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Recovery of Hydroxytyrosol from Olive Mill Wastewater Using the Promiscuous Hydrolase/Acyltransferase PestE

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Olive mill wastewater (OMWW) is produced annually during olive oil extraction and contains most of the health-promoting 3-hydroxytyrosol of the olive fruit. To facilitate its recovery, enzymatic transesterification of hydroxytyrosol (HT) was directly performed in an aqueous system in the presence of ethyl acetate, yielding a 3-hydroxytyrosol acetate rich extract. For this, the promiscuous acyltransferase from *Pyrobaculum calidifontis* VA1 (PestE) was engineered by rational design. The best mutant for the acetylation of hydroxytyrosol (PestE_I208A_L209F_N288A) was immobilized on EziG^2 beads, resulting in hydroxytyrosol conversions between 82 and 89% in one hour, for at least ten reaction cycles in a buffered hydroxytyrosol solution. Due to inhibition by other phenols in OMWW the conversions of hydroxytyrosol from this source were between 51 and 62%. In a preparative scale reaction, 13.8 mg (57%) of 3-hydroxytyrosol acetate was extracted from 60 mL OMWW.

Introduction

Olive mill wastewater (OMWW) is an industrial sewage produced besides olive oil and solid pomace in three-phase olive mills. Annually, 30 million m^3 of OMWW are produced worldwide within a few months (October to February). The wastewater causes environmental problems as it has a high polluting organic load, including polyphenolics, sugars, and lipids.[1] In addition to the acidity of the OMWW (pH ~5), the phytotoxic and antimicrobial activities of polyphenolics hinder the biodegradation of organic compounds.[2,3] Therefore, in addition to illegal disposal, incubation of OMWW in open ponds to decompose the organic matter is still widespread. Besides the unpleasant odor and the land requirements of open pond OMWW treatment, the economic value of the phenols is also lost. The phenols of olives have several health-promoting properties, e.g., neuroprotective, which are associated with their antioxidant and anti-inflammatory benefits.[4–8] Several methods have been developed to facilitate the degradation or extraction of phenolic compounds, such as chemical oxidation, solvent extraction, membrane systems or adsorbents.[9–20] However, all these systems require several laborious steps. In the case of solvent extraction, several extraction steps are required due to the hydrophilic nature of the contained phenols, e.g., the major phenolic compound 3-hydroxytyrosol (HT).[11] Lipophilization of HT by acetylation could facilitate the extraction and increase the bioavailability of the extracted health-promoting 3-hydroxytyrosol acetate (HTA).[4,21] However, acetylation/transesterification in an aqueous medium is challenging, as the hydrolysis of the formed ester and acyl donor is thermodynamically favored and thus pure organic solvents must be used for lipase-catalyzed acetylation.[22] As an alternative, promiscuous acyltransferases are able to catalyze the acetylation of nucleophiles with an acyl donor in a kinetically controlled manner in an aqueous system, thus favoring acetylation over hydrolysis.[22] The most studied promiscuous acyltransferase from *Mycobacterium smegmatis* (MsAcT) has been shown to catalyse the acetylation of HT.[23] However, MsAcT cannot be used in this process, because the pH optimum of MsAcT is in the basic range[24] and the pH of OMWW is acidic. The hyperthermostable esterase from the archaeon *Pyrobaculum calidifontis* VA1 (PestE) is a very robust biocatalyst exhibiting activity down to pH 3.5.[25] Our group recently discovered PestE as a promiscuous hydrolase/acyltransferase and demonstrated high acetylation activity and acyltransferase efficiency toward monoterpene alcohols.[26,27] Therefore, PestE was used in this study to catalyze the acetylation of HT in untreated aqueous OMWW in order to facilitate direct extraction of the HTA formed. Extraction of HT(A) from OMWW could provide an alternative to petrol-based chemical synthesis pathways of these compounds.[28,29]

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Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbic.202200254

