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Immobilised peroxidases from *Asparagus acutifolius* L. seeds for olive mill waste water treatment

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ABSTRACT

This work compares the main enzymatic parameters of a cationic peroxidase (AaP-1-4), purified and characterized from *Asparagus acutifolius* L. seeds (*Mol Biotechnol*, 2014, 56:738-746) and its immobilised form (Eup-AaP-1-4), on Eupergit® CM with Horseradish peroxidase. The optimum in the pH-activity profile was pH 4.0 and pH 3.0 for AaP-1-4 and Eup-AaP-1-4, respectively, Ca^{2+} cation enhanced both enzymatic activities, however, when submitted to a temperature stress (120 min at 50°C) Eup-AaP-1-4 lost only 20% activity while AaP-1-4 70%. Furthermore, AaP-1-4 was proved to be able to remove polyphenol in olive mill waste water (OMW), with hydrogen peroxide electron donor. The Eup-AaP-1-4 kinetic proprieties were investigated and the operational stability evaluated in a continuous stirred membrane bioreactor. AaP-1-4 appears to be a novel non-expensive source of peroxidases suitable for biotechnological applications in the environmental field for the removal of aqueous (poly)phenols produced from several industrial processes.

Keywords: *Asparagus acutifolius* L.; Biotechnological applications; Environmental field; Peroxidases; Waste waters treatment.

1. Introduction

Peroxidases contain a common structure consisting of a haem group formed from Fe^{3+} and protroporphyrin IX that is capable of reducing H_2O_2 using different donor substrates [1]. Haem peroxidases include two super-families: one found in bacteria, fungi, plants and the other found in animals. Ubiquitous in vascular plants are certainly crucial to growth and development [2]. The role played by individual peroxidase isoenzymes has been difficult to assess using standard biochemical techniques, due in part to the many substrates and peculiar enzymatic proprieties. In general, considering the biochemical and cytological approaches, the peroxidases are implicate in many physiological processes in plant such as: i) the generation of reactive radicals and H_2O_2 to reduce pathogen attack in seeds germination [3]; ii) the control of H_2O_2 levels as the generated reactive radicals can cleave polysaccharides in cellular growth and cell wall loosening [4]; iii) the formation of different linkages necessary to create physiological barrier or cell wall stiffening formation as well as lignification, suberisisation, formation of diferulic linkages between polysaccharides-bound lignins or polysaccharides in cell wall reinforcement, etc [5]; and iv) the induction of O_2 and H_2O_2 during the development of senescence stage [6].

Simultaneously, both academic and industrial interests in this class of enzymes either for their potential biotechnological applications [7] or for food quality are well documented, as peroxidases have been implicated in excessive browning and fiber formation in harvested fruits and vegetables [8, 9]. In the framework of environmental applications peroxidases are investigated as they are able to polymerize and precipitate aqueous phenols [7, 10]. Phenolic wastes are nowadays produced in significant quantities from several industrial processes [11]. In particular, in the Mediterranean basin and also in many other countries throughout the world where olive oil is produced, a very large

amount of phenol-polluted waters is produced [12, 13]. The wastes of this process, named olive mill waste water (OMW), are known for their phytotoxic effects causing very serious problems when spread on farmland. Thus, the identification of novel sources of peroxidases and the characterization in experimental studies of their abilities to reducing the amount of phenols and polyphenols in OMW are of interest. They could represent an important field for the environment protection especially where olive oil manufactures are settled [14]. Furthermore, several investigators proposed a recovery of these waters as some constituents of OMW have been considered reusable (excipients, sweeteners, whilst pectinolyti, mannitol, sugars, polysaccharides, phenolic substance etc.) may be used in the pharmaceutical and agronomic sectors [15]. In particular, the recovery of phenolic substances can be considered important economical sources of natural antioxidants [16].

In a previous work, we purified some novel peroxidases from seeds of *Asparagus acutifolius* L., named AaP-1, AaP-2, AaP-3 and AaP-4, and developed a fast method to obtain this pool, hereafter named AaP-1-4, for possible biotechnological use [17]. The *A. acutifolius* is a wild plant growing in the Mediterranean basin and well known in Campania, a southern Italy region [18, 19], rich in olive oil producers.

The aim of this study was to investigate the use of AaP-1-4 to remove phenols in waste waters. The optimal reaction conditions were evaluated by using a standard solution of phenol in water or OMW. Furthermore, an immobilisation procedure was carry out to obtained an Eupergit® CM immobilised AaP-1-4 (Eup-AaP-1-4), and the optimum operating conditions to perform the reaction either in batch or continuous processes were investigated. The continuously stirred UF-membrane reactor, more complex and costly compared to a batch reactor configuration, was adopted to investigate some important operational conditions, which make the scale-up of the process of potential industrial interest.

This application could be useful, because in Campania region a large amount of OMW as well as the wild asparagus used for regional sprout sales is available. Thus, it is possible to consider this cultivation as a useful and cheap source of seed peroxidases to be used in the search of biotechnological approaches for the removal of phenols either in OMW or similar waste waters.

2. Experimental

2.1. Plant material, chemicals and reagents

Seeds were harvested from *A. acutifolius* L. at the end of November in Caserta vecchia (Southern Italy; geographical coordinates: 41°05′48″N 14°21′59″E) in 2011, dried and stored at -20 °C until use. Materials for chromatography were described elsewhere [17, 20]. ABTS [2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid], guaiacol, ascorbate acid, chlorogenic acid, and phenols were purchased from Sigma-Aldrich (Milan, Italy). For enzyme immobilisation, Na-phosphate dibasic, Na-phosphate monobasic, adipic dihydrazide, Na-periodate, ethylene glycol, Eupergit® CM and Horseradish peroxidase (HRP) were purchased from Sigma-Aldrich.

Cathecol, hydroxytyrosol, tyrosol, vanillic acid, caffeic acid, coumaric acid and ferulic acid were purchased from Sigma-Aldrich or Fluka.

2.2. Basic peroxidases purification

The basic peroxidase pool (named hereafter AaP-1-4) was purified according to the procedure already reported [17, 21]. This latter, an affinity chromatography, takes advantages of specific interaction between the basic peroxidase and the S-Sepharose matrix [22].

2.3. Analytical methods

Peroxidase activity was determined spectrophotometrically according to previously reported procedures [20]. One unit of enzyme was defined as the amount (μg)

of substrate (ABTS) consumed in 1 min. Total protein content was estimated using the Bradford method with bovine serum albumin as standard. Homogeneity of isolated proteins was determined by SDS-PAGE with a Mini-Protean II mini-gel apparatus (Bio-Rad; Milan, Italy), using 6% (w/v) stacking polyacrylamide gel and 15% (w/v) separation gel [23].

2.4. Immobilisation of AaP-1-4

Briefly, three main steps were required to AaP-1-4 immobilisation that were: i) oxidation of the peroxidases by Na-periodate (periodate method); ii) pre-treatment of the polymer with adipic dihydrazide to make "hydrazido beads"; and iii) coupling of the oxidized enzyme to the hydrazido Eupergit® CM support as previously reported [24]. The effect of the immobilisation of AaP-1-4 was compared with HRP as reference, both dialyzed against 100 mM Na-phosphate buffer, pH 7.2. During immobilisation process, free peroxidase activities were checked by spectrophotometric assay using the ABTS method (Guida et al., 2011) in the same experimental conditions using 10 mM Na-acetate buffer, pH 4.0, for AaP-1-4 and 10 mM Na-phosphate buffer, pH 7.2, for HRP. The effect of the enzyme loading was investigated by varying, between 25 and 500 µg, the amount of each enzyme to be fixed to the support (10 mg). The immobilisation procedure was stopped when the activity of the free enzyme in the solution was constant.

