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Bio-based and cost effective method for phenolic compounds removal using cross-linked enzyme aggregates

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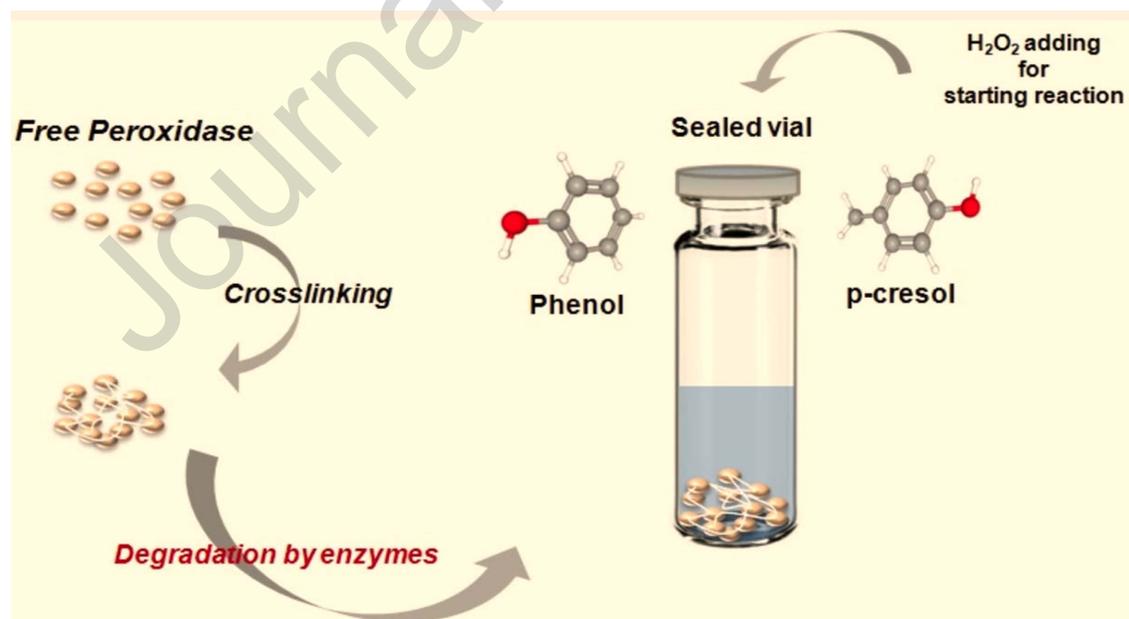
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Abstract

This work aimed at presenting a green method using a new source of peroxidase isolated from *Raphanus sativus var. niger* (RSVNP) in immobilized form, for the treatment of wastewater. To ensure stability and enzymatic activity in the biodegradation process, RSVNP was immobilized as a cross-linked enzyme

aggregate (CLEAs). With more than 29% of recovered activity and 85% aggregation yield, acetone was selected as the best precipitating agent. The formed protein aggregates required 2% (v/v) of glutaraldehyde (GA) concentration and a ratio of 9:1 (v/v) enzyme (E) amount to cross-linker (E/GA). Compared to the free enzyme, RSVNP-CLEAs were found more chemically and thermally stable and exhibited good storage stability for more than 8 weeks. In addition, RSVNP-CLEAs were evaluated for their ability to remove phenol and p-cresol from aqueous solution by varying several operating conditions. A maximal yield (98%) of p-cresol conversion was recorded after 40min; while 92% of phenol was degraded after 1h duration time. The reusability of RSVNP-CLEAs was tested, displaying 71% degradation of phenol in the third batch carried out and more than 54% was achieved for p-cresol after four successive reuses in the presence of hydrogen peroxide at 2 mM concentration.

Graphical abstract



Highlights

- A new source of peroxidase was immobilized for phenolic compounds treatments.
- Crosslinked peroxidase showed better physical and thermal stability compared to the free enzyme.
- Immobilized peroxidase retained 100% activity after storage at 4°C for 8 weeks.
- RSVNP-CLEAs were able to remove 92% of phenol and 98% of p-cresol.
- More than 71% degradation of phenols was achieved after 3 successive reuses.

Keywords:

Peroxidase Immobilization; Phenols; CLEAs; Enzymatic transformation; green process

1. Introduction:

Modernization and economic development have led to changes in the various industrial sectors to ensure the comfort and well-being of humans. On the other hand, they participated negatively and irreversibly to the deterioration of our environment (**Bhandari, 2015**). Intensive industrialization also have a direct effect on ecosystems and severe climate changes (**Chopra, 2016; Jhariya et al., 2019**). Industrial activity releases numerous toxic chemicals compounds into water, air and soil (**Mielke and McLachlan, 2020**). This hazardous waste represents a significant risk to human health and can be in some cases an additional source of cancer development (**Jagai**

et al. 2017). The effluents generated by the petrochemical industry are characterized by a strong concentration of toxic organic products, as well as oil and grease (**Abd El-Gawad 2014; Bahri et al. 2018**). Indeed, this industry uses a large volume of water for cooling systems and refining of crude oil; thus causing, a continuous discharge of pollutant-laden wastewater (**Bustillo-Lecompte et al., 2018; Tian et al., 2019**). Generally, wastewater from petrochemical refineries contains sulfides, hydrocarbons, phenols, oils and greases (**Abdelwahab et al., 2009; Altaş and Büyükgüngör, 2008; Dat and Chang, 2017**). The US Environmental Protection Agency (EPA), Agency for toxic Substances and Disease Registry (ATSDR) and the National Institute for Occupational Safety and Health (NIOSH) consider phenols as priority pollutants and dangerous for health or life at a concentration of 250 mg.L⁻¹ (**Tandjaoui et al. 2019**). Due to their solubility, phenolic compounds are widely present in sewage treatment plants discharged by petroleum refineries. Indeed, the concentration of phenolic compounds varies from 40 mg.L⁻¹ to 185 mg.L⁻¹ in the wastewater of oil refinery (**Naguib DM 2019**). For example, they can reach a concentration of 185 mg.L⁻¹ for raw outlets sour water, 50 mg.L⁻¹ for catalytic cracker and 80 mg.L⁻¹ for post-stripping (**Al Hashemi et al., 2014**). Moreover, several authors have revealed the undesirable effects of phenols and their derivatives on aquatic organisms. It has been reported that exposure of algae and fish to phenol concentrations of 72.29 mg.L⁻¹ and 14 mg.L⁻¹ respectively, lead to their death (**Duan et al., 2017; Patra et al., 2007**). The same observations have been reported for exposure to p-cresol at concentrations of 45.8 mg.L⁻¹ for algae and 28.6 mg.L⁻¹ for fish (**Aruoja et al., 2011; DeGraeve et al., 1980**). This is why, in order to protect the

receiving environment, many wastewater treatment processes are implemented to reduce their impact on the environment (**Villegas et al., 2016**).

The use of microorganisms, enzymes and immobilized cells of organisms for the degradation of phenol and p-cresol have been the subject of several studies (**Bayramoğlu and Arica, 2008; Edalli et al., 2016; Günther et al., 1995; Tallur et al., 2009**). However, the treatment of pollutants with microorganisms may show certain limits due to the drastic conditions in which bacterial growth must be ensured (**Karigar and Rao, 2011**). Therefore, the use of enzymes may be a more attractive approach to overcome these problems. Several studies have demonstrated the potential of peroxidases for wastewater bioremediation by involving free radicals, thus generating polymerized or oxidized products (**Bansal and Kanwar, 2013**). In addition, through work carried out by researchers on horseradish peroxidase (HRP) and soybean peroxidase (SBP), it turns out that peroxidases are an effective tool in the degradation of phenolic compounds (**Bayramoğlu and Arica, 2008; Steevensz et al., 2014**). Despite their certain efficacy in the treatment of these compounds, free enzymes also encounter problems which can lead to their denaturation and a loss of catalytic activity (**Brady and Jordaan, 2009**). Immobilization or insolubilization of enzymes, can give rise to a more stable biocatalyst, resistant to extreme conditions, recyclable and having the capacity to operate continuously (Brady and Jordaan 2009). Four traditional methods of enzymes immobilization can be distinguished mainly, including entrapment, encapsulation, fixation on support and cross-linking (**Sheldon and van Pelt, 2013**). Among them, cross-linking is a promising technique since the immobilization of the enzyme is possible by using bifunctional cross-linking

agent to bind the enzyme molecules without using a support (**Brady and Jordaan, 2009**). In addition, the other techniques show some drawbacks. In the case of entrapment, the molecules of the enzymes are free but the gel which surrounds them limits their movement and act as a barrier to mass transfer, which impact reaction kinetics (**Brady and Jordaan, 2009**). For encapsulation, its use is limited to enzymes with substrates of small molecular size due to the diffusion limits (**Hanefeld et al., 2009**). As for attachment to support by adsorption, the enzyme seems to retain certain mobility; which allows it to retain its catalytic properties. However, it has a major drawback because the interactions between the enzyme and the support are weak. The enzyme can thus be released over time, especially under industrial conditions (strong agitation). On the other hand, the ionic and covalent bindings are generally stronger but the fact remains that this technique is more expensive due to the use of the support (**Sheldon and van Pelt, 2013**). Therefore, the synthesis of cross-linked enzyme aggregates (CLEAs) is a simple alternative technique in its implementation; it allows the reduction of the immobilization cost compared to immobilization on support while retaining a considerable rate of initial enzyme activity. Many advantages are associated to CLEAs technology in comparison with soluble enzyme, including stability to denaturation caused by high temperature, organic solvents and proteolysis (Sheldon, 2007). It also offers many benefits, such as volumetric productivities and recoverability, superior operational stability following the formation of a rigid structure which prevents the breakdown of the aggregated enzyme by the multipoint attachment of enzyme molecules (Park et al., 2012; Sheldon, 2011). Despite several advantages, carrier-free immobilized enzymes have some drawbacks which can make its application at industrial scale complicated. The