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Results and Discussion

In a first screening, the PestE wild type (wt) and the variants PestE_H95 A, PestE_I208A, and PestE_N288F, created in a previous study to improve the activity of PestE towards monoterpene alcohols,[26] were screened for HT acetylation with ethyl acetate. All PestE variants were found to be active, but the weak spots on thin-layer chromatography (TLC) indicates only low acyltransferase activity (Figure S1). Among the tested variants, PestE_I208A showed a stronger spot, indicating a higher activity and was therefore included in further investigations. The N288 position putatively has an impact on the water network and increases the conversions achieved with ethyl acetate as an acyl donor.[26] However, docking HT in the binding pocket of PestE revealed that N288 may interact with the phenolic alcohol groups of HT (Figure S2). To find a compromise between the conflicting requirements for water network suppression and polar interactions with HT, the N288A mutant was created. Additionally, the binding of HT in the active site was investigated by molecular docking, as Kazemi *et al.* found that a high binding affinity is the basis of promiscuous hydrolases/acyltransferase.[30] To increase the binding affinity of HT, the structures of several PestE variants were modelled and analyzed. Molecular docking suggested that PestE_G86A and PestE_L209F bind the substrate stronger than the wild-type enzyme (Figure S2).

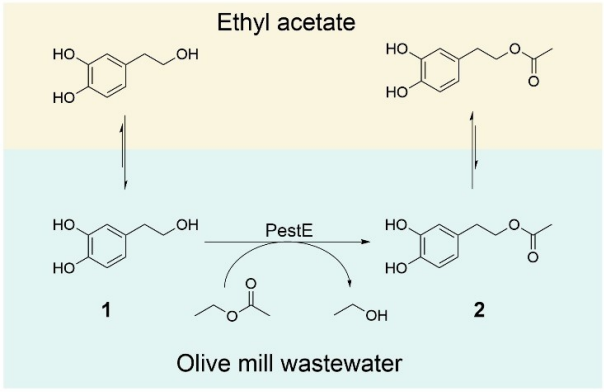
All PestE variants were studied in an aqueous/organic two-phase system (2:1) with ethyl acetate (EtOAc; Scheme 1). EtOAc is proved to be the best solvent for the extraction of the OMWW phenols[11] and it can act simultaneously as an acyl donor for PestE. In contrast to vinyl acetate, which is a much better acyl donor, EtOAc is less toxic to aquatic organisms and therefore more suitable for OMWW treatment and subsequent use of the extracted phenols in the food industry.[22,31]

Mutations I208A, L209F, and N288A improved HT conversion from 18% (PestE_wt) to 22, 52, and 21%, respectively, while G86A was neutral. It was demonstrated by thin-layer chromatography (TLC) that the maximum conversions were not reached at an earlier time point examined (data not shown). To investigate possible synergistic effects, mutations were combined iteratively and the resulting variants were used for HT acetylation (Figure 1).

Although G86A is a neutral mutation, combination with I208A and L209F resulted in a decreased turnover. Only PestE_G86A_N288A showed higher turnover than the single mutant alone. Since PestE_G86A_N288A was still less active than other double mutants and the combination with other mutations was deleterious, G86A was not further included in the combinatorial approach. PestE_I208A_L209F_N288A showed the best performance among the tested variants, reaching 86% conversion after 24 h (Figure 1). Further investigation revealed that over 80% conversion was reached already after 4 h and no hydrolysis of hydroxytyrosol acetate was observed even after 24 h (Figure S3).

When using acyltransferases for the treatment of OMWW, the enzyme production would be a major economical cost factor.

Enzyme immobilization can reduce these costs by allowing easy separation and reuse of the catalyst.[32] Therefore, immobilization on EziG beads was investigated. EziG beads have different surface polarities and bind the target proteins via their polyhistidine tag. EziG^1 has a hydrophilic surface, EziG^2 is coated with a hydrophobic polymer, while the surface polarity of EziG^3 is intermediate. Further immobilization properties according to the product specification are listed in the SI (Table S3). The immobilization efficiency using EziG^1 beads was very low, so EziG^2 and EziG^3, both of which had an immobilization efficiency of about 50% (0.3 $mg_{enzyme}/mg_{carrier}$), were further evaluated. PestE_I208A_L209F_N288A immobilized on EziG^2 beads (PestE_I208A_L209F_N288A-EziG^2) showed slightly better performance over ten reaction cycles studied compared to PestE_I208A_L209F_N288A immobilized on EziG^3 beads and was used for the following experiments (Figure 2).