2.5. Peroxidase assay on immobilised enzyme

The immobilised enzyme activity was carried out using the ABTS method with some modifications to analyse solid particles. In a first step, 15 mL of assay mixture (as

for the free enzyme) were added to about 300 μL of a suspension containing the immobilised enzyme-Eupergit® CM. The mixture was very quickly stirred and, on appropriate time intervals, 1 mM Na-azide was added to an aliquot of the supernatant solution (50 μL) to stop the reaction [25]. Then, the absorbance at 414 nm was measured at 30 s intervals over 5 min. The linear relationship of absorbance *versus* time allowed calculating the reaction rate. After the assay, the solid was filtered, washed and then dried until dry weight constant.

2.6. Optimum pH, stability and thermal stability studies

Optimum pH (for both free and immobilised enzymatic forms) was analysed in the pH range 2-11 using 10 mM of the following buffers: Na-citrate, pH 2.0-3.5; Na-acetate, pH 4.0-5.0; Na-phosphate, pH 6.0-7.0; Tris•Cl, pH 8.0; Na-borate, pH 9.0; Na-carbonate, pH 10.0-11.0. pH stability was evaluated using aliquots of Eup-AaP-1-4 were kept for 24 h at 25 °C in the appropriate buffer under agitation. The residual activity was evaluated at optimum pH. The free and immobilised peroxidase activities were also determined by varying the EDTA concentration (0-50 mM). Furthermore, dependence of the peroxidase activity from divalent cations was determined in presence of Ca^{2+} and Mg^{2+} (0-50 mM) [26].

The thermal stability of free and immobilised AaP-1-4 was assayed in the temperature range 35-75 °C [27]. Enzyme aliquot (1 μg) was assayed in a 0.5 mL final volume using 10 mM Na-citrate, pH 4.0, for free enzyme and 10 mM Na-acetate, pH 3.0, for immobilised enzyme, respectively. After 10 min incubation, the samples were rapidly cooled in ice-water (10 min), and residual peroxidase activity was immediately determined at 25 °C considering initial activity value of AaP-1-4 as 100% [28]. All experiments were performed in triplicate and the standard deviations were <5%.

2.7. Substrate specificity and enzyme kinetics for H_2O_2

The enzymatic activity of free and immobilised peroxidases toward various reducing substrate concentrations was performed, at 25 °C, in 10 mM Na-acetate pH 4.0 (final volume 1.5 mL), and in 10 mM Na-citrate pH 3.0 (final volume 15 mL), respectively.

The following wavelengths and extinction coefficients were used: *p*-guaiacol, 470 nm, ε= 6700 M^{-1} cm^{-1}; ferulic acid, 318 nm, ε= M^{-1} cm^{-1}; ascorbic acid, 290 nm, ε= 2.8 M^{-1} cm^{-1}; chlorogenic acid, 410 nm, ε= 2100 M^{-1} cm^{-1}; caffeic acid, 450 nm, ε= 2010 M^{-1} cm^{-1}. Peroxidase activity with phenol was analysed according to the 4-aminoantipyrine (4-AAP) method [29]. 3 nM enzyme was used in all determinations, and the addition of 5 mM H_2O_2 started the reaction.

Kinetic constants for free and immobilised enzymes were calculated by using ABTS assay as described in the preceding paragraph, at the optimum pH. K_M value was obtained from the Lineaweaver-Burk plot for the two-substrate mechanism followed by peroxidase [30]. The Michaelis-Menten constant K_M and V_{max} values for H_2O_2 were determined as the reciprocal absolute values of the intercepts on the x- and y-axes, respectively, of the linear regression curve (with a correlation coefficient , R^2 = 0.948). Kinetic constants (H_2O_2 K_M and v_{max} values) for immobilised enzyme were determined in the same reaction conditions reported above. All experiments were performed at least in duplicate and the standard deviations were <5%.

2.8. Operational thermo-stability

Free and immobilised peroxidase activities were studied in stirred ultrafiltration-membrane reactor, operated continuously [31] and equipped with PM 10 UF-membranes (MWCO 10,000 Da) which ensured total retention of free enzyme. The reactor, 70.0 mL volume, and stirred at 250 rpm, was loaded either with 50 μg of native enzyme or with 100 μg of Eup-Aap-1-4. Substrate solutions, prepared in a buffer A (10 mM of Na-acetate, pH 4.0, for free enzyme, and 10 mM of Na-citrate, pH 3.0, for immobilised enzyme), containing 2 mM ABTS, and a solution of 5 mM H_2O_2 (kept separated to avoid spontaneous formation of $ABTS^+$) were fed (at 20 mL h^{-1}), using a peristaltic pump (Gilson Italy, Cinisello Balsamo, MI). The module was fully immersed in a water bath and the temperature was controlled (±0.1 °C) by means of a thermostat. The reaction product absorbance was monitored, for 72 h at λ414 nm, spectrophotometrically using a continuous flow quartz cuvette. Product concentration was calculated using a ε=36 mM^{-1} cm^{-1}. A fraction collector collected the outflowing stream.

2.9. Phenol removal

The reaction of phenol in the presence of H_2O_2 was also performed in a batch system with both free and immobilised enzymes using 10 mM Na-acetate, pH 4.0, and 10 mM of Na-citrate, pH 3.0, respectively. The reaction was started by addition of 5 mM H_2O_2 to the phenolic solution (2.0 mM) containing free and/or immobilised enzymes and was stopped adding 10% phosphoric. In all experiments, samples (1 mL) were withdrawn at different reaction times, centrifuged and the supernatant analysed by HPLC. The rate of non-enzymatic reactions was ascertained by monitoring the rate of

phenol production after addition of H_2O_2 [32]. The activity of peroxidase on phenol was also determined in the presence of Ca^{2+}.

2.10. Enzymatic treatments of olive-mill waste water (OMW)

The peroxidase treatment of OMW was carried out with free peroxidase (purified AaP1-4) and compared to that performed with HRP. The OMW was clarified by vigorous shaking and then filtered through Miracloth paper (Calbiochem, Milan, Italy). Reagents were added in the following order: 50 mM buffer, (Na-acetate, pH 4.0 for free peroxidase, Na-phosphate, pH 7.2 for HRP), enzyme (20, 50 and 100 μg) and OMW solution. Reactions, performed at 25 °C, in of 1.5 mL and under constant stirring conditions, were initiated adding 5 mM H_2O_2. Substrate volatilization and/or spontaneous transformation, were evaluated in tests carried out without enzymes. After incubation, samples were analysed for polyphenol concentration and residual enzymatic activity. For polyphenol concentration, 1 mL of samples and their corresponding controls without peroxidase were added to 100 μL of 10% phosphoric acid to stop the enzymatic reaction. The samples were, then, centrifuged for 15 min at 12000 *g*. The supernatants were filtered through 0.45 μm filters (Acrodisc LC 13 PVDF, Sigma-Aldrich) and analysed by high-performance liquid chromatography (HPLC). To calculate residual peroxidasic activity 250 μL of each sample was immediately assayed using ABTS, under standard conditions.

2.11. Qualitative analysis of the phenol and OMW's polyphenol concentration.

The phenol concentration of the liquid samples and the supernatants of OMW treatments were analysed and quantified using HPLC Agilent Technologies 1100 Series

(Cernusco s/N, MI, Italy) using a C-18 column (i.d. 0.4×25 cm, Beckman Coulter, Cassina De' Pecchi, Milan, Italy). The best resolution was achieved, at constant flow rate of 1 mL min^{-1}, using an elution concentration gradient and a mobile phase made of 1% glacial acetic acid (solvent A) and methanol (solvent B). The mobile phase composition started at 25% B for 5 min, followed by a linear increase in B to 95% over 5 min. A return step to the initial conditions lasting 15 min was necessary before the next run. The detection of phenol compound was performed with an UV with diode array detector (DAD) at λ272 nm, the maximum for phenol absorbance.

Linear calibration curves relating peak area to analyte concentration (within the range 0.2 -10 mM) were constructed. The polyphenol concentration in OMW was determined using these calibration curves. The mobile phase (1.0 mL min^{-1}), a mixture of solvent A and solvent B had a gradient change in the composition: 5% B for 5 min, to 75% B in 20 min and then 95 % B for 5 min. The UV detector was set at 272 nm.

2.12. Total phenol content determination

Total phenol amounts were quantified colorimetrically by using Folin-Ciocalteau (Sigma-Aldrich) assay [9]. As standard was used the tyrosol. All samples were analysed in triplicate. All experiments were performed in triplicate and the standard deviations were <5%.

3. Results and discussion

3.1. OMW enzymatic treatment with free AaP-1-4

The investigation on a possible biological treatment, to reduce the toxicity of the OMW, was carried out in batch reactors using peroxidases as biocatalyst. To this end, we studied the reactivity of the AaP-1- on OMW; AaP-1-4 are the purified basic peroxidases obtained from seeds of *A. acutifolius* following the procedure previously reported in Guida et al., 2014. In a preliminary study, using different amounts of free AaP-1-4 and free horseradish (HRP) peroxidases (20, 50 and 100 μg), the concentrations of OMW were kept constant. The phenol concentration in OMW at the different enzyme concentration and at different time are reported in **Table1** and were obtained in a batch reactor configuration. The data clearly show that, using the same amount of enzyme for all the assayed concentrations, higher conversion percentages can be obtained with free AaP-1-4 rather than with free HRP. In fact, both Folin-Cioccalteau and HPLC analyses showed about 50% transformation of the total phenolic content, compared to the relative control, after 24 h incubation with AaP-1-4 (50 μg). Although the AaP-1-4 mediated transformation was lower than that expected on the basis of results achieved with single phenols (**Table 2**), it was however higher than that observed with horseradish peroxidase, thus confirming a better catalytic efficiency of AaP-1-4 for the reaction even in the presence of a more complex phenolic sample.

The catalytic efficiency of peroxidase was evaluated also with a second addition of peroxide hydrogen to the reaction medium, and after a further incubation of 24 h (data not shown). The data validate its applicability for OMW de-phenolization as shown in **Fig. 1** that compares the HPLC profiles of untreated and treated with AaP-1-4 OMW-

samples. The reaction catalysed by AaP-1-4 progresses faster than the HRP reaction that suddenly stops after 24h incubation. AaP-1-4 maintains its activity longer, so attaining better percentages of phenolic removal. This behaviour might be due to a faster inactivation process of HRP. After 48 h treatment with 100 µg of AaP-1-4, the total phenolic content decrease was higher than 55%. These results suggest that HRP shows a lower catalytic efficiency, when applied to a more complex phenolic mixture, than *A. acutifolius* peroxidase.

A rational validation of the speculated removal through polymerization and precipitation of phenols, generally reported in the literature [14], could derive from the investigation of the polyphenolic profile. Better results of an HPLC analyses of the sample, exhibiting the amount of polyphenol remained (**Table 2**), revealed consistant alteration of the peroxidase treated waste when compared to the untreated waste (**Fig. 1**).

In this study, we found that treatment with native AaP-1-4 (100 µg) led to a significant reduction in the total phenol content in accordance with the results of the quantitative analyses described before. Particularly, vanillic, caffeic and coumaric acids, and cathecol were completely degraded after 24 h of reaction, while the hydroxytyrosol, tyrosol and ferulic acid persisted after the treatment, and the remaining compounds (not identified) were also reduced in amount. The *Asparagus* peroxidase catalysed reactions, on phenol degradation in olive-mill waste water, enable reductions higher than 80% for hydroxytyrosol and tyrosol, and 40% for ferulic acid. In waste water sample treated with the HRP (100 µg), only the vanillic and caffeic acid were degraded, while it was observed that the peak areas of substances detected at 275 nm was decreased with a reduction of 60% for cathecol, 60% for coumaric acid, 65% for hydroxytyrosol and tyrosol, and only the 30% for ferulic acid, after 24 h of reaction (data non shown). Both

enzymatic reactions, for the removal of phenolic compounds were achieved through the formation of insoluble polymers.

It is important to note that studies on polyphenol identification during enzymatic catalysed reactions are scarce in the literature, as most of them make use of selected microorganisms in order to evaluate phenol degradation and its impact on olive-mill wastes detoxification. Certainly we are aware that the efficient OMW remediation cannot be based on a bioprocess using free enzymes such as *Asparagus* peroxidase. It is irrefutable that the extraordinary burden of OMW in phenolic compounds would not permit any oxidative enzyme to function to a satisfactory extent. Nevertheless, enzymes can be used as an additional means of removing toxic substances, thus contributing to an integrated treatment of OMW and similar effluents. However, the exploitation of by-products for producing peroxidase-active preparations might merit a higher attention as a material with promising prospect in bioremediation, as the use of AaP-1-4 provides an economical alternative to HRP-catalysed removal of phenols from waste waters.

3.2. The immobilisation of AaP-1-4

The decrease of polyphenols content in OMW with AaP-1-4 prompted us to explore its possible use as immobilised form. The immobilisation method is based on the use of Eupergit® CM resin as previously described [24].

The progress of enzyme immobilisation onto the matrix was monitored by measuring the activity of the remaining free enzyme (either AaP-1-4 or HRP) present in the solution. We assumed that, during immobilisation process, as long as free linkers were present in the support, enzyme activity in the solution diminished and thus the enzyme was still binding. Only once all linkers were saturated, the unbound enzyme in the solution remained constant and thus, its measured activity was also constant. The

activity profile *versus* time obtained for the AaP-1-4 enzymatic activity in the solution during the immobilisation is shown in **Fig. 2 A**. These results were compared to those obtained, performing in identical conditions the same experimental procedure on HRP enzyme (**Fig. 2 B**). The immobilisation parameters for HRP were higher than those for AaP-1-4. In both experiments, when 25 µg of enzyme was used, almost the entire amount of free enzyme was coupled within 5 h to 10 mg of support. The increase of enzyme concentration required more time to complete the immobilisation procedure, indeed, with 500 µg and 24 h incubation, about 50% of AaP-1-4 was unbound to the support, while for the HRP the unbound enzyme was about 20%. Therefore, the amount of immobilised enzyme per gram of dry support was for AaP-1-4 24.8 $mg_{AaP\text{-}1\text{-}4}\ g^{-1}$, roughly half that obtained with HRP, 40 $mg_{HRP}\ g^{-1}$

3.3. Optimum pH and stability of immobilised enzyme

The optimum pH for any peroxidase depends on the hydrogen donor and buffer solutions used in the activity assay. Using ABTS as H donor, free and immobilised AaP-1-4 showed maximum activity at approximately pH 4.0 and pH 3.0, respectively (**Fig. 3**). This shift of the pH optimum towards more acidic pH-values was attributed to the new microenvironment that might be quite different from that of free enzyme in the bulk medium. Immobilisation introduces changes in the surrounding environment of enzyme, due either to the physical and chemical nature of the support or to the interactions with substrates or products of the reaction. Most likely these new electrostatic interactions and hydrogen bonds might significantly contribute to enzyme stability in presence of resin support. Immobilised AaP-1-4 on Eupergit® CM, remained stable (data not shown) over a wide pH range, between pH 3.0 and 8.0, and retained ≥ 80% of its original activity. However, by lowering the pH of the solution below 2.8, a

sharp decline in stability occurs, and at a pH 2.2 the enzyme loses all its activity. This loss has been attributed, for other peroxidases, to the dissociation of the haem prosthetic group from the polypeptide chain [33].

The presence of Ca^{2+} and Mg^{2+} in the solution modulates the activity of many peroxidases and thus the free and immobilised enzyme activity was also investigated in their presence at different concentrations. Free AaP-1-4 showed an increasing activity in the presence of Ca^{2+} up to 20 mM, but at higher concentrations (up to 50 mM) the activity was constant. On the contrary, while the presence of lower concentration of Mg^{2+} (20 mM) increased the activity, higher concentrations decreased it (**Fig. 4 A**). In particular, the activity of free AaP-1-4 increased in the presence of Ca^{2+} *approx.* 4-fold with respect to Mg^{2+} at pH 4.0. The protective effect of Ca^{2+} at pH 3.0 is also observed on Eup-AaP-1-4 (**Fig. 4 B**) indeed, incubated up to 1 mM Ca^{2+}, 1.4-fold increase was evaluated with respect to Mg^{2+}. At concentration higher than 1 mM of each ion, the activity decreased apparently with the same rate, but the Ca^{2+} assured a higher activity. The major role played by these bound calcium ions appears to be the maintenance of the protein structure around the haem pocket. The storage stability of the Eup-AaP-1-4 was also tested. After 1 month storage at 4 °C in water, *ca.* 90% initial activity was retained by Eup-AaP-1-4. Under the same conditions, *ca.* 20% initial activity was lost for the free AaP-1-4. Thus the immobilised form of AaP-1-4 appears to be more stable during storage being the activity loss lower.

Free AaP-1-4, exhibited average thermal stability, because about 30% of the initial activity was retained after heating at 55 °C for 10 min at pH 4.0 (**Fig. 4 C**). Midpoint for free AaP-1-4 inactivation temperature (T_m), defined as the temperature at which 50% of enzyme activity is lost, was 45 °C. Immobilised enzyme showed higher resistance than free AaP-1-4, with ~80% retention of initial activity after the same heating time at pH 3.0; the T_m value was in this case 65 °C.

The higher stability of the immobilised enzyme was further supported by the results achieved in a long-term experiment performed at constant temperature (50 °C), and illustrated in **Fig. 4 D**. As shown the activity of the free AaP-1-4 decreased dramatically increasing the incubation time; two hours later, only ~30% of the initial activity was retained. Interestingly, under the same conditions, the activity of Eup-AaP-1-4 decreased slowly and only ~20% of the initial activity was lost after 2 h incubation **Fig. 4 D**. The improved thermal stability for immobilised enzyme should be due to the multipoint attachment between AaP-1-4 and the Eupergit surface, which makes the most likely AaP-1-4 tridimensional structure more stable. This improved thermal stability of Eup-AaP-1-4 is of great interest for practical and economically viable industrial applications. These latters usually require repetitive use of enzymes, long process time in continuous operations, high temperature to enhance reaction rate, conversion and solubility of some reactants and products.

3.4. Substrate specificity and kinetic parameter determination

Peroxidases showed different specificity over substrates normally involved in lignin biosynthesis [34]. Thus, the substrate specificity of free AaP-1-4 was investigated (**Table 3**). Free AaP-1-4 displayed a high oxidizing capacity of phenols, in the presence of H_2O_2, which may be important for the removal of phenolic compounds from waste waters. The high activity for phenols was showed for the immobilised enzyme, despite a loss of 40% with respect to the free enzyme. Moreover, both free and immobilised AaP-1-4 showed important ascorbate peroxidase activity. Lower specific activity for free and immobilised AaP-1-4 was detected in presence of ferulic acid, caffeic acid, chlorogenic acid and guaiacol.

Lineweaver-Burk double-reciprocal plots for free and immobilised AaP-1-4 showed parallel lines, indicating that peroxidase isoenzymes follow ping-pong mechanisms [30]. Kinetic parameters for free and immobilised AaP-1-4 were determined using H_2O_2 as substrate. The V_{max} values for free and immobilised AaP-1-4 were found to be 10.20 μM min^{-1} and 5.21 μM min^{-1}, respectively, whereas the K_M values 1.01 mM and 4.17 mM, respectively.

The V_{max} value was decreased significantly upon covalent immobilisation on the support. The affinity of the substrate for the enzyme appeared slightly affected in the case of Eup-AaP-1-4 being the K_M value higher. Indeed, the K_M value for the immobilised enzyme increased about 4 fold compared to the free form. Diffusional limitations that give rise to a local gradient concentration, different from bulk concentration, steric hindrance modulating the access of the substrates to the active site of AaP-1-4, and/or the change of the active enzyme conformation after covalent immobilisation on the beads are factors regulating this affinity decrease. These same factors could also explain the change of V_{max} value, the lower turnover number (kcat, substrates molecules consumed per second per molecule of peroxidase) and the catalytic efficiency (kspe = kcat/Km) values after the immobilisation. Finally, free and immobilised AaP-1-4 showed an enzyme turnover (k_{cat}) values of 5.72 10^{-3} s^{-1} and 3.83 10^{-4} s^{-1}, respectively.

3.5. Phenol removal from water with immobilised AaP-1-4

The high substrate specificity of AaP-1-4, prompted us to investigate the opportunity of using Eup-AaP-1-4 for phenol removal from aqueous solutions. The investigation was performed with both free and immobilised forms of AaP-1-4 and the results were compared. The time course of free and immobilised AaP-1-4 activity in

phenol removal was determined in batch experiments carried out under constant stirring conditions. The reaction medium, containing 2 mM phenol, 5 mM H_2O_2 and buffer solution, was incubated with different amounts of Eup-AaP-1-4, ranging from 0.75 to 3.0 μg (**Fig. 5 A**). The batch reactor configuration allowed reaching an average value of residual phenol , around 60% after 1 h of reaction and 3.0 μg of Eup-AaP-1-4e. This value of phenol conversion was almost the same one obtained using 2 μg of free enzyme (**Fig. 5 B**) in 20 minute reaction (40% phenol removal). Furthermore, as shown in **Fig. 5**, the degree of phenol oxidation is influenced by the initial enzyme concentration. The addition of the optimum concentration of Ca^{2+}, increased the degree of phenol oxidation by the two forms of the enzyme. In fact, with 1 mM of $CaCl_2$, the activity for 3.0 μg Eup-AaP-1-4 increased reaching 60% phenol removal. Instead, 2 μg of free AaP-1-4 in the presence of 20 mM Ca^{2+} increased up to b 70% removal, leaving nearly 30% of residual phenol, after 60 minutes. The inactivation of free AaP-1-4 at 2 μg mL^{-1} concentration was evident after 30 minutes incubation and the addition of fresh enzyme, 2.00 μg, re-started the removal of the phenolic compounds as shown by the change of slope in **Fig 5 B**.

3.6. Operational stability

The operational stability of both free and immobilised AaP-1-4 was investigated, at 25 °C, for process time as long as 70 h, in a continuous stirred UF-membrane reactor. This reactor configuration was successfully adopted for the investigation of inhibiting effects of lignin-derived compounds, similar to those present in OMW [35]. The reactor was loaded either with 50 μg of free AaP-1-4 or 100 μg of Eup-AaP-1-4 and fed with a buffered solution of 2 mM ABTS. The product formed, which was continuously monitored measuring the optical density at 414 nm, was at most 1.3% of the inlet

substrate concentration, thus assuring the reactor to be operated in differential conditions. The time course of the enzymatic activity decay, shown in **Fig. 6,** gave rise to exponential curves, linearized in a semi-log plot, that allowed the hypothesis of a first-order deactivation mechanism [31]. Interestingly, rather rapidly both reactors reached the steady-state regime, being the mean residence time (reactor volume to flow rate ratio) roughly 3.5 h. It is evident that the activity of Eup-AaP-1-4 remains almost constant during the whole period regardless the adopted operational conditions of temperature, stirring speed, and concentration of substrates and reaction products. The first-order operational inactivation rate constant, k_i, of the Eup-AaP-1-4 was calculated from above data as $k_i = 1 \times 10^{-3}$ h with an half-life of 693 h. The free AaP-1-4 exhibited a different behaviour being its k_i an order of magnitude higher (0.3×10^{-2} h) giving rise to a lower half-life of 30 h. Consequently, as shown in **Fig. 6**, the difference in the inactivation process, most likely due to the protective effects introduced by the immobilisation, allowed to preserve for a longer time a higher reaction rate for the immobilised AaP-1-4, even though the preparation loaded in the reactor (100 μg) exhibited a lower specific enzyme activity at time zero (0.089 versus 0.254 μmol $(min*mg_{Enzyme})^{-1}$. The potential applicability of this new enzyme in a real process is supported by the above data that also clearly show the higher performance of the continuous reactor operated with the immobilised form of AaP-1-4 from the 45th hour onwards, thus allowing the use of cascade reactors to reach high-yield conversion [36].

4. Conclusions

A new peroxidase (AaP-1-4 from *A. acutifolius* seeds), with high catalytic efficiency in OMW removal, was immobilised on Eupergit® CM. The support binding capacity was 40.0 and 24.8 mgE/g support. Comparing the two enzymatic forms, the immobilised one (Eup-AaP-1-4) exhibited broader pH range and higher operational stability. The Eup-AaP-1-4 half-life was 693 h, 23 fold higher than that of free AaP-1-4. This work supports AaP-1–4 as a candidate enzyme for biotechnological applications in an integrated treatment of OMW or similar wastewaters, representing an alternative useful tool for removing contaminating polyphenol compounds. However, further studies should assess the economical impact of this approach.

Acknowledgements

This study was made possible by care and abnegation of all participants. This research was supported by funds from the Second University of Naples and University of L'Aquila.

References

1 M. Gajhede, *Biochem Soc Trans*, 2001, **29**, 91-98.
2 R. Malinowski and M. Filipecki, *Cell Mol Biol Lett*, 2002, **7**, 1137-1151.
3 T. Kawano, *Plant Cell Rep*, 2003, **21**, 829-837.
4 P. Schopfer, C. Plachy and G. Frahry, *Plant Physiol*, 2001, **125**, 1591-1602.
5 S. Hiraga, K. Sasaki, H. Ito, Y. Ohashi and H. Matsui, *Plant Cell Physiol*, 2001, **42**, 462-468.
6 C. Cosio and C. Dunand, *BMC Genomics*, 2010, **11**, 528.
7 B. J. Ryan, N. Carolan and C. O'Fagain, *Trends Biotechnol*, 2006, **24**, 355-363.
8 A. K. Raimbault, P. A. Marie-Alphonsine, J. P. Horry, M. Francois-Haugrin, K. Romuald and A. Soler, *J Agric Food Chem*, 2011, **59**, 342-348.
9 V. Guida, G. Ferrari, G. Pataro, A. Chambery, A. Di Maro and A. Parente, *LWT - Food Science and Technology*, 2013, **53**, 569-579.
10 A. M. Kilbanov, B. N. Alberti, E. D. Morris and L. M. Felshin, *Journal of Applied Biochemistry*, 1980, **2**, 414-421.
11 J. Z. Liu, H. Y. Song, L. P. Weng and L. N. Ji, *Journal of Molecular Catalysis B: Enzymatic*, 2002, **18**, 225-232.
12 P. Paraskeva and E. Diamadopoulos, *Journal of Chemical Technology and Biotechnology*, 2006, **81**, 1475-1485.
13 M. Arienzo, A. De Martino, R. Capasso, A. Di Maro and A. Parente, *Phytochem Anal*, 2003, **14**, 74-82.
14 C. I. Justino, R. Pereira, A. C. Freitas, T. A. Rocha-Santos, T. S. Panteleitchouk and A. C. Duarte, *Ecotoxicology*, 2012, **21**, 615-629.
15 H. K. Obied, M. S. Allen, D. R. Bedgood, P. D. Prenzler, K. Robards and R. Stockmann, *J Agric Food Chem*, 2005, **53**, 823-837.
16 F. Visioli, A. Romani, N. Mulinacci, S. Zarini, D. Conte, F. F. Vincieri and C. Galli, *J Agric Food Chem*, 1999, **47**, 3397-3401.
17 V. Guida, M. Cantarella, A. Chambery, M. C. Mezzacapo, A. Parente, N. Landi, V. Severino and A. Di Maro, *Mol Biotechnol*, 2014, **56**, 738-746.
18 L. Ferrara, R. Dosi, A. Di Maro, V. Guida, G. Cefarelli, S. Pacifico, C. Mastellone, A. Fiorentino, A. Rosati and A. Parente, *Journal of Food Composition and Analysis*, 2011, **24**, 326-333.
19 A. Di Maro, S. Pacifico, A. Fiorentino, S. Galasso, M. Gallicchio, V. Guida, V. Severino, P. Monaco and A. Parente, *Food Research International*, 2013, **53**, 180-188.
20 V. Guida, G. Criscuolo, R. Tamburino, L. Malorni, A. Parente and A. Di Maro, *BMB Rep*, 2011, **44**, 64-69.
21 A. Di Maro, P. Valbonesi, A. Bolognesi, F. Stirpe, P. De Luca, G. Siniscalco Gigliano, L. Gaudio, P. Delli Bovi, P. Ferranti, A. Malorni and A. Parente, *Planta*, 1999, **208**, 125-131.
22 M. P. Allen, S. H. Choo, T. M. Li and R. F. Parrish, *Anal Biochem*, 1991, **192**, 453-457.
23 A. Di Maro, R. Berisio, A. Ruggiero, R. Tamburino, V. Severino, E. Zacchia and A. Parente, *Biochemical and biophysical research communications*, 2012, **421**, 514-520.
24 L. Pramparo, F. Stuber, J. Font, A. Fortuny, A. Fabregat and C. Bengoa, *Journal of Hazardous Materials*, 2010, **177**, 990-1000.
25 B. Porstmann, T. Porstmann and E. Nugel, *J Clin Chem Clin Biochem*, 1981, **19**, 435-439.

26 A. Di Maro, I. Terracciano, L. Sticco, L. Fiandra, M. Ruocco, G. Corrado, A. Parente and R. Rao, *J Biotechnol*, 2010, **147**, 1-6.
27 E. Agostini, M. I. Medina, S. R. Milrad de Forchetti and H. Tigier, *J Agric Food Chem*, 1997, **45**, 596-598.
28 H. Qiu, L. Lu, X. Huang, Z. Zhang and Y. Qu, *Bioresource Technology*, 2010, **101**, 9415-9420.
29 T. Araiso and H. B. Dunford, *J Biol Chem*, 1981, **256**, 10099-10104.
30 M. A. Duarte-Vazquez, S. Garcia-Padilla, B. E. Garcia-Almendarez, J. R. Whitaker and C. Regalado, *J Agric Food Chem*, 2007, **55**, 10396-10404.
31 L. Cantarella, A. Gallifuoco, A. Malandra, L. Martínková, F. Pasquarelli, A. Spera and M. Cantarella, *Enzyme Microb Technol*, 2010, **47**, 64-70.
32 J. Yu, K. E. Taylor, H. Zou, N. Biswas and J. K. Bewtra, *Environ Sci Technol*, 1994, **28**, 2154-2160.
33 L. Vamos-Vigyazo, *Crit Rev Food Sci Nutr*, 1981, **15**, 49-127.
34 G. H. Onsa, N. Saari, J. Selamat and J. Bakar, *Food Chemistry*, 2004, **85**, 365-376.
35 M. Cantarella, C. Mucciante and L. Cantarella, *Bioresour Technol*, 2014, **156**, 48-56.
36 L. Cantarella, A. Gallifuoco, A. Malandra, L. Martinkova, A. Spera and M. Cantarella, *Enzyme Microb Technol*, 2011, **48**, 345-350.

Figure Captions

Fig. 1. HPLC-profiles of OMW samples untreated and treated 24 h with 100 μg of AaP-1-4 named in the graph untreated and treated, respectively. Identified polyphenols: (1) cathecol, (2) hydroxytyrosol, (3) tyrosol, (4) vanillic acid (5) caffeic acid, (6) coumaric acid, (7) ferulic acid.

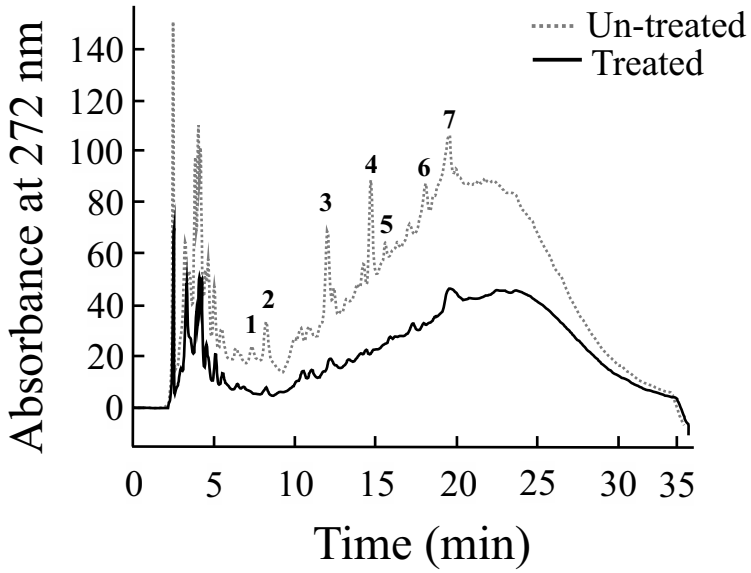
Fig. 2. Time course of free peroxidase activity during immobilisation of different amounts of AaP-1-4 (A) and HPR (B) on 10 mg Eupergit® CM, in 0.1 M phosphate buffer, pH 7.2, kept at 4 °C. Standard deviations were always <5% for each experiment.

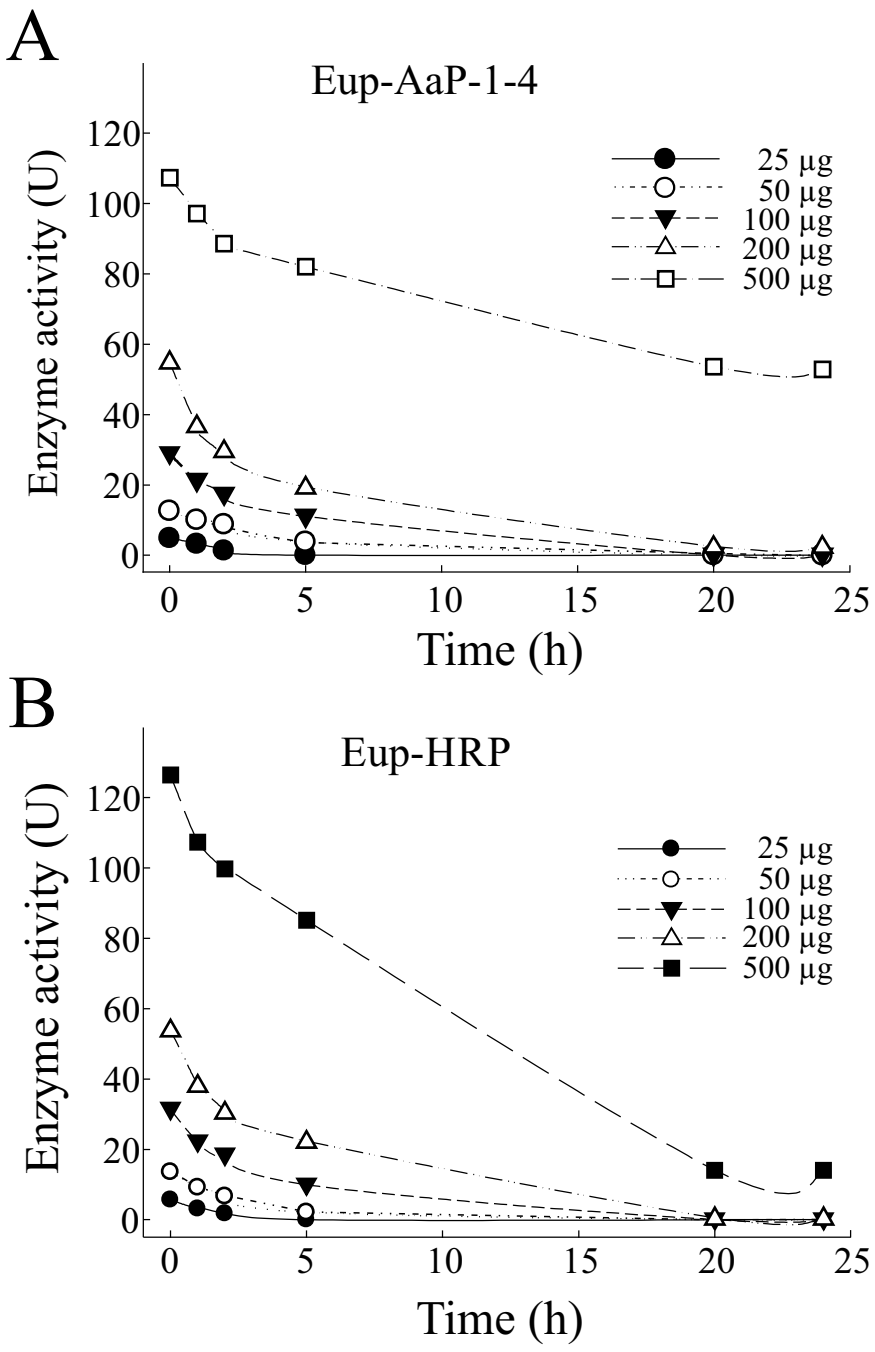
Fig. 3. Optimum pH of AaP-1-4 and Eup-AaP-1-4, using ABTS as substrate. Buffers: 10 mM Na-citrate, pH 2.0-3.5; 10 mM Na-acetate, pH 4.0-5.0; 10 mM Na-phosphate, pH 6.0-7.0; 10 mM Tris•Cl, pH 8.0; 10 mM Na-borate, pH 9.0; 10 mM Na-carbonate, pH 10.0-11.0. Standard deviations were always <5% for each experiment.

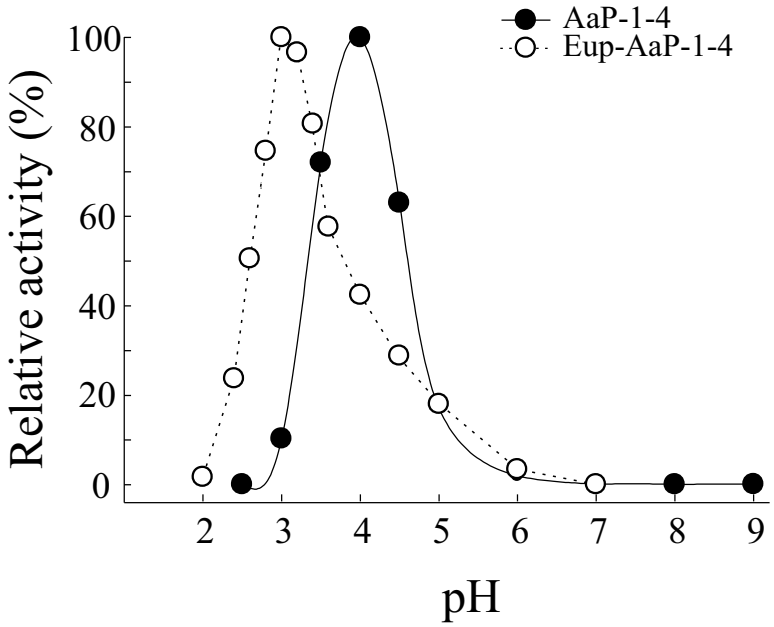
Fig. 4. A and B effect of Ca^{2+} and Mg^{2+} ions on the peroxidase activity on AaP-1-4 and Eup-AaP-1-4, respectively. C, percent of residual activity of AaP-1-4 and Eup-AaP-1-4 upon 10 min incubation at pH 4.0. D, time course of residual activity of AaP-1-4 and Eup-AaP-1-4 at 50 °C. Standard deviations were always <5% for each experiment.

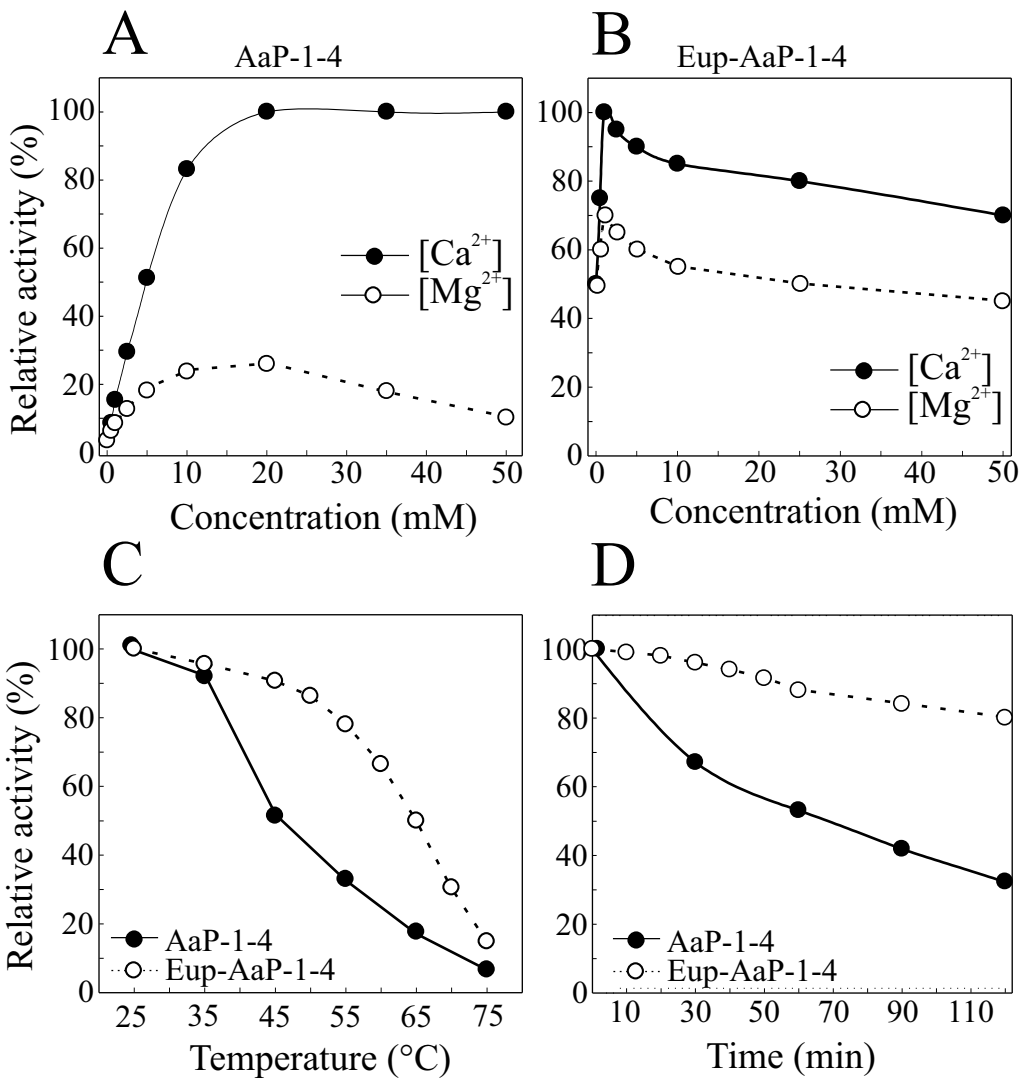
Fig. 5. Time course of phenol removal from water solutions with different concentrations of AaP-1-4 (A) and Eup-AaP-1-4 (B). The arrow in B represents the addition of fresh enzyme, 2.0 μg of AaP-1-4. The data point are mean values of at least duplicate measurements and the standard deviation is always <5% for each experiment.

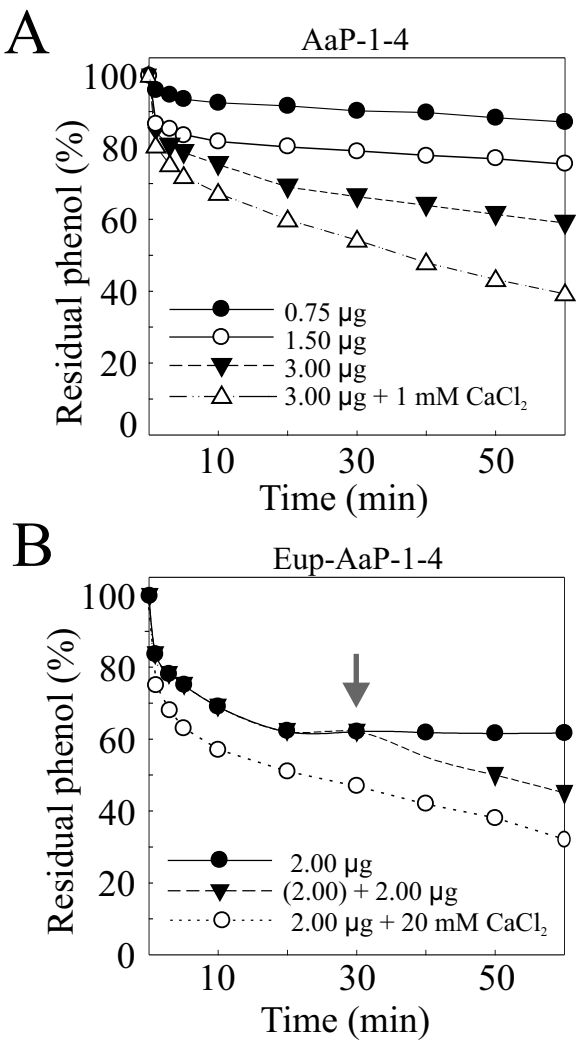
Fig. 6. Time course of reaction-rate, at 25 °C, in a continuous stirred UF-membrane reactor, stirred at 250 rpm, loaded with 50 μg of AaP-1-4 (○) or 100 μg of Eup-AaP-1-4 (●), and fed with a buffered solution of 2 mM ABTS**.** The reaction rate equation using the kd-values obtained from the interpolating data at steady state are quoted in the figure. The correlation coefficient, R^2, was 0.6634 and 0.9919 for free and Eup-AaP-1-4 enzyme, respectively. Standard deviations on product evaluation were always <5%.











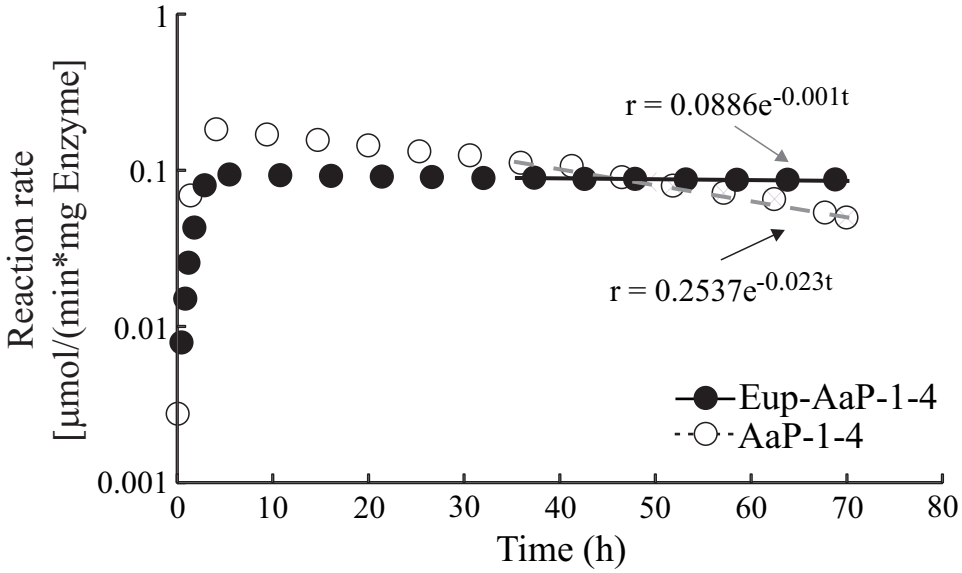


Table 1: Reduction of polyphenol in OMW after treatment with peroxidase from *A. acutifolius* (AaP-1-4) and horseradish (HRP) peroxidases at 4, 24, and 48 hours of incubation as indicated in Materials and methods. Removal of polyphenol is reported as percentages of reduction with respect to the untreated OMW. Values are means (±SD) of triplicate analyses (n = 3).

Enzyme amount (mg)*	***Polyphenol reduction (%)***					
	AaP-1-4	**HRP**	**AaP-1-4**	**HRP**	**AaP-1-4**	**HRP**
20 µg	28.9±2.0	14.3±1.2	33.6±2.1	23.0±1.4	43.9±2.2	23.9±1.3
50 µg	36.9±2.2	17.4±1.0	40.9±2.1	27.6±1.4	48.8±2.1	28.1±1.2
100 µg	40.2±2.1	21.0±2.0	46.8±1.2	31.0±1.2	54.2±2.2	33.9±1.3
	(4 h)		(24 h)		(48 h)	

Reaction volume 1.5 mL.

Table 2: Degradation of phenols in OMW by peroxidase from *A. acutifolius* and horseradish. Values are means (±SD) of triplicate analyses (n = 3).

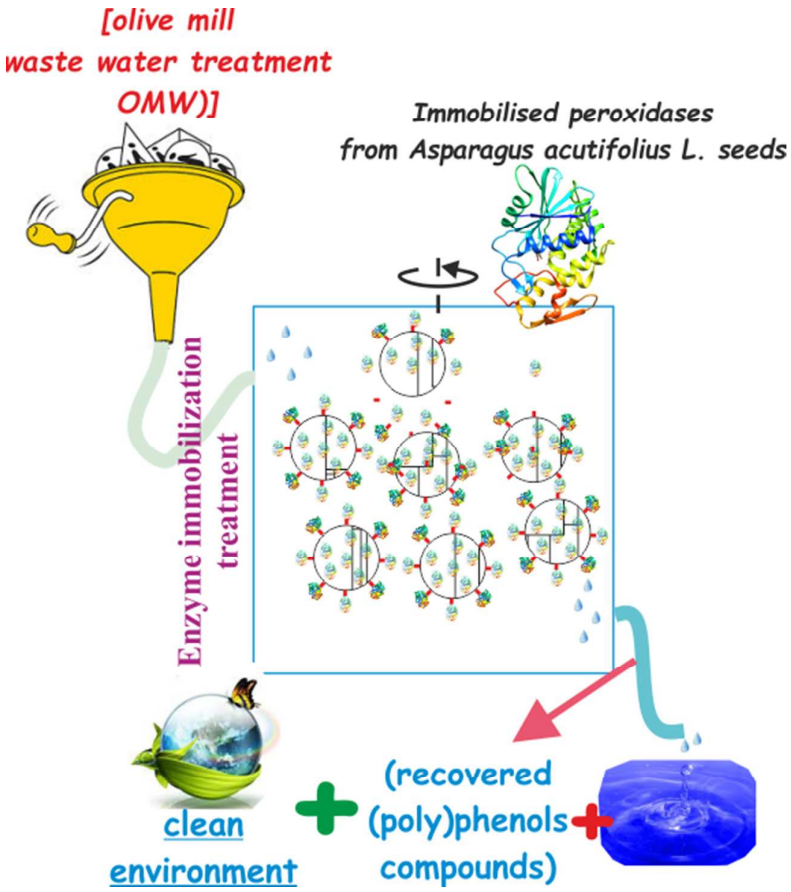
Polyphenolic compounds	Retention time (min)	Untreated OMW (mg L^{-1})	Treated OMW with 2 nM of enzyme	
			AaP-1-4 (mg L^{-1})	HRP (mg L^{-1})
1. Cathecol	7.2	4.1±0.13	n.d.	1.7±0.04
2. Hydroxytyrosol	8.3	10.6±0.26	1.5±0.02	3.8±0.01
3. Tyrosol	12.1	20.0±0.39	4.6±0.16	6.9±0.27
4. Vanillic acid	15.1	22.1±0.47	n.d.	n.d.
5. Caffeic acid	15.8	2.5±0.03	n.d.	n.d.
6. Coumaric acid	18.2	6.2±0.19	n.d.	2.6±0.04
7. Ferulic acid	19.5	10.2±0.32	6.0±0.15	7.3±0.23

Table 3: Substrate specificity of free and immobilized AaP-1-4 over different substrates. Values are means (±SD) of triplicate analyses (n = 3).

Substrate	λ (nm)	ε (M^{-1} cm^{-1})	Substrate (mM)	Specific activity (mM min^{-1} mg Eup-AaP-1-4^{-1})	Specific activity (mM min^{-1} mg AaP-1-4^{-1})
Ferulic acid	318	31100	0.04	0.38±0.01	58.61±1.38
Caffeic acid	450	2010	0.05	1.22±0.03	91.42±1.45
Ascorbic acid	290	2.8	0.5	10040±260	12894±159
Chlorogenic acid	410	2100	4	35.98±0.53	1410±30
Guaiacol	470	6700	10	16.12±0.37	18.27±0.39
Phenol	510	6.58	0.085	17106±310	29134±423

Manuscript entitled “Immobilised peroxidases from Asparagus acutifolius L. seeds for olive mill waste water treatment” by Vincenzo Guida et al. for publication on RSC Advances.

Graphical abstract



Highlights

1. AaP-1-4 peroxidase from *A. acutifolius* L. was immobilised on Eupergit®;
2. Eup-AaP-1-4 was proved to be able to remove polyphenol in olive mill waste water;
3. Operational stability of Eup-AaP-1-4 was evaluated in membrane bioreactor.
4. Eup-AaP-1-4 is an economic source for removal phenols from industrial processes.