lack of control on their particle size, mass-transfer issues related to recovery operations such as filtration and centrifugation, make the CLEAs particles only partially recycled (Cui and Jia, 2013). To overcome these constraints, several researchers have developed techniques to improve the mechanical stability of CLEAs and facilitate their recovery, by encapsulating, coating and trapping them in polymers and matrices. Penicillin G acylase have been successfully co-aggregated with amino-functionalized superparamagnetic iron oxide nanoparticles and then cross-linked, thus allowing its efficient and rapid magnetic decantation (Kopp et al., 2013). Similarly, by adopting an innovative approach Cui et al. (2016) developed a hybrid magnetic CLEAs (HM-CSL-CLEAs) using lipase and unfunctionalized magnetite nanoparticles. The resulting CLEAs enhanced the enzyme stability to elevated temperature, increased storage stability while allowing their separation in a simple and easy manner. The same observations were concluded in the synthesis of spherical CLEAs with biosilica shell which turned out stable against denaturants, while maintaining 70% of its activity after 13 cycles (Cui et al., 2017). In addition, the combination of traditional techniques with CLEAs technology may prove useful, as in the case of the adsorbed cross-linked phenylalanine ammonia lyase aggregate on the crude-pored microspherical silica core. The immobilized CLEAs can be precipitated naturally without filtration or centrifugation, thus reducing mass-transfer limitations (Cui et al., 2014).

However, it is important to take into consideration the biochemical properties and protein structure of each enzyme before proceeding to any immobilization (Yamaguchi et al., 2018).

In a circular economy objective, the present study relates to the use of a green method for the degradation of phenolic compounds, whose component is a new source of peroxidase which could succeed to SBP and HRP. In this context, *Raphanussativus var. niger* peroxidase was extracted and immobilized in the form of cross-linked enzyme aggregates (RSVNP-CLEAs). After preparation, optimization and characterization of RSVNP-CLEAs, the latter were evaluated for their potential in the degradation of phenol and p-cresol while investigating the parameters that can influence their elimination. This approach could provide an ecological and cost-effective alternative for a large-scale enzyme treatment.

2. Materials and methods

2.1. Chemical reagents

Phenol (purity 99%), p-cresol (purity 99%), 4-aminoantipyrine, hydrogen peroxide solution (30% w/w) and glutaraldehyde (GA) were supplied by Sigma-Aldrich (Saint Quentin Fallavier, France). Sulfuric acid (purity ≥ 95) was purchased from AcrosOrganics (Thermo Fisher Scientific, Geel, Belgium). All solutions were prepared with ultrapure water and all reagents used for experiments were obtained from Acros and Sigma.

2.2. Protocol for RSVNP-CLEAs preparation

The enzyme was isolated from a fresh black radish using a juice extractor. The solution of crude peroxidase was filtered by four layers cheesecloth after what the solution was used to develop cross-linked aggregates of *Raphanussativus var. niger* peroxidase without any purification beforehand. The RSVNP-CLEAs were

synthesized using a standard preparation method described by **(Sheldon 2011)**. Briefly, the preparation is carried out in two steps (i) the precipitation which consists in the addition of a solvent (acetone, methanol, 2-propanol, acetonitrile, and ethanol); then, three times volume of pre-cooled solvent are added dropwise to the enzyme solution. After a continuous stirring (250 rpm) for 1 hour at 4 °C, (ii) the cross-linking step occurs, glutaraldehyde (GA) using different volume concentrations, 1%, 1.5%, 2%, 2.5%, 3%, are added to the total volume solution and then allowed to react for 3 hours. The CLEAs were recovered by centrifugation at 4000g for 10 min. After each preparation, the CLEAs were washed and stored in a phosphate buffer solution (pH 7±0.2, 50 mM) at 4 °C.

For an optimal preparation of immobilized enzyme, several parameters were studied, such as the optimal precipitating agent, the enzyme and the cross-linking agent, taking into account activity recovery (Eq. (1)) and the aggregation yield (Eq. (2)) defined as reported by **(Bilal et al., 2017a)**.

Activity recovery (%)

$$= \frac{\text{Total activity of RSVNP-CLEAs (U)}}{\text{Total activity of free RSVNP used for CLEAs preparation (U)}} \times 100 \quad (1)$$

Aggregation yield (%)

$$= \left[1 - \frac{\text{Total activity of the residual solution (U)}}{\text{Total activity of free RSVNP used for CLEAs preparation (U)}} \right] \times 100 \quad (2)$$

2.3. Peroxidase activity assay

Free and immobilized enzyme activity was assayed using hydrogen peroxide and phenol as substrates; the reaction with 4-aminoantipyrine (4-AAP) forms a colored product which absorbs at ($A_{510 \text{ nm}}$) (Nicell and Wright, 1997). The mixture assay contained 1.4 mL of 4-AAP/phenol solution at a concentration of 0.0025 M and 0.17 M respectively; 0.1 mL of free enzyme or 2 mg of CLEAs and 1.5 mL of 0.0017 M hydrogen peroxide. One unit (U) of peroxidase activity corresponds to the amount of enzyme that oxidizes 1 μmol of hydrogen peroxide per min at room temperature and pH 7 ± 0.2 (molar extinction coefficient, $7100 \text{ M}^{-1}\text{cm}^{-1}$).

2.4. Protein quantification

The determination of the protein amount was made by the Bradford method (Bradford, 1976) using bovine serum albumin as a standard for the calibration curve.

2.5. Characterization of RSVNP-CLEAs

2.5.1. Effect of pH and temperature

Different values of pH and temperature ranging from 3 to 9 and 25 °C to 60 °C respectively were assayed to evaluate the tolerance of CLEAs compared to the free enzyme under extreme conditions. Samples were incubated without the presence of any substrate for 15 min in the previously mentioned different cases. Then, the residual activity of the samples was analyzed.

2.5.2. Effect of organic solvents

Given the interest of using enzymes in organic media, RSVNP-CLEAs were evaluated by incubating a quantity of 10 mg in 10 mL of the different selected solvents with different polarities (**table 1**): methanol, chloroform, acetone, dimethyl sulfoxide (DMSO), hexadecane and silicone oil or polydimethylsiloxane (PDMS) V20. Tests were conducted at room temperature for 72 h. After their recovery, the CLEAs were washed and dried then the residual activity was determined at pH 7 ± 0.2 and 25 °C.

2.5.3. Storage stability

Incubation of free and immobilized enzyme for 60 days in a phosphate buffer solution (pH 7 ± 0.2) at 4 °C was carried out to evaluate their stability over time. The percentage of initial activity (100%) of the two enzymes was considered as control.

2.6. Phenolic compounds degradation by RSVNP-CLEAs

The RSVNP-CLEAs potential to degrade selected phenolic compounds was studied in 22 mL sealed glass sacrificed vials for a given time in order to establish the degradation kinetics. The medium consisted of phosphate buffer solution (50 mM, pH 7 ± 0.2) containing phenol or p-cresol at a given concentration, in which was added the required amount of RSVNP-CLEAs to achieve the desired activity. The reaction was started by adding gradually hydrogen peroxide (5 μ L) four times every 10 min through the rubber septa. Vials were kept under permanent magnetic agitation (300

rpm) and constant temperature at 25 °C. At the end of the reaction, solutions were centrifuged at 4000 rpm for 5 min and the supernatant was acidified by a few drops of 6 M H₂SO₄ solution to stop the reaction. After filtration, samples were taken for the determination of the residual phenol using HPLC and TOC analysis.

Biodegradation kinetics was studied by varying several parameters such as: initial p-cresol and phenol concentrations in the range of 20 to 160 mg.L⁻¹, hydrogen peroxide dose from 0.25 to 20 mM and initial enzyme activity varied from 0.1 to 1.8 U.L⁻¹. The objective was to determine optimal conditions under which maximum efficiency for phenol and p-cresol degradation was realized.

Experiments were conducted in duplicate.

In order to quantify abiotic degradation, control tests were performed under the same conditions without enzyme or without hydrogen peroxide. An insignificant degradation yield of 3±1% was observed in both cases.

Degradation yield (DY) and initial degradation rate (IDR) were calculated using the (Eq. (3)) and (Eq. (4)) respectively.

$$DY (\%) = \left(\frac{c_0 - c_t}{c_0} \right) \times 100 \quad (3)$$

$$IDR (\text{mg.L}^{-1}\text{min}^{-1}) = \frac{c_0 - c_t}{t_f - t_0} \quad (4)$$

Where,

C₀ = Initial concentration before enzymatic treatment (mg.L⁻¹)

C_t = Final concentration after enzymatic treatment (mg.L^{-1})

t_0 = Initial time of reaction (min)

t_f = Final time of reaction (min)

2.7. Reusability tests

Four consecutive cycles were performed to evaluate the reusability of RSVNP-CLEAs under fixed optimal conditions. Each batch was carried at room temperature $25\text{ }^\circ\text{C}$ for a given time of phenol and p-cresol degradation followed by centrifugation (4000 rpm for 5 min) of the reaction media to separate the immobilized enzyme from the solution. The recovered enzyme was reused directly without any treatment or washing in the next cycle. The efficiency of RSVNP-CLEAs in each batch was calculated by means of the degradation percentage according to (Eq. (3)).

2.8. High performance liquid chromatography (HPLC) and Total organic carbon (TOC) analysis

The residual concentration of phenol and p-cresol was measured using an Alliance UV controller HPLC system equipped with waters 717 plus autosampler. The column was a Waters C-18 ($4.6\text{ mm} \times 250\text{ mm} \times 5\text{ }\mu\text{m}$). The mobile phase for phenol concentration determination was a mixture of ultrapure water/acetonitrile (30/70 v/v) with 0.1% of formic acid and the flow rate was kept at 1 mL min^{-1} . A ratio of (20/80 v/v) ultrapure water/acetonitrile containing 0.1% formic acid was used as the mobile phase for the p-cresol amount and set at a flow rate of 0.5 mL min^{-1} . The phenolic compounds were detected at 275 nm.

TOC concentration was determined using a TOC-meter (Shimadzu TOC-VCSH).

2.9. Assessment of Michaelis-Menten reaction kinetics

Immobilized peroxidase was used to determine the initial reaction rates of phenol and p-cresol at various initial concentrations in the range from 20 to 160 mg.L⁻¹. From the data of established kinetics, Michaelis-Menten and Lineweaver-Burk curves were constructed to obtain Michaelis-Menten kinetics constant (K_m) and the maximal velocity (V_{max}).

2.10. Scanning electron microscopy of RSVNP-CLEAs

The surface morphologies of the enzyme before and after degradation essays were envisaged using a JSM IT 300 apparatus from JEOL. Samples were prepared by drying and coating with gold/palladium mixture. The SEM images were obtained with 2000 × magnification.

3. Results and discussion

3.1. Preparation of RSVNP-CLEAs

Results from this study represent two novelties. First, cross-linking and the characterization of the resulting CLEAs of RSVNP, as a new source of plant peroxidase. On the other hand, the capacity of RSVNP-CLEAs to degrade phenols under specified conditions. First step was to implement an aggregation protocol by testing different precipitant agents. Previous studies have shown the importance in the choice of the precipitation solvent which plays an important role in the preservation of enzyme activity (Šekuljica et al., 2016; Sheldon, 2011). For this

purpose, five organic solvents (acetone, acetonitrile, methanol, ethanol and 2-propanol) were tested as precipitant using 1:3 (v/v) ratio of enzyme solution/precipitant. After proteins precipitation, the formed aggregates were cross-linked with glutaraldehyde and the results are shown in Fig. 1a. The low recovered activity of RSVNP-CLEAs was observed in the case of different solvents (acetonitrile, ethanol, methanol and 2-propanol) used as precipitants, displaying a value not exceeding 10%. However, acetone showed better results yielding 20% of the initial activity recovery and 83% of aggregation yield. Loss of activity is probably due to highly denaturing effect of solvents. Hence, considering the retention of the initial activity, which was two times higher compared to the solvents selected for enzyme precipitation, acetone was adopted for further experiments. Similar results were found (Bilal et al., 2017b; Šekuljica et al., 2016) in the synthesis of HRP-CLEAs by opting for acetone as a precipitant of choice. The impact of the precipitation solvent varies from one enzyme to another and can affect the catalytic activity of the biocatalyst by denaturing the protein (Schoevaart et al., 2004). Therefore, the choice of a precipitating agent strongly depends on the tertiary structure and the properties of the protein and this is also valid for the same type of enzyme extracted from different sources (Talekar et al., 2013), which explains the different ways to prepare cross-linked enzymes.

Another parameter that can influence the preparation of CLEAs is the initial enzyme concentration. Thus, different dilutions of RSVNP mother solution were introduced and mixed with the cross-linking agent (GA:E, v/v) (1:3, 1:6, 1:9 and 1:12) using acetone for physical aggregation of proteins, which were subsequently cross-linked by

glutaraldehyde at 2% concentration. Recovered activity and aggregation yield of the resulting CLEAs are illustrated in Fig. 1b. The results revealed that the activity recovery increases up to 29 % for 1:9 (GA:E, v/v) ratio, while it was decreasing for higher values. This concentration was therefore chosen for the rest of the experiments.

The second step in the preparation of RSVNP-CLEAs is the cross-linking of the precipitated proteins with GA. The concentration of GA can be defined by the number of aldehyde groups present in the solution. Increasing the concentration leads to a large number of free aldehyde groups that react with the amine groups of the enzyme. Accordingly, it is important to quantify the amount of cross-linker required to successfully form CLEAs with tunable catalytic performance. In addition, an excess or a deficit in glutaraldehyde can influence the enzyme activity, stability, and particle size of the resulting CLEAs (**Sheldon, 2011**). Low cost, commercial availability, gentle toxicity and ease of manipulation made glutaraldehyde the ultimate choice for CLEAs preparation (**Talekar et al., 2013**). The influence of GA concentration on the activity recovery and aggregation yield of RSVNP-CLEAs is depicted in Fig. 1c. At less than 2% glutaraldehyde concentrations, a considerable amount of the enzyme was not cross-linked and lost in the supernatant or during the washing operation. The increase in the cross-linker quantity, at a rate of 0.5%, resulted in the formation of a noticeable load of CLEAs. At an optimal level of 2% GA, 29% of peroxidase activity was retained due to the presence of a large number of cross-linking agent molecules. Beyond this value (2%), the recovered activity decreased owing to the loss of enzyme flexibility, which is necessary for the catalytic activity of CLEAs (**Sheldon,**

2011). It was also observed that there was no significant change in the aggregation yield above 2% (v/v) of GA; its value remained around 85%.

3.2. Characterization of RSVNP-CLEAs

3.2.1. Effect of pH and temperature on free and immobilized enzyme

Activity test can be carried out under different pH conditions in order to study the chemical stability of cross-linked enzyme. Results, are shown in Fig. 2a. RSVNP-CLEAs showed better stability against variations in pH compared to the free enzyme. The relative activity of RSVNP-CLEAs was about 60% at pH 3 while it did not exceed 10% for the free enzyme. The optimum pH range was between 6 and 7 for both forms of the enzyme. Previously, (Bashir et al., 2018) reported similar findings with crosslinked alkaline protease from *Bacillus licheniformis* compared to the free enzyme. Loss of activity under extreme pH values could be explained by a change in the charges of the active site as well as the entire surface of the enzyme and leads to an alteration in the stereochemistry of the enzyme (Verma et al., 2019). Enzyme aggregation plays a protective role against such phenomenon.

Temperature can have two contradictory effects in enzyme catalysis. On the one hand, it can increase the speed of the reactions by increasing the number of collisions between the substrate and the enzyme and on the other hand it can cause enzyme deactivation. The influence of the temperature variation is presented in Fig. 2b; it was found that the RSVNP-CLEAs retained more than 88% of their initial activity in an interval of 25 °C to 50 °C compared to the free enzyme which posted a significant drop at a temperature of 50 °C by preserving only 45% of its initial activity. These results were confirmed by several authors who explained that this decrease in

catalytic activity is attributed to a progressive denaturation of the protein, while the cross-linked enzyme would be protected by the presence of the strong covalent bonds (Bilal et al., 2016; Verma et al., 2019).

3.2.2. Effect of organic solvents on RSVNP-CLEAs

The nature of the solvent, the size, the morphology and the microenvironment of the biocatalyst can affect the enzymatic activity in organic solvents (Sangeetha and Emilia Abraham, 2008; Xu et al., 2011). The effect of organic solvents on immobilized enzyme is illustrated in Fig. 2c. Considering the activity of CLEAs in the buffer solution pH 7 ± 0.2 as a maximum value (100%), it was found that the RSVNP-CLEAs retained 68 and 63% of their initial activity after 72 hours of incubation in hexadecane and silicone oil, respectively; while RSVNP-CLEAs retained only 38, 33, 29 and 19% of their catalytic activity in methanol, acetone, chloroform and DMSO. The drop in activity is due to the competition between the enzyme and the solvent to react with the essential water layer which is on the surface of the enzyme because polar solvents tend to peel off the water layer surrounding the enzyme by establishing bonds with the water molecules (Dordick, 1989; Fathali et al., 2019). These data indicate that cross-linking reduces the deformation of the protein structure by strengthening the rigidity of the enzyme molecules. The same conclusions were reported by Sangeetha and Emilia Abraham (2008).

3.2.3. Storage stability

The activity of the two forms of enzyme was monitored regularly for 60 days at 4 °C and results are presented in Fig. 2d, considering an initial activity at 100%. Free

peroxidase activity was almost totally lost after 30 days of storage. A biological degradation phenomenon may be involved. In contrast, a stable activity was observed with RSVNP-CLEAs, maintaining 100% of their activity over 60 days of storage, and even an increase of 10% after 30 days of follow-up. This stability can be explained by the rigidity of CLEAs which limits enzyme structural changes (**Verma et al., 2019**).

3.3. Biodegradation of p-cresol and phenol

3.3.1. Influence of the initial concentration

The degradation capacity of RSVNP-CLEAs was evaluated for different initial concentrations of p-cresol and phenol under constant temperature of 25 °C and pH 7 ± 0.2 . At fixed enzyme activity ($0.9 \text{ U} \cdot \text{mL}^{-1}$), the hydrogen peroxide was added at $t=0$ min to initiate the enzymatic reaction so as to obtain a final concentration of 0.5 mM in the reaction medium. The residual concentration of phenol and p-cresol over time is shown in Fig. 3a and Fig. 3b respectively. It is obvious that an increase in compound concentration led to a decrease degradation capacity due to saturation of active sites of CLEAs. Consequently the reaction kinetic became zero order for high concentrations (**Tandjaoui et al., 2019**). Another observation, which can be drawn from this experiences that RSVNP-CLEAs show probably more affinity for p-cresol than for phenol. For example, for an initial concentration of $100 \text{ mg} \cdot \text{L}^{-1}$ only 18% of phenol was removed within 20 min. In contrary, 63% of p-cresol degradation was obtained in 10 min of reaction time. The same deduction were made in another study, which investigate the oxidation of various phenolic compounds by *Luffa aegyptiaca*

peroxidase (Yadav et al., 2017). A distinction can be made from Fig. 3a and Fig. 3b where the degradation of phenol is less significant than for p-cresol.

3.3.2. Effect of co-substrate (H₂O₂)

The oxidation of phenolic compounds requires the use of a co-substrate which is hydrogen peroxide. A change in the oxidative state of the enzyme occurs and leads to a generation of intermediate radicals after reaction with the aromatic compound to form insoluble polymers and consequently a reduction of medium toxicity (Veitch, 2004). The effect of hydrogen peroxide dose variation (0.1-20 mM) on initial degradation rate and biodegradation efficiency of phenolic compounds by RSVNP-CLEAs was explored and responses are shown in Fig. 4a 4b, 4c and 4d. For this study, the H₂O₂ concentration was varied for each initial concentration of phenol and p-cresol (only data for 100 mg.L⁻¹, 120 mg.L⁻¹, 140mg.L⁻¹ and 160 mg.L⁻¹ are shown) by introducing the co-substrate discontinuously in four aliquots at 10 min intervals to reach the desired amount. The reaction time was extended by two times i.e., 40 min for phenol and 20 min for p-cresol in order to examine the influence of gradual addition mode. It was observed in both cases that degradation rate and conversion yield increased with increasing concentration of hydrogen peroxide. An optimal concentration between 2 and 3 mM of H₂O₂ led to a maximum dephenolization and an increase in the rate of degradation by achieving at least a yield of more than 78 and 96 ± 2% with an initial rate of 3.04 ± 0.078 mg.L⁻¹.min⁻¹ for phenol and 7.43 ± 0.15 mg.L⁻¹.min⁻¹ for p-cresol respectively. This is valid for each concentration of phenol and p-cresol investigated. Moreover, a decrease in phenol conversion was noted at co-substrate amounts greater than 3 mM. This decline was due to the inhibitory effect

exerted by H_2O_2 on the active site of peroxidase (**Bilal et al., 2016**). The same phenomenon was described in researches focusing on phenol biodegradation by horseradish peroxidase (**Alemzadeh and Nejati, 2009; Xia et al., 2016**). It was also found that the crescent addition of the oxidant improved the catalytic performances by increasing the degradation yield to 25% for phenol and 70% for p-cresol (100 mg.L^{-1}) with 0.5 mM of H_2O_2 . It seems that hydrogen peroxide was an impacting parameter for biodegradation process. Effectively, a control in co-substrate delivery while adjusting it according to the reaction rate plays an important role in oxidation procedure (**Velde et al., 2001**).

It can also be noticed that RSVNP-CLEAs showed good stability at large doses of oxidant, due to its protection against denaturing agents.

3.3.3. Effect of RSVNP-CLEAs activity on phenols removal

A study was also carried out on the activity of RSNVP-CLEAs by varying it from 0.1 to 1.8 U.mL^{-1} , at initial concentrations of H_2O_2 (2mM), phenol (100 mg.L^{-1}) and p-cresol (160 mg.L^{-1}). From Fig 5a, it was noticed that the degradation of phenol increased with enzyme dose until it reached 92 % of phenol removal with an initial rate of $2.28 \text{ mg.L}^{-1}.\text{min}^{-1}$ at 0.9 U.mL^{-1} . No further improvement was observed by increasing the amount of CLEAs introduced into the reaction medium. A dramatic decrease in phenol degradation (65% of conversion yield with a degradation rate of $1.6 \text{ mg.L}^{-1}.\text{min}^{-1}$) occurred when enzyme dose decreased to 0.45 U.mL^{-1} . This might be justified by a decrease in the availability of active sites ready to with the target compound (**Tandjaoui et al., 2019**). The experiments also revealed that a lower amount of CLEAs was sufficient to achieve more than 98% of p-cresol degradation

(Fig. 5b). Indeed, it was found that equilibrium was reached for an initial activity of $0.2\text{U}\cdot\text{mL}^{-1}$, namely 4 times lower than that used for phenol degradation, by achieving more than 98% conversion yield of p-cresol with an initial degradation rate of $7.43\text{mg}\cdot\text{L}^{-1}\cdot\text{min}^{-1}$.

3.4. Kinetics and reusability of RSVNP-CLEAs under optimal conditions

After examining the effects of the concentration of phenol and p-cresol, hydrogen peroxide and enzyme activity on the degradation process, a kinetic study was conducted under optimal conditions. RSVNP-CLEAs showed their effectiveness in the biodegradation of phenolic compounds, as illustrated in Fig. 6a and 6b. After 1 hour of reaction time; the enzymatic transformation of phenol was recorded showing a value of 92% and a yield of 78% of TOC eliminated. Additionally, 98% of the p-cresol was treated after 40 min with elimination of more than 76% of TOC. In an earlier study, immobilized polyphenol oxidase was evaluated for the degradation of p-cresol and almost complete degradation was achieved after 30 hours of transformation with the enzyme entrapped in sodium alginate-polyvinyl alcohol-silver nanoparticles matrix showing an activity of 208 Units/2g beads (**Edalli et al., 2016**). Evenly, researchers described the removal of phenol by immobilized HRP in porous calcium alginate where the maximum conversion yield was 50% with an activity of $7.5\text{U}\cdot\text{mL}^{-1}$. after 100 min of treatment (**Alemzadeh and Nejati, 2009**).

The ease of recovery and recycling of the biocatalyst is one of the advantages of enzyme immobilization for efficient use. This property was verified in the case of RSVNP-CLEAs during the biodegradation process of the two phenolic compounds over 4 successive cycles. As it appears from Fig. 7a and 7b, the performance stability

of RSNVP-CLEAs was revealed and this without any washing operation before their reuse, achieving more than 71% degradation of phenol after 3 successive batches against more than 54% elimination of p-cresol operating on 4 repeated uses. Beyond the third cycle, the decline in phenol conversion was presumably due to the polymer generated from enzymatic transformation on the CLEAs surface leading to lower activity. The use of CLEAs in stirred batch systems presents some drawbacks. In particular, a risk of damage to the cross-linked structure due to agitation and consequently a loss of enzyme (**Šekuljica et al., 2016**). In addition, a break in the prepared form causes exposure of the enzyme to the substrate and it has been reported that this phenomenon leads to an inhibition of enzyme activity (**Bilal et al., 2017b**). Finally, to overcome these disadvantages, a use of CLEAs in packed-bed reactor is strongly recommended to avoid CLEAs destructuring by passing the solution to be treated through the layer of the fixed bed.

3.5. Kinetics parameters of RSVNP-CLEAs

The kinetic parameters of phenol and p-cresol degradation by RSVNP-CLEAs were determined after obtaining profiles of the initial rate evolution as a function of substrate concentration. It has been shown that in both cases, the kinetic typically follows the Michaelis-Menten model. The Michaelis-Menten kinetics constant (K_m) and the maximal velocity (V_{max}) were determined using a Lineweaver-Burk double reciprocal plot. It was found that the K_m values for phenol 125.99 mg.L^{-1} and p-cresol 115.60 mg.L^{-1} were almost at the same order of magnitude. As for V_{max} , a value of $2.30 \text{ mg.L}^{-1}.\text{min}^{-1}$ was found for phenol and $13.70 \text{ mg.L}^{-1}.\text{min}^{-1}$ for p-cresol, respectively (**table 2**).

3.6. Morphology of RSVNP-CLEAs

The analysis of shape, size and morphology of CLEAs could be an interesting way to explore the factors influencing the formation of enzyme molecules aggregates, therefore, it allows in some way to predict their impact on the catalytic activity. Controlling these parameters would pave the way for the transformation of CLEAs from a laboratory phenomenon into a mature catalytic process (Schoevaart et al., 2004).

Images of RSVNP-CLEAs before and after using in successive batch treatment of the target compound are portrayed in Fig. 8. Before use, the cross-linked peroxidase shows an amorphous structure with a clean surface and many cavities. At the end of the fourth batch, a decrease in phenol conversion rate by RSVNP-CLEAs was observed; this could be explained by the partial inhibition of CLEAs caused by products deposition of the catalytic transformation on their surface. The picture after use confirmed this suggestion by revealing the presence of small particles on the surface of CLEAs; their morphology also differed to that of RSVNP-CLEAs.

4. Conclusion

The efficacy of immobilized RSVNP-CLEAs in the form of cross-linked enzyme aggregates was investigated for the biodegradation of phenolic compounds. The immobilization step consisted in the preparation and optimization of a robust biocatalyst with efficient catalytic performances, as well as great chemical and thermal stability compared to the free enzyme. The cross-linked enzyme showed

interesting characteristics, retaining 100% of its activity after 60 days of storage and significant operational stability in hydrophobic solvents. RSNVP-CLEAs showed promising capacity for dephenolization under mild conditions. According to the results, a total conversion of p-cresol was obtained after 40 min of treatment in the presence of hydrogen peroxide as co-substrate. Phenol was also eliminated by more than 71% after 3 consecutive batches. Given the ability of RSNVP-CLEAs to transform more than one phenolic compound, it might be a possible alternative to conventional biological treatment methods. To confirm these results, a large-scale study on similar compounds and then on industrial effluents would be useful.

Conflict of interest:

We declare that we have no conflict of interest.

Ethical statement:

We declare that there are no ethical issues form human or animal rights in the work presented here.

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Figures:

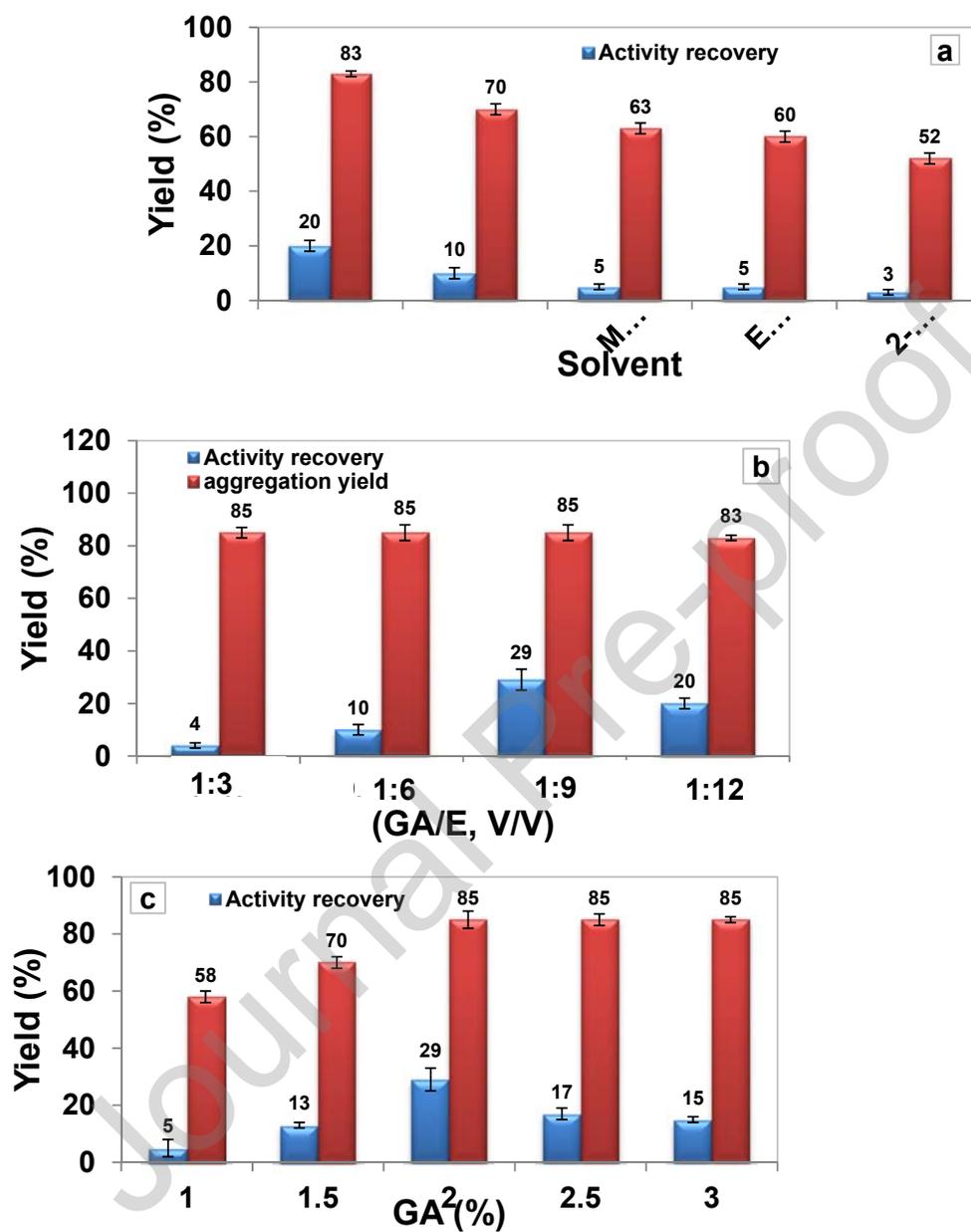
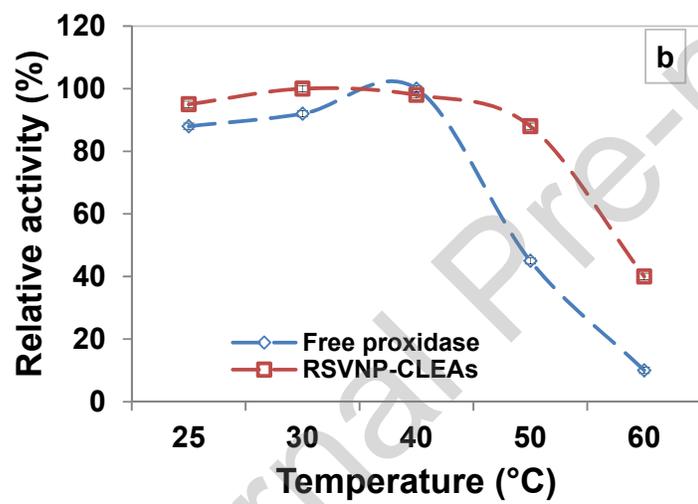
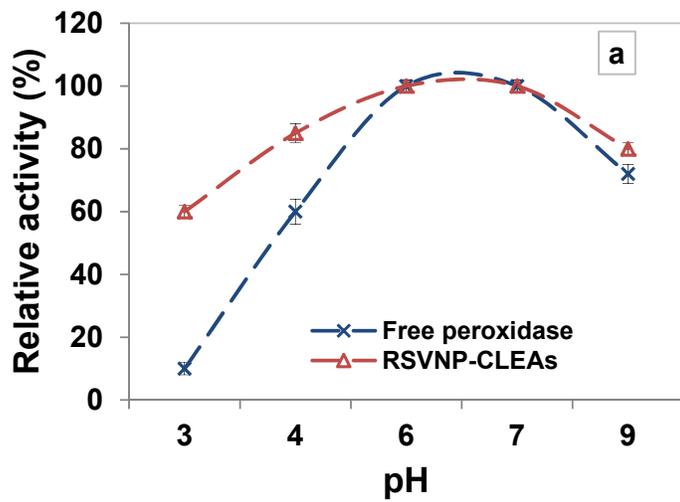


Fig 1.Effect of (a) the precipitation agent, (b) the enzyme concentration, and (c) the glutaraldehyde concentration on activity recovery and aggregation yield



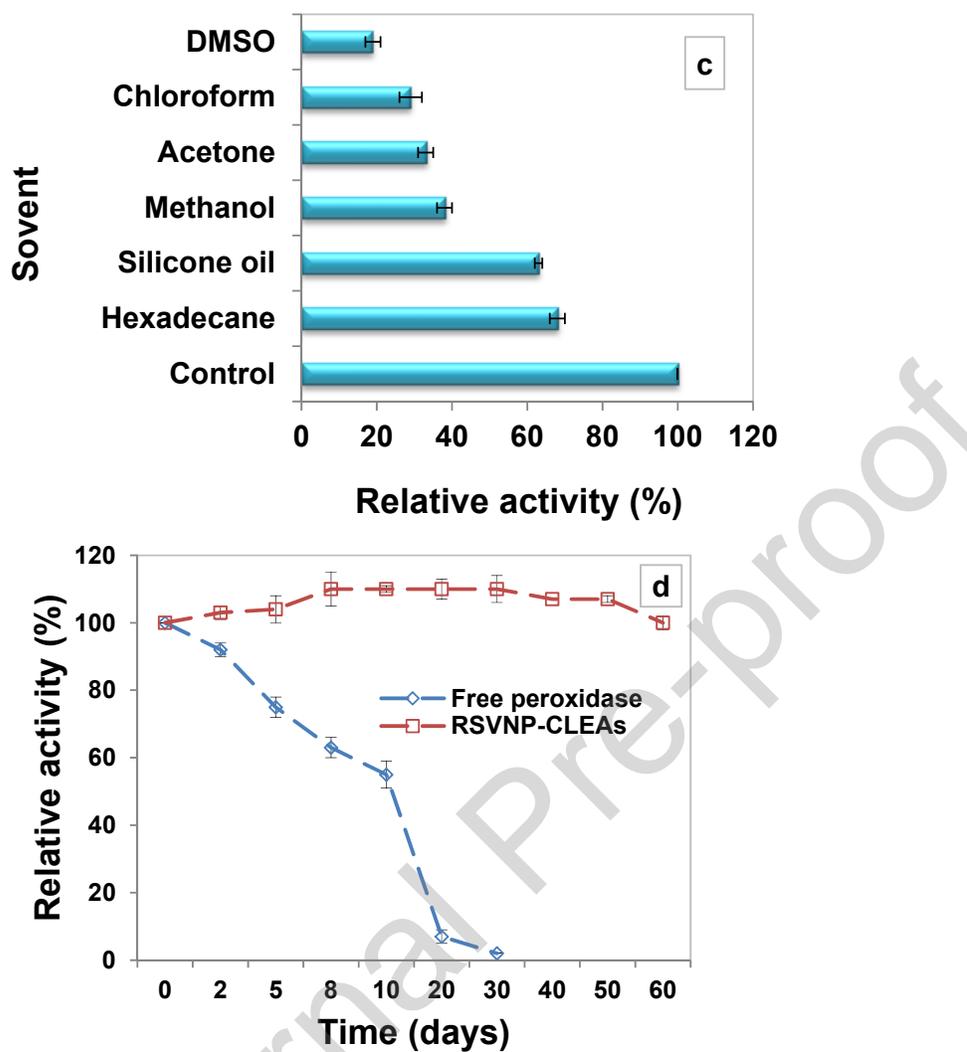


Fig 2. Biochemical characterization of RSVNP-CLEAs: effect of pH **(a)**, and temperature **(b)** on free and immobilized peroxidase, effect of different organic solvents on RSVNP-CLEAs **(c)**, and storage stability of free and immobilized peroxidase **(d)**

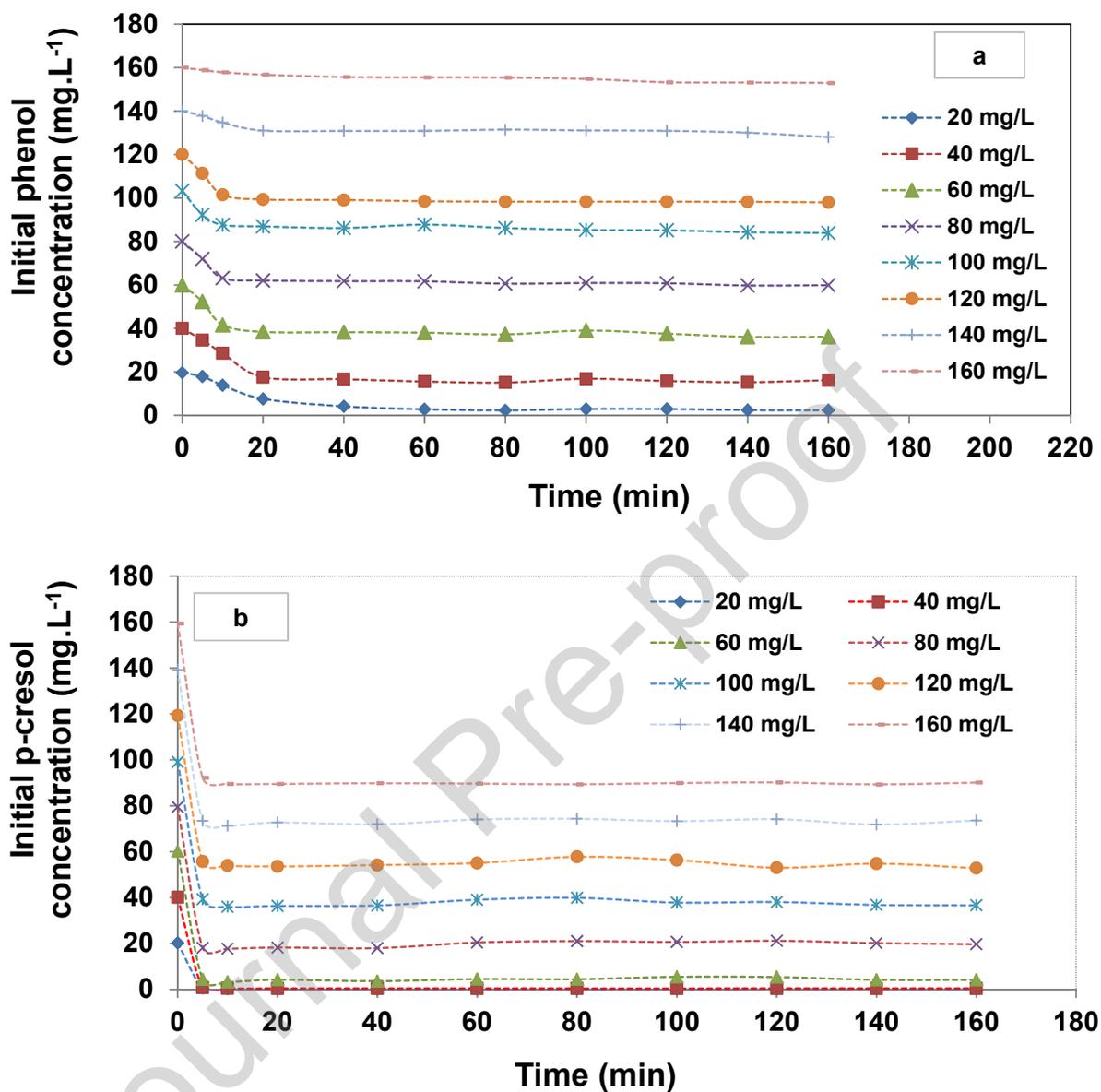
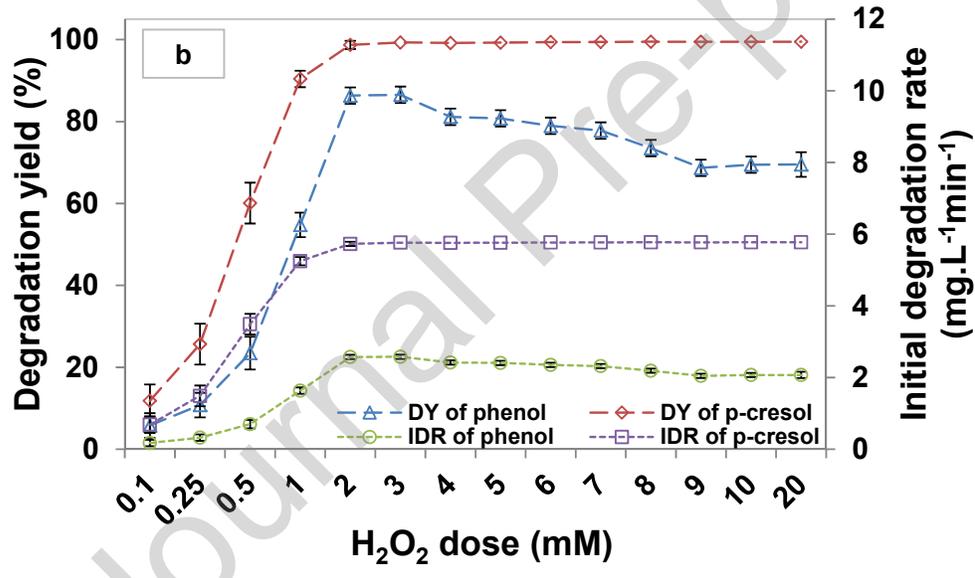
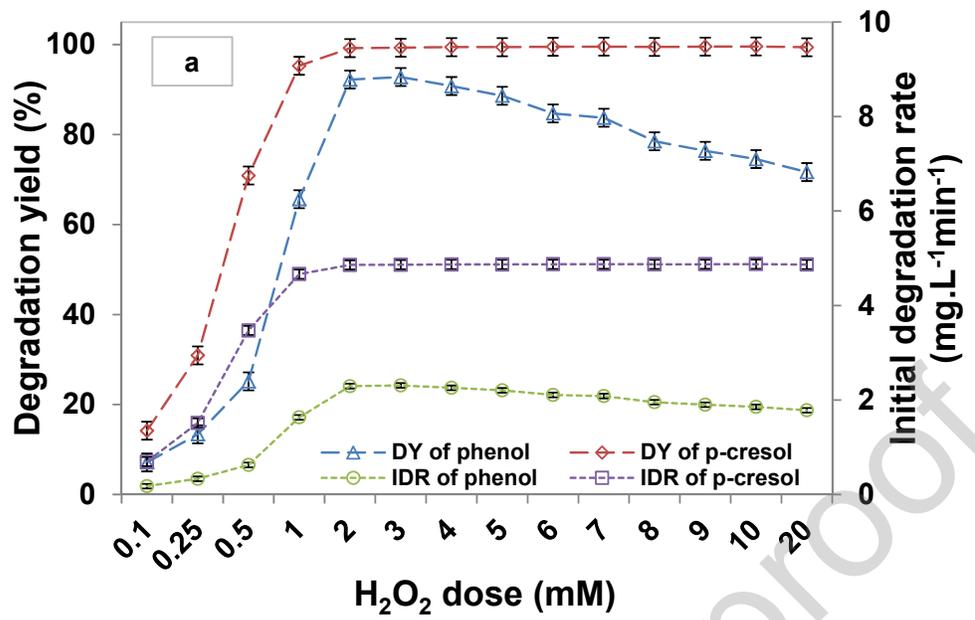


Fig 3. Effect of phenol (a) and p-cresol (b) concentration on kinetics of enzymatic transformation by RSVNP-CLEAs. Reaction mixture: 5 mL of phosphate buffer (pH 7), [H₂O₂] = 0.5 mM, 0.9 U.mL⁻¹ enzyme activity.



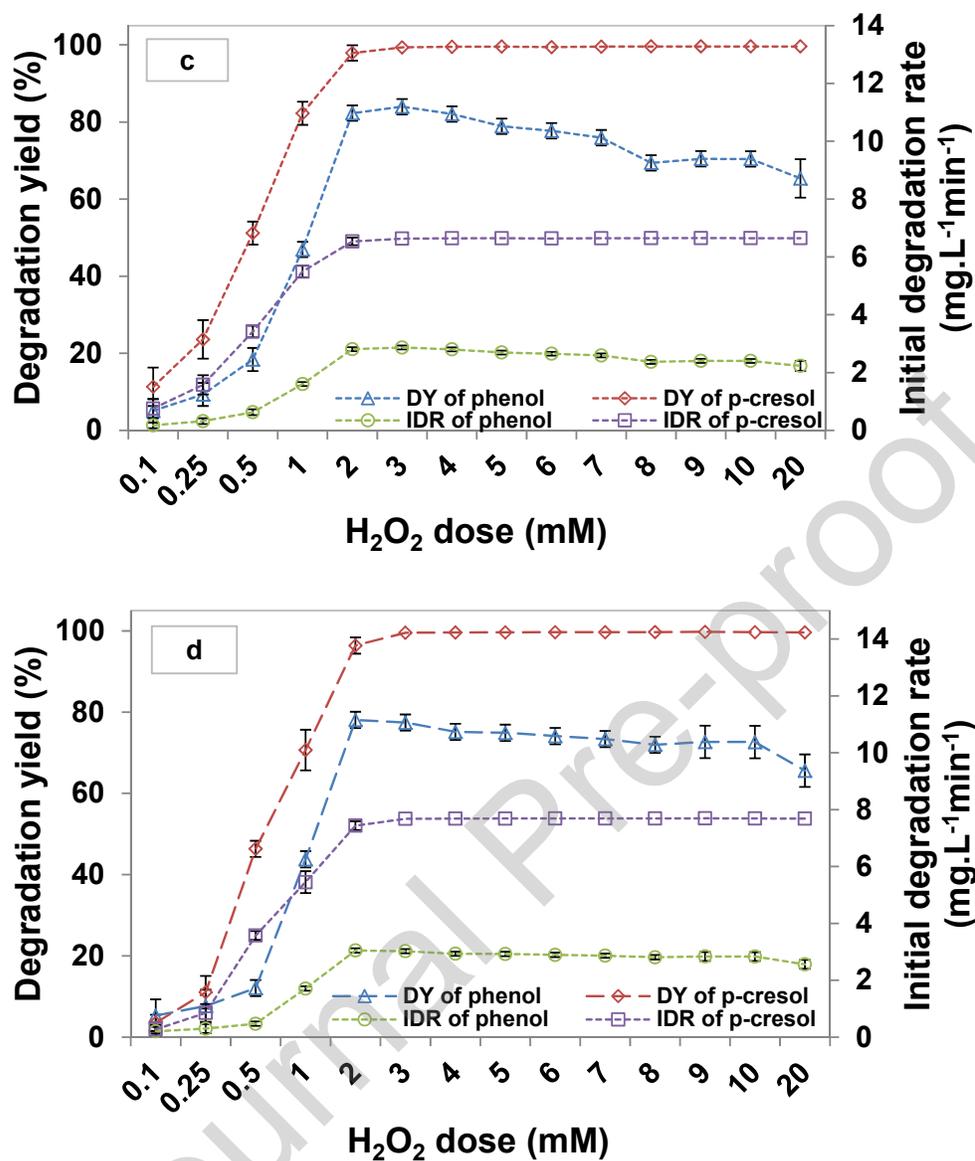


Fig 4. Effect of H₂O₂ concentration on phenol and p-cresol degradation by RSVNP-CLEAs using different pollutant initial concentration (a) 100 mg.L⁻¹, (b) 120 mg.L⁻¹, (c) 140 mg.L⁻¹, (d) 160 mg.L⁻¹. Reaction conditions: 5 mL of phosphate buffer (pH 7), 0.9 U.mL⁻¹ of initial enzyme activity.

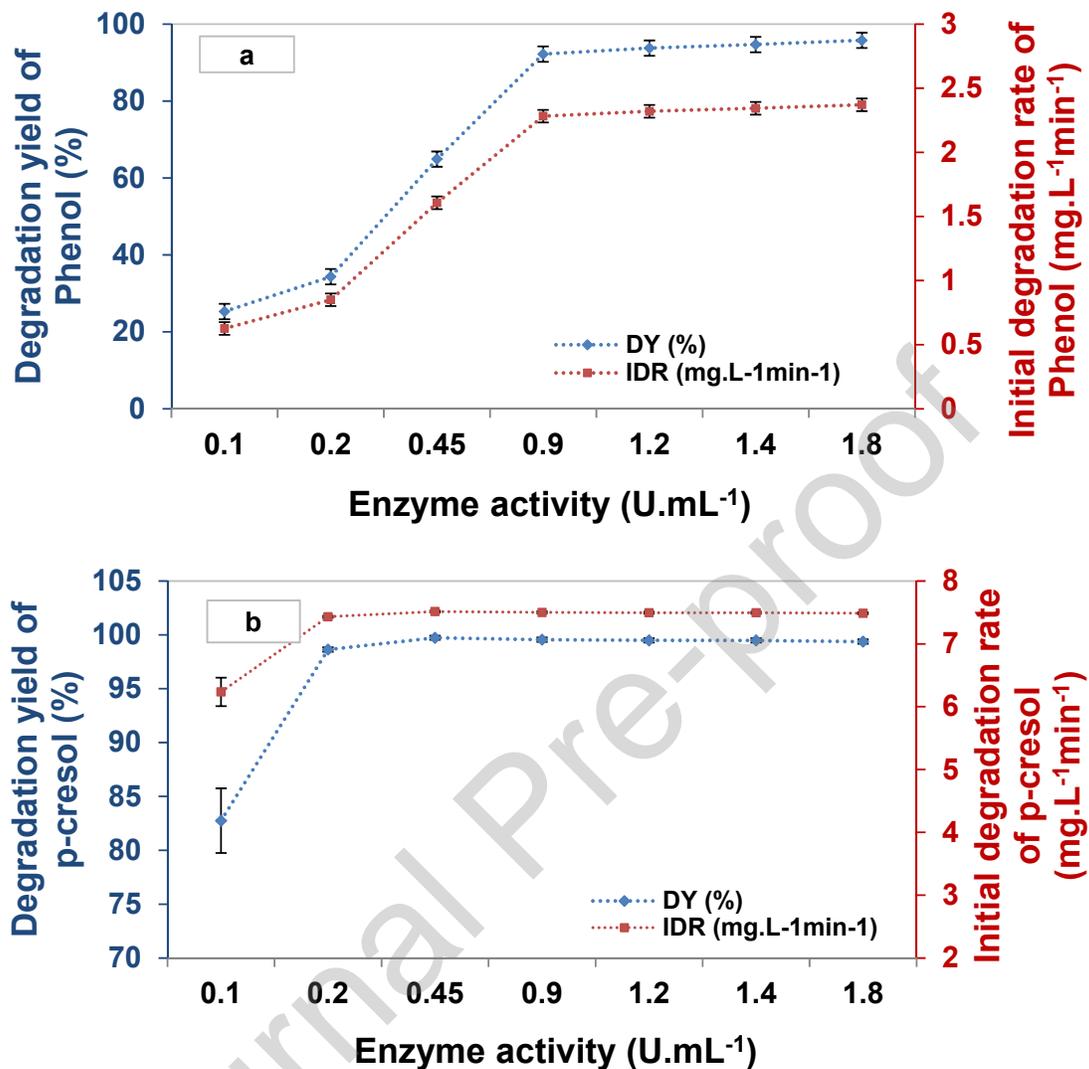


Fig 5. Effect of RSNVP-CLEAs on phenol (a) and p-cresol (b) degradation. Reaction mixture: 5 mL of phosphate buffer (pH 7), [H₂O₂] = 2 mM. 100 mg.L⁻¹ of phenol and 160 mg.L⁻¹ of p-cresol.

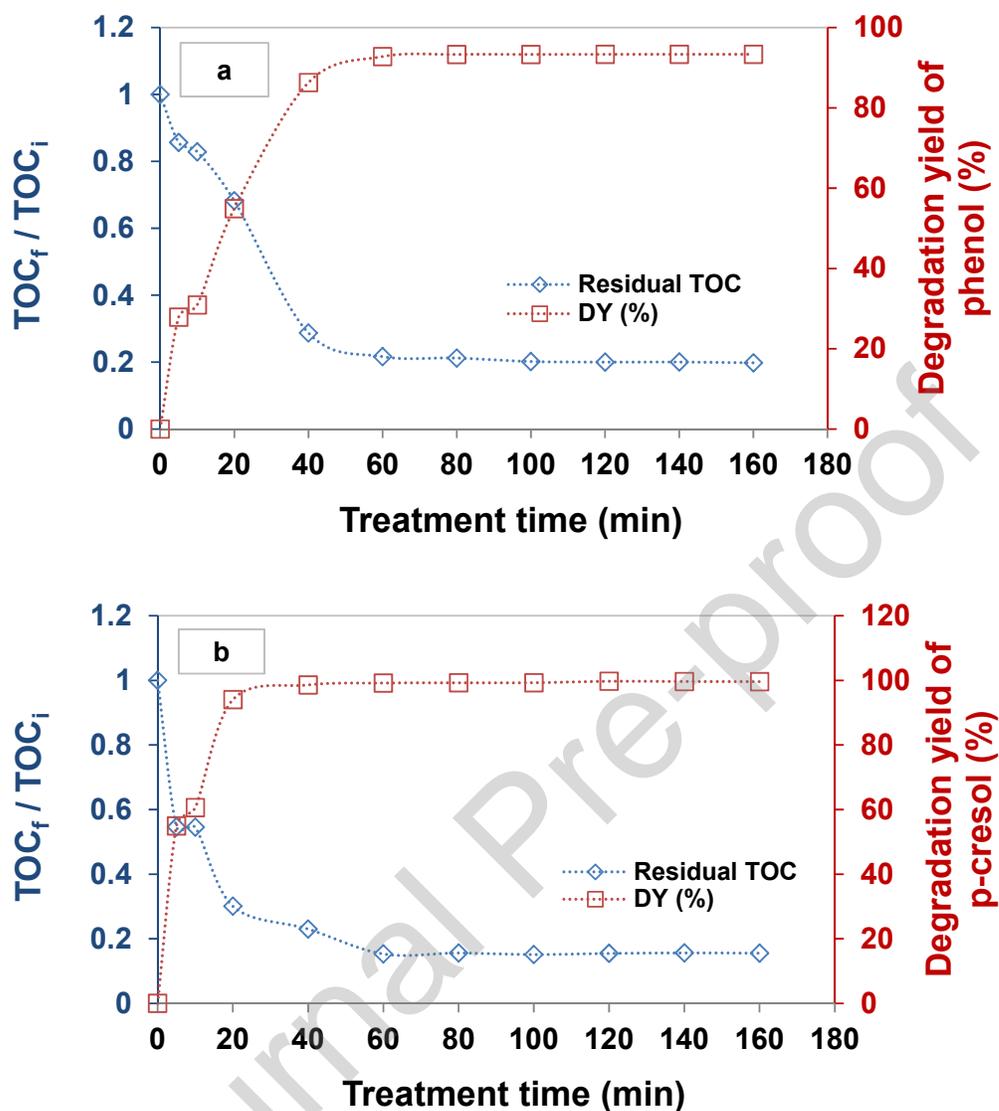


Fig 6. Enzymatic treatment of phenol (a) and p-cresol (b) under optimal conditions. Reaction mixture: 5 mL of phosphate buffer (pH 7), [H₂O₂] = 2 mM. 100 mg.L⁻¹ of phenol and 160 mg.L⁻¹ of p-cresol. 0.9 U.mL⁻¹ of RSVNP-CLEAs for phenol and 0.2 U.mL⁻¹ for p-cresol

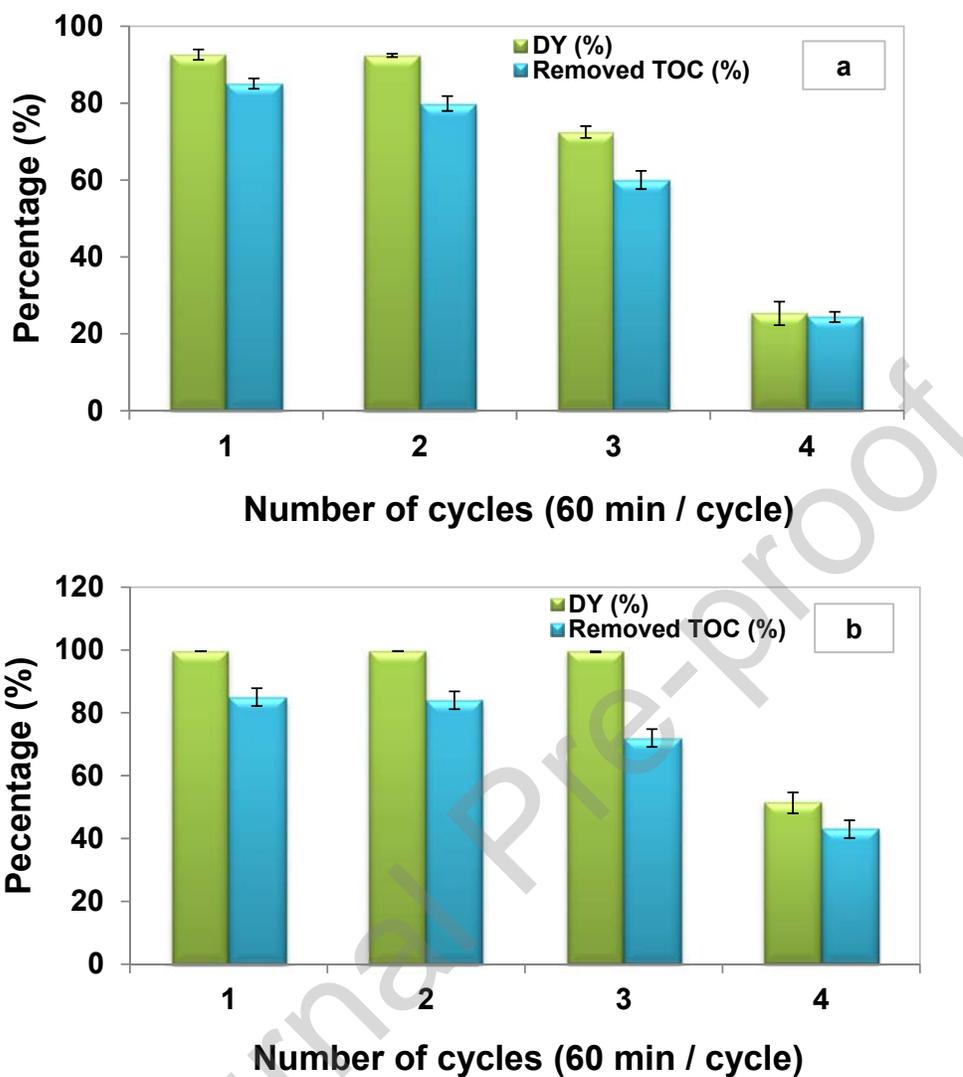


Fig 7. Reusability of RSVNP-CLEAs for phenol (a) and p-cresol (b) removal in batch process. Reaction conditions: 5 mL of phosphate buffer (pH 7), $[H_2O_2] = 2$ mM. 100 $mg.L^{-1}$ of phenol and 160 $mg.L^{-1}$ of p-cresol. 0.9 $U.mL^{-1}$ of RSVNP-CLEAs for phenol and 0.2 $U.mL^{-1}$ for p-cresol

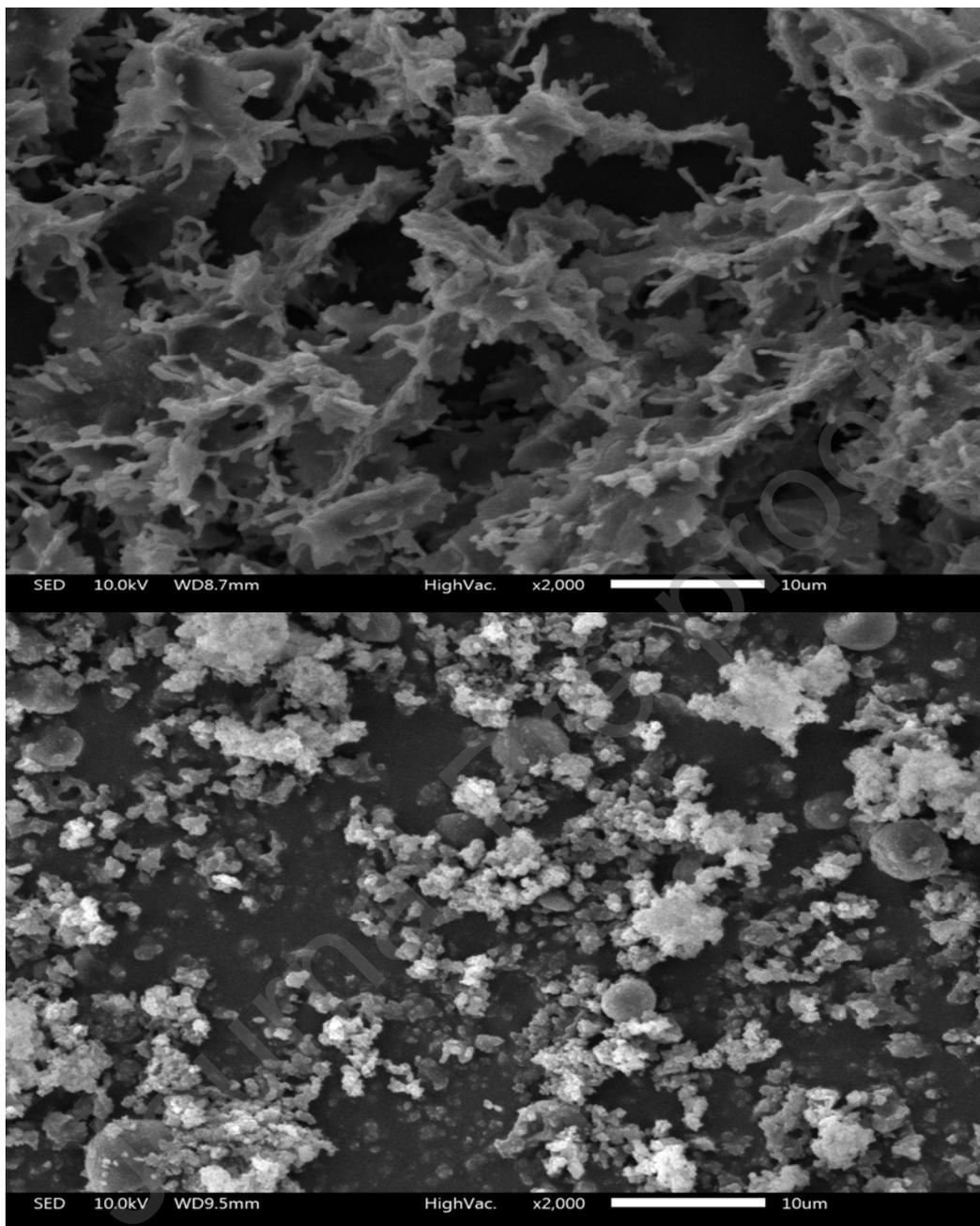


Fig 8. SEM of RSVNP-CLEAs at 2000 × of magnification, before and after use for phenol degradation.

Tables

Table 1. Characteristics of the solvents used for CLEAs stability test (Darracq et al., 2010; Fathali et al., 2019; Hahn et al., 1993; Sangeetha and Emilia Abraham, 2008; Tandjaoui et al., 2019).

Solvent	Dielectric constant ϵ	Log P
Water	80.2	1
Hexadecane	2.01	8.5
Silicone oil (V20)	2.6	4.25
Methanol	32.7	-0.74
Acetone	21	-0.1
Chloroform	4.8	2.3
DMSO	47	-1.3

Table 2. Kinetic constants of Michaelis-Menten model for phenol and p-cresol degradation.

Substrate	K_m (mg.L⁻¹)	V_{max} (mg.L⁻¹.min⁻¹)	R^2
Phenol	125.99	2.30	0.97
p-cresol	115.60	13.70	0.97