Scheme 1. Two-phase system for acetylation of HT (**1**) in OMWW by PestE and extraction of HTA (**2**) into the ethyl acetate phase.

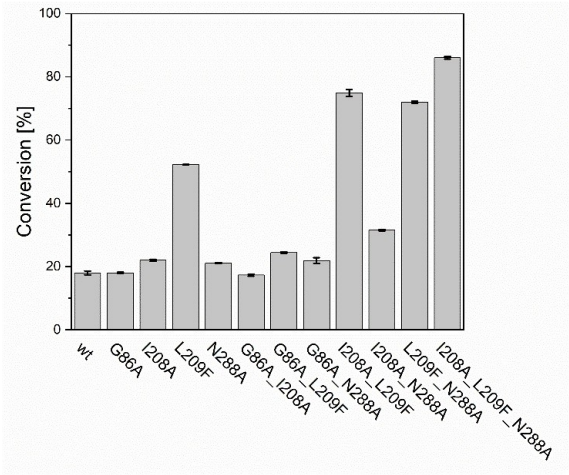


Figure 1. Conversion of HT to HTA after 24 h at 25 °C (1000 rpm) by 0.1 mg mL^{-1} of each PestE variant in a two-phase system with EtOAc.

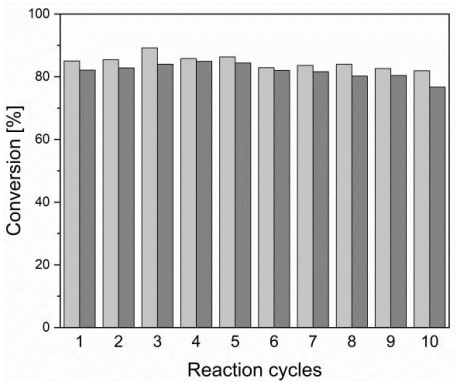


Figure 2. Conversion of HT to HTA by PestE_I208A_L209F_N288A immobilized on EziG^2 (light gray) or EziG^3 (dark gray) in a two-phase system with EtOAc over ten reaction cycles. Reactions were performed for 1 h at 25 °C and 1000 rpm with 1.0 mg mL^{-1} enzyme (in the aqueous phase).

Batch reactions with promiscuous hydrolases/acyltransferases usually result in maximum conversion before the product formed is hydrolyzed again.[22] By using ethyl acetate as the organic phase, a large excess of the acyl donor is present and the product is removed from the reaction. Both lead to a shift of the reaction equilibrium and a flattening of the reaction curve after reaching the maximum conversion (Figure S3). However, the maximum conversion of the reaction still depends on the efficiency of the acyltransferase. A comparison of the conversions achieved with PestE_I208A_L209F_N288A and MsAcT, which was used by Annunziata *et al.* for HT acetylation,[23] shows that our engineered PestE variant is a much more efficient acyltransferase for HT acetylation in water. Annunziata *et al.* could not overcome 29% conversion in their batch process with MsAcT, while PestE_I208A_L209F_N288A allows conversion of over 80%. Moreover, the pH of this model system was adjusted to pH 5.0 with respect to the OMWW application, while Annunziata *et al.* worked at the optimal pH of MsAcT, around pH 8.0.[23,33] Therefore, PestE_I208A_L209F_N288A is a much better candidate than MsAcT for acetylation of HT in OMWW.

However, OMWW is a challenging reaction medium because it is unbuffered and contains numerous other compounds.[1] Therefore, it is necessary to determine whether immobilized PestE_I208A_L209F_N288A can indeed be used to acetylate HT in OMWW. Therefore, OMWW was obtained from three different three-phase olive mills in Crete (Greece) and used as HT source. The pre-test of the HT acetylation in OMWW already showed that the PestE activity in OMWW is strongly reduced (data not shown). Since the HT conversions were still increasing up to 24 hours, an inactivation of the enzyme in OMWW could be excluded. However, inhibition by structurally similar phenols to HT, e.g., ferulic acid, could explain the reduced activity. To confirm this theory, PestE activity was examined in the presence of ferulic acid. The hydrolysis of *p*-nitrophenyl acetate was decreased by up to 60% (Table S1). Consistently, the acyl transfer reaction of HT was decreased by 27% in the presence of 1 mM ferulic acid, although no acetylation of ferulic acid was observed. Considering that other OMWW phenols besides ferulic acid might have an inhibitory effect on PestE, the reduced activity could be thus explained.

To compensate for the decreased activity, the reaction temperature was increased to 35 °C, the approximate temperature of fresh OMWW (Table S2), and the reaction time was extended to 24 h. Nevertheless, the conversions measured with HT in OMWW were lower than in reactions with HT (Figure 3).

Due to the large variety of potential inhibitors in OMWW, it might be difficult to address the inhibition by rational design. Nevertheless, a preparative scale reaction with 60 mL OMWW was performed to show the potential of using PestE for OMWW valorization. Using 72 μg mL^{-1} heat shock enriched PestE_I208A_L209F_N288A on EziG^2, 13.8 mg of HTA could be extracted, corresponding to over 57% of the HT contained in the OMWW. Together with the 2.1 mg HT in the extract, 15.9 mg hydroxytyrosol derivatives were extracted. This value corresponds to 73 mol% of HT and HTA in the investigated OMWW mixture, which is comparable to the extraction yields reported in literature,[10,11] although here only a simple one step liquid/liquid extraction procedure was performed.

However, the absolute amount of HT derivatives extracted per liter OMWW is relatively low.[10,11] This could be explained by the ripeness status of olives at the time of sampling in the middle of the season (late November). During ripening, HT is released from oleuropein, so HT would have been higher in the late season.[34] Accordingly, the darkest OMWW had the highest HT content (Figure S4; Table S2). Further studies could be conducted to couple HT-releasing enzymes, such as β-glucosidase, with PestE, to form HTA from the HT precursor, oleuropein.[35] This could lead to better HTA yields regardless of the maturity of the olives. In addition, procedural measures, such as the application of the immobilized enzymes in a flow application, could increase the productivity and activity of PestE_I208A_L209F_N288A.[18]

Although several other compounds were present in the organic extract, HTA is the major compound, as revealed by the ^{1}H-NMR study (Figure S5). Phenol-rich extracts are used as

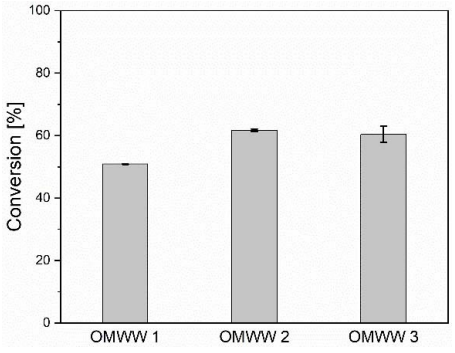


Figure 3. Conversion of HT in OMWW with EtOAc after 24 h at 35 °C (1000 rpm). PestE_I208A_L209F_N288A (0.5 mg mL^{-1}) on EziG^2 was used as catalyst.

dietary supplements, e.g., in fermented sausages, to stabilize the product or increase the health value of the product.[36–38] HTA-enriched functional foods could have neuroprotective, anticoagulant, arthritis preventive and other health-promoting properties.[4,8,39,40] For example, olive oil that contains 250 mg kg^{-1} hydroxytyrosol derivatives can be called health-promoting according to an EU regulation (EU Commission Regulation No. 432/2012). However, further purification of the extracts may be required to meet the criteria for food applications. Taking into account that three to five liters of OMWW are produced per kilogram of olive oil, about 810 to 1,340 mg of HT and HTA can be obtained from the OMWW of one kilogram of oil. Using the example of the olive mills visited that produce 200,000 to 1,300,000 t olive oil annually, 160 to 1,740 t HT derivatives could be produced.

The recyclability of the immobilized enzymes is thus a first step towards a possible industrial application. The use of a simple heat shock enrichment of PestE could also help to reduce the cost of the process. However, further process engineering measures, e.g., recycling of ethyl acetate and more sophisticated extraction procedures, would be required to make the process economically viable and sustainable.

Conclusions

Since only 2% of olive phenols remain in olive oil and 53% are found in the OMWW, which is causing environmental problems, new methods of recovering the health-promoting phenols are needed to utilize the full power of the olive.[41] Lipophilization of phenols such as HT using acyltransferases could be an important step to facilitate the recovery of phenols from OMWW. Immobilization and rational optimization of PestE led to the recyclable catalyst PestE_I208A_L209F_N288A-EziG^2, which is also active in untreated OMWW. We demonstrated that the phenol-rich extract obtained from OMWW provides sufficient HT and HTA to enrich the same amount of olive oil to phenol-rich olive oil according to the EU health claim.

Experimental Section

Enzyme preparation

Expression of PestE variants was performed according to a protocol previously described by our group.[26]

Mutant screening

For screening of PestE variants, 160 μL of 50 mM citrate buffer pH 5.0 and 40 μL enzyme solution (0.1 mg mL^{-1} final) were mixed. Then, 10 μL 200 mM HT in ethyl acetate (EtOAc) and 90 μL EtOAc were added. The reaction was incubated at 25 °C (1000 rpm) for 24 h. Reaction controls of 1 μL of the organic phase (OP) of each reaction were taken after 1, 2, 3, 4, 8, and 24 h and analyzed by TLC after 24 h to exclude that higher conversions were achieved at an earlier time point. TLC was performed using silica plates (Merck, Darmstadt, Germany) as solid and EtOAc as mobile phase. The staining was done with iodine (R_f(HT) = 0.71; R_f(HTA) = 0.91). After 24 hours, the reaction was stopped by adding 50 μL 2 M HCl. Subsequently, the reactions were extracted three times with 200 μL EtOAc each. The organic phase was dried over sodium sulfate, and analyzed by GC-FID.

Selection and reuse of immobilization carriers

To a reaction tube, 5 mg EziG^1, EziG^2, and EziG^3 were added and then 500 μL PestE_I208A_L209F_N288A solution (2.98 mg mL^{-1} in 20 mM NaP_i with 500 mM NaCl pH 8.0). Immobilization was performed according to the manufacturer's protocol (https://enginzyme.com/wp-content/uploads/2015/05/EziG%E2%84%A2-Detailed-Instruction-manual.pdf; April 25, 2022). Briefly, incubation was performed for 2 h at 25 °C (1000 rpm) and the beads were washed twice with 500 μL 50 mM citrate buffer pH 5.0. Loading was calculated based on the residual protein concentration in the supernatant. Biocatalytic test reactions with the immobilisate were performed as described for mutant screening and stopped after 1 h by transferring the supernatant to a new vial containing 50 μL of 2 M HCl. The supernatant was extracted and analyzed by GC-FID, as described in the respective paragraph. The beads were reused by washing with 200 μL of citrate buffer pH 5.0, centrifuging and discarding of the supernatant. The reaction was repeated nine more times.

PestE_I208A_L209F_N288A immobilization for preparative scale reaction

Expression of PestE_I208A_L209F_N288A was performed according to a protocol previously described on a 400 mL scale.[26] Deviating from this, the lysis was performed in 4 mL loading buffer (20 mM NaP_i + 500 mM NaCl, pH 8.0) and the enzymes were enriched in the lysate using only heat shock (40 min, 80 °C). The enriched lysate was immobilized on 400 mg EziG^2™ beads (EnginZyme, Solna, Sweden) as described above. After washing twice with 1 mL loading buffer (20 mM NaP_i + 500 mM NaCl, pH 8.0), the immobilized material was stored moist at 4 °C before further use.

Small scale extraction of OMWW with PestE_I208A_L209F_N288A-EziG^2

30 mg PestE_I208A_L209F_N288A-EziG^2 immobilisate (0.1 mg enzyme) was submitted in a reaction tube. Then, 200 μL of the filtered OMWW was added and incubated at 35 °C (1000 rpm) for 24 h after the addition of 100 μL ethyl acetate. Subsequently, the aqueous and organic phases were separated and each diluted tenfold in HPLC running medium (ddH_2O + 2% v/v AcOH : methanol; 25 : 75). The samples were analyzed by reverse phase high performance liquid chromatography (RP-HPLC). Analogous reaction approaches without enzyme were performed to compare HT conversions and extraction. All experiments were performed in duplicates.

Preparative scale extraction of OMWW with PestE_I208A_L209F_N288A-EziG^2

In a 250 mL round bottom flask, 1.310 g of the PestE-EziG^2 immobilisate (4.32 mg enzyme) was placed and 20 mL each of the filtered OMWW 1, 2, and 3 were added, to a total volume of 60 mL. After the addition of 30 mL of ethyl acetate, the reaction mixture was incubated for 24 h under vigorous stirring. A preparation without enzyme immobilisate was performed analo-

gously. The temperature was adjusted to 35 °C by an oil bath and controlled with a ground-glass thermometer. Subsequently, the organic phase was separated via a separating funnel, dried over magnesium sulfate, filtered, and the solvent was evaporated in vacuum. After additional drying for 16 h into a lyophilizer, the weight of the extracted substances (117.0 mg and 104.3 mg in the control without enzyme, respectively) was determined. The brown, highly viscous oil exhibited a spicy-bitter odor. The extract was dissolved in deuterated methanol (MeOD) and a ^{1}H-NMR spectrum was recorded. The HT and HTA content were quantified by gas chromatography with flame ionization detector (GC-FID). The natural HTA content in the OMWW was subtracted from the HTA measured in the organic phase, which would correspond to a complete extraction of the natural HTA. Accordingly, the conversion is to be evaluated as minimum conversion.

GC-FID analytics

Samples in 50 μL ethyl acetate were derivatized by adding 40 μL *N,O*-bis(trimethylsilyl)trifluoroacetamide and 10 μL pyridine. Analysis was performed with GC-FID (GC-2010, Shimadzu, Kyoto, Japan) equipped with a BPX5 column (25.0 m × 0.25 mm, 0.25 μm film thickness, Trajan Scientific and Medical, Ringwood, Australia). Injector and detector temperature was 250 °C, and 1 μL sample was injected. The column temperature was held at 150 °C for 3 min, increased to 220 °C with 14 °C min^{-1}, and held 2 min. Compounds were identified with authentic standards (retention times: HT 8.0 min; HTA 8.5 min).

RP-HPLC analytics

An Agilent 1260 Infinity II with a Lichosphere RP18-5 (250 × 46 mm, 5 μm) column was used for RP-HPLC analysis. For the first 30 minutes of the run, the flow was maintained at 0.5 mL min^{-1} and 25% (v/v) methanol (MeOH). The flow was gradually decreased to 0.4 mL min^{-1} and 50% (v/v) MeOH until 40 minutes and held for ten minutes. In the following five minutes, the MeOH was reduced again to 25% (v/v). Then the flow rate was gradually increased to 0.5 mL min^{-1} until 60 min, followed by a final hold time of five minutes. Compounds were identified with authentic standards (retention times: HT 10.4 min; HTA 44.3 min). Naturally occurring HTA was subtracted to calculate HT conversions.

Acknowledgements

H.T. was funded by the Leibniz Association's strategic networking funding program Leibniz ScienceