPA is used in manufacturing polycarbonate plastic and epoxy resins, which are used in manufacturing food containers, bottles, baby bottles, and coating to protect canned foods and water pipes [1,2]. Due to low concentrations in the order of μg/L, they go unnoticed, but exposure and their ability to accumulate represent a threat to health. Several studies have shown that BPA can mimic some hormones, such as estrogen, and consequently cause various health problems associated with sex-specific neurological development, uterine cancer, immunological toxicity, neurotoxicity, and interference of cellular pathways [1,3,4].

The use and consumption of BPA in the different products translate to an estimated daily exposure of 0.16 μg/kg of body weight for people in the United States and between 0.04 and 0.08 μg/kg for people in Japan. Although there are multiple international studies on the permitted concentration of BPA in food products, Mexico remains without regulations to control its use [2,4,5].

The methods of degradation and transformation of BPA are the center of attention within the scientific community. Currently, there

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exist two large groups: physicochemical methods and biological methods. Within the physicochemical methods, we can find fenton-oxidation, ultrasonic-oxidation, photocatalytic-oxidation, ozonation, and combination methods, characterized by being expensive, slow, and producing substances equal to or more harmful than BPA. On the other hand, biological processes consist of biotransformation using microorganisms and specific enzymes without producing toxic products [6].

Laccases are part of the enzymes used in the degradation processes of BPA. Thanks to its high oxidation capacity, it is used for the degradation of pollutants and manufacturing biosensors. However, the lack of long-term operational rigidity, reusability, and short service life make them susceptible to different operating conditions. These disadvantages can be solved using immobilization systems such as adsorption, trapping, covalent bonding, and cross-linking. Works carried out by immobilizing laccase in Cu alginate beads using the trapping method managed to degrade 9612 ppm of BPA after 60 min [7]. Other authors have reported the degradation of 10 ppm of BPA using laccases immobilized on chitosan beads cross-linked with glutaraldehyde after 150 min of reaction [8].

When enzymes are immobilized in a microfluidic device, several advantages are obtained, such as integrating several channels connected in parallel, better integrating multiple functions and processes in a single device, improvements in the mixing process, the reaction process, and even the sample treatment. There are few reports of immobilized enzymes in microfluidic devices [9,10]. In these reports, the enzymes were passively immobilized on the devices, transketolase and transaminase being examples of this passive immobilization, laccase was also passively immobilized for the enzymatic reaction of phenolic compounds, catechol, and L-DOPA [9].

BPA degradation using immobilized laccases in microfluidic offers an operational advantage since these devices allow high reaction homogeneity. To date, no reports of devices have been found. Therefore, this study aimed to produce and test a biocatalytic reactor that can degrade BPA by immobilized laccases.

2. Materials and methods

An economic soft lithography variant fabricated a microfluidic reactor. First, a mold was produced with laser engraving on a 3 mm PMMA plaque. Then, a second mold was created with polydimethylsiloxane (PDMS). Then, a replicate from the PDMS mold was done with PDMS to get the shape of a serpentine and then functionalized by laccase on its inner walls (details in **SI**). In general, The immobilization process was tested for duplicate with three different methods -ethyl-3-(3-dimethylamino propyl) carbodiimide (EDC), Glutaraldehyde (GA), and passive adhesion (PA). The laccase enzymatic activity was measured through the oxidation reaction for the substrate 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) by absorbance measurement. Similarly, the BPA transformation was measured by absorbance with a wavelength of 228 nm.

Poly (methyl methacrylate) (PMMA) was acquired from Polymershapes, Chihuahua, Mexico, and polydimethylsiloxane (PDMS) Sylgard 184 elastomer Kit, absolute ethanol, phosphate citrate buffer pH 3, ABTS pills of 5 mg, Dichlorodimethylsilane (99.5 %), 1-ethyl-3-(3-dimethylamino propyl) carbodiimide (EDC), and Glutaraldehyde (GA) were acquired from Sigma Aldrich, San Louis, USA. The laccase enzyme was produced by the Sustainable and Applied Biotechnology Group from Tecnológico de Monterrey using the fungus *Pycnoporus sanguineus* CS43.

2.1. PMMA sheet design

For the biocatalytic reactor a serpentine shape design was used (drawn in AutoCAD) shown in Fig. 1. The PMMA sheet milling was done with a CO_2 laser cutter with 30 W of power and a cutting speed of 400 mm/s. Milling was done on the x- and y-axis. Once the mold was obtained from the pre-recorded PMMA sheet, this one was brushed and washed with Extran® (50 % v/v, with bi-distilled water). Then it was dried for 2 min at 100 °C to evaporate the residual water, subsequently, it was exposed to chloroform vapor for 12 min to erode surface debris and dried for 2 min at 100 °C.

2.2. PDMS device fabrication

The PDMS there was mixed with a base of PDMS and its cross-linking agent in a 10:1 ratio in grams, respectively, degassed in a LABCONCO vacuum chamber for 20 min. The mixture was poured over the PMMA mold to avoid bubble formation. Subsequently, cured for 12 min at 100 °C. The PDMS layer was peeled, and the procedure was repeated now using the new PDMS sheet as the mold.

A fine layer of PDMS is poured into a glass slide (75 mm × 25 mm) and cured for 2 min at 100 °C. The PDMS replicate is carefully placed with slight pressure over the thin PDMS layer and cured for 10 min, at 100 °C. Finally, the input and output were punched out,

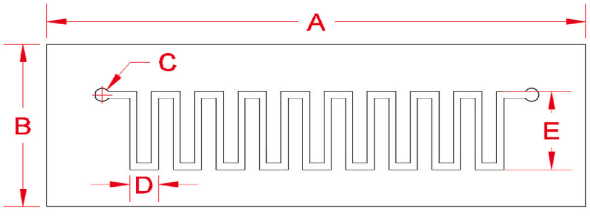

Fig. 1. AutoCAD design for PMMA pre-recorded sheet. A - Sheet length 75 mm, B - Sheet width 25 mm, C - In and out diameters 2 mm, D - Horizontal trajectory 4 mm, E – Vertical trajectory 12 mm. The design has a total linear trajectory of 292 mm and a channel width of 1 mm.

and then the hoses were introduced on each puncture. The enzyme immobilization process is described in subsection 2.4.

2.3. Enzymatic activity

The laccase enzymatic activity was measured through the oxidation reaction for the substrate ABTS in a microplate spectrophotometer BGM labtech Model FLUOstar at 420 nm. From the absorbance read the enzymatic activity was computed with Eq. (1).

$$UL^{-1} = \frac{m \times Vt \times Df \times 10^6}{\varepsilon \times d \times Vs} \quad \text{(Eq. 1)}$$

where m is the change of absorbance over time ($\Delta A/t$), Vt is the total reaction volume (200 μL), Vs is the enzyme volume (10 μL), Df is the dilution factor (500), d is the cell length (1 cm), automatic correction from the equipment and, ε is the molar extinction coefficient (36,000 $Mol^{-1}*cm^{-1}$).

2.4. Enzymatic immobilization

For the enzymatic immobilization, 1-ethyl-3-(3-dimethylamino propyl) carbodiimide (EDC) and Glutaraldehyde (GA) were employed. Also, passive immobilization was evaluated (PA), in which no agent was used for immobilization. The methodology employed for each one of the immobilizations (EDC, GA, and PA) is described ahead. The solutions were introduced to the channels using a Fusión 200 Chemix Inc. pump. The enzyme immobilization was determined through an enzymatic activity comparison between the entrance and output flows (Eq. (2)).

$$Reactor's\ enzymatic\ activity = AE_i - \sum AE_f \quad \text{(Eq. 2)}$$

where, AE_i is the Initial Enzymatic Activity, and AE_f is the Final Enzymatic Activity.

EDC Immobilization was done by a mixture of absolute ethanol and DMSO to 99.5 %. Then, the channel is cleaned with the mixture for 10 min at room temperature. A drying step is done by submitting the devices to the stove at 100 °C for 1 h. A solution of anhydride succinic acid (SSA) with a pH of 6.0 is pumped through the channel for 2 h to prepare the surfaces. The device channel is washed with buffer phosphate citrate with a pH of 3.0 and dried with nitrogen. Later, the EDC 20 μg/mL is left inside the device for 1 h at room temperature to functionalize the surface prior to the laccase enzyme (Enzyme activity 3312 U/mL) being introduced for 1 h at room temperature. Finally, buffer Tris-HCl with a pH of 8.0 to stop the cross-linking reaction, and washed the microfluidic with three times the volume of the microfluidic device capacity so it wouldn't have enzyme activity. The residual enzyme activity from this final washed was quantification to guarantee the device wouldn't have enzyme release in the time.

GA Immobilization was done with a mixture of absolute ethanol and DMSO 99.5 %. Then, the channel is cleaned with the mixture for 10 min at room temperature. Another cleaning step is done with absolute ethanol of 96 %. A drying step is done by submitting the devices to the stove at 100 °C for 1 h. Then, a solution of Glutaraldehyde 2.5 % is pumped through the device's channel. The channel is washed with buffer Tris-HCl with a pH of 8.0 and then dried with nitrogen. The concentrated laccase enzyme is introduced and left inside the device for 2 h at room temperature. Finally, the channel is activated with Glutaraldehyde 0.1 % for 1 h and washed with Tris-HCl pH 8.0 to guarantee the not release of the enzyme from the microfluidic device as it had described previously.

Passive Immobilization was done by a first wash with absolute ethanol. Finally, the laccase enzyme was introduced and left inside the device for 1 h at room temperature and washed with Tris-HCl pH 8.0 as in the previous description.

2.5. Degradation of ABTS and quantification of BPA

The oxidizing capacity of the system was assessed using ABTS. For this purpose, an ABTS solution was prepared by dissolving a 5 mg tablet in 3.3 mL Milli-Q water. The solution was introduced into the microfluidic device with the immobilized enzyme and its

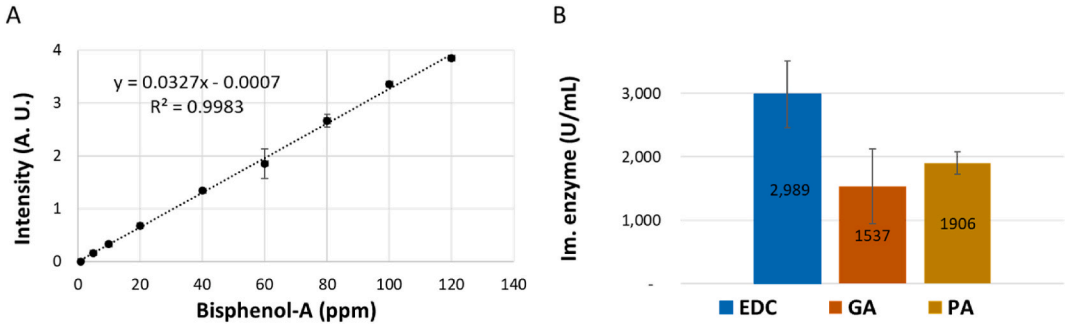

Fig. 2. A) Standard curve obtained for the Bisphenol A quantification. **B)** Enzyme immobilization through the different cross-linking agents EDC, and GA contrasted with passive immobilization method (PA).

duplicate. The ABTS solution was passed through the microreactor at different flow rates to ensure various retention times (1, 10, and 100 min). Finally, the output solution from the microreactor was collected and quantified using spectrophotometry.

The quantification curve of Bisphenol A was constructed using a spectrophotometer at 280 nm and employing a BPA standard. To assess the transformation capacity of the immobilized laccase, solutions containing 20 ppm and 100 ppm of BPA were passed through the device for duplicate, maintaining the flow rate to ensure retention times of 1, 10, and 100 min. Finally, the collected solution was analyzed for the residual amount of untransformed BPA, subtracted from the initial concentration.

3. Results

3.1. BPA quantification and laccase immobilization

Bisphenol A was tested by spectrophotometry using the evaluation of a standard curve defined by the slope of 0.0327 and initial intensity of −0.0007 and a correlation of 0.9983 (R^2) (Fig. 2 A). The experiments to test the transformation capacity were done with two different concentrations (100 ppm and 20 ppm) at three different retention times (1, 10, and 100 min). The degradation of pollutant BPA was measured by a spectrophotometer. A standard curve was constructed for the quantification of BPA through spectrophotometry using a spectrophotometer of the brand Thermo Scientific model Varioskan Flash at a wavelength (λ) of 228 nm (Fig. 2 A).

The transformation of ABTS is a common practice in biotechnology laboratories. In this work, it was used to test the system's capacity to oxidize ABTS by laccase enzyme. A concentrated solution of ABTS (5 mg pill of ABTS solubilized in 3.3 mL of MiliQ water) was pumped through the channels of the microfluidic devices. The outlet fluid was collected and then the oxidized ABTS was quantified by spectrophotometry.

Among the different immobilization agents tested, EDC presented the greatest immobilization capacity for the laccase enzyme in comparison with PA and GA immobilization (Fig. 2 B). The evaluation of enzymatic activity employing ABTS as indirect laccase immobilization. Among the different immobilization agents tested, EDC presented the most significant immobilization capacity for the laccase enzyme in comparison with PA and GA immobilization with values of 2989, 1537, and 1905 U/mL, respectively (Fig. 2 B). The BPA transformation tests were made using higher immobilized laccase (EDC method).

3.2. Indirect enzymatic activity with ABTS

The results obtained using the EDC devices show that the biocatalytic reactor has the capacity to oxidize ABTS with an enzymatic reaction rate of 0.3624 $\frac{U/mL}{min}$ (Fig. 3).

3.3. BPA transformation

The most significant degradation percentage of BPA was obtained at a retention time of 100 min, in which the treatment with an initial concentration of 100 ppm of BPA reached a transformation percentage of 55 %. In contrast, the treatment with an initial concentration of BPA of 20 ppm reached a percentage of 41 %. The results of the percentage of degradation for each one of the treatments at the different retention times are shown in Fig. 4 (A and B).

Among the evaluated treatments, the high reaction rate presented was at an initial concentration of BPA of 100 ppm (0.5039 $\frac{U/mL}{min}$), 10 times greater than the presented at an initial concentration of BPA of 20 ppm (0.0536 $\frac{U/mL}{min}$) Fig. 4 (C and D).

4. Discussion

The objective to build and test a biocatalytic reactor Bisphenol A transformation was achieved. In this case, it was used PDMS since it is a cheap, transparent, and biocompatible biopolymer and can be used in biotechnological processes [11]. Moreover, the methods

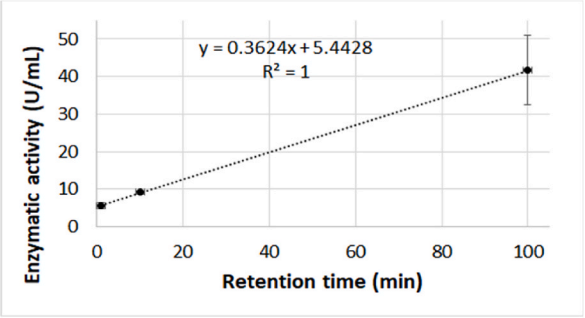

Fig. 3. ABTS oxidation in the biocatalytic reactor of enzymes immobilized with EDC.

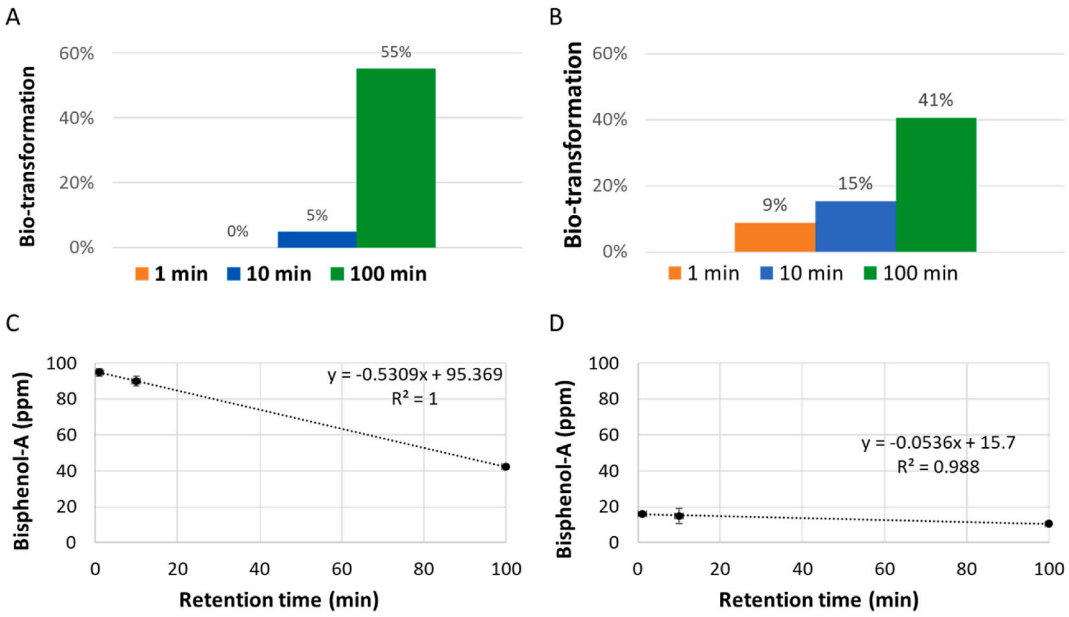

Fig. 4. Final bisphenol A (BPA) transformation percentage at retention times of 1, 10, and 100 min with an initial concentration of **A)** 100 ppm., and **B)** 20 ppm. Bisphenol A (BPA) transformation at 1, 10, and 100 min retention times with an initial concentration of **C)** 100 ppm., and **D)** 20 ppm.

and experiments were done with attainable technology in contrast to other complex and expensive technology [12,13]. The design of the device's channel with a serpentine shape allows a satisfactory distribution of the immobilized enzyme and a greater contact area between the substrate and the enzyme.

Among the immobilizing agents tested, GA did not favor immobilization and only retained a concentration equivalent to an enzyme activity of 1084 U/mL, which was lower than that of the passive method (PA) that trapped 1415 U/mL. Although GA has been reported as a compound that favors the adhesion of enzymes due to the exposure of its aldehyde group that forms a bond with the amino group of the enzymes, the hydrophobic nature of PDMS after polymerization and crosslinking means that when using GA there is less enzymatic stability due to the proximity between the hydrophobic surfaces and what causes incorrect conformations of the enzymes since, in general, well support for the anchoring of GA must have a hydrophilic surface. The PDMS can become a good support for GA by functionalizing its surface with plasma oxidation, ultraviolet irradiation, chemical deposition, or cathodic coating of metallic compounds [14,15]. For its part, passive immobilization could be favored by surface binding by nonspecific forces such as van der Waals forces, hydrogen bonds, and hydrophobic interactions [10]. The enzymatic activity obtained for the system immobilized with EDC was much higher than the previously named methods, with a retained enzymatic activity of 2989 U/mL. The superiority of this method could be due to the activation of the carboxyl and amine groups of the proteins. In addition, hydrophobic interactions provide stabilizing characteristics by incorporating hydrophobic residues that facilitate the crosslinking of amino acids [16].

The use of immobilizing agents brings the disadvantage of loss in enzymatic activity compared with the enzymatic activity or the total enzyme adhered to the system due to the use of short agents. As a result, a short distance between the support and the enzyme affects the enzymatic activity, so the enzymatic immobilization is not a guarantee of having a system with high enzymatic activity [17], as can be observed by comparing the theoretical enzymatic activity of the system (activity obtained with **Eq. (2)**) vs the authentic enzymatic activity (measure with ABTS, Fig. 3). The linearization allows determining the enzyme's reaction rate (0.3622 $\frac{U/mL}{min}$) when ABTS is used as the substrate.

The BPA transformation capacity inside the biocatalytic reactor presented a significant variability when the initial concentration of substrate was 100 ppm in comparison to the initial concentration of 20 ppm. Under the concentration of 100 ppm, the system could degrade an equivalent of 55 ppm with a retention time of 100 min. In comparison, at a concentration of 20 ppm, it could degrade only an equivalent of 8.2 ppm. The obtained degradation rate of BPA at a concentration of 100 ppm is 10 times greater than one with a concentration of 20 ppm (0.5309 and 0.0536 $\frac{U/mL}{min}$, respectively). (shown in Fig. 4).

Since the retention time was 100 min at different concentrations (20 and 100 ppm), this retention time is significantly high in a microfluidic system, which could influence the reaction rate. For the BPA degradation, it must be transported through the channels and reach the walls containing immobilized laccase. Under this regime, mass transport primarily depends on diffusion processes and is not significantly influenced by convection, as with shorter retention times. This behavior implies that at higher analyte concentrations, the reaction rate accelerates due to a greater availability and concentration of the analyte diffusing within the system [18].

Results found for the degradation of BPA (100 ppm) coincide with the one obtained for laccases produced by the fungi strains *Coriolopsis gallica*, *Bjerkandera adusta*, and *Trametes versicolor* studied in bulk conditions with degradation after 100 min was 78, 58, 48 % respectively [19]. Other laccases immobilized produced with different strains of *T. versicolor,* and *P. sanguineus* do not exceed the 20

% degradation of BPA for retention times of 6 h [20].

Some advanced methods of immobilizing laccases for BPA transformation have achieved only the degradation of 5 and 10 ppm within 60 min [7,21]. On the other hand, other methods allow for the reutilization of the immobilized enzyme for up to 10 cycles without experiencing a considerable loss of its catalytic capacity (<25 %) [22]. The transformation obtained in this study is comparable to that observed in research involving free enzymes, with the advantage of extending the enzyme's lifespan through immobilization and subsequent reuse. Compared to immobilization systems, the primary advantage lies in the absence of additional methods to recover the enzyme-support complex from liquid media, enabling continuous operation.

Furthermore, it can be done with precise flow control and process retention time. However, during this work, pH and temperature were not evaluated. There is a possibility to increase catalytic transformation capacity by exploring its correlation. The working range can further optimize the system operation. Additionally, it can be used to predict further immobilization strategies dedicated to particular tasks, i.e., wastewater, soil, and air pollution treatments, or direct applications to industrial activities.

5. Conclusions

It successfully fabricated a biocatalytic reactor integrated into a microfluidic system, which can transform a high concentration of BPA at a reaction rate of 0.5309 $\frac{U/mL}{min}$ when the immobilization is performed using EDC. Despite the results in pollutant transformation through time and the concentration of pollutants removed being satisfactory concerning the ones found in the literature, the process can be optimized if factors such as pH and temperature are evaluated. Modifying the microfluidic device design with two or more inputs will allow the determination of kinetic parameters such as the maximum reaction rate, substrate affinity, and inhibitory substrate concentration. These parameters are difficult to estimate from traditional methods due to the high consumption of reagents and the prolonged research times.

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Data availability

Data generated in this study can be found in the article.

CRediT authorship contribution statement

Juan Eduardo Sosa-Hernández: Writing – review & editing, Writing – original draft, Supervision, Software, Resources, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Elsa M. Gutierrez:** Writing – original draft, Methodology, Investigation. **Jhosseph S. Ochoa Sierra:** Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation. **Osvaldo Aquines:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Felipe Robledo-Padilla:** Writing – review & editing, Writing – original draft, Conceptualization. **Elda M. Melchor-Martínez:** Writing – review & editing, Writing – original draft, Conceptualization. **Hafiz M.N. Iqbal:** Writing – review & editing, Writing – original draft, Conceptualization. **Roberto Parra-Salvídar:** Writing – review & editing, Writing – original draft, Conceptualization.

Declaration of competing interest

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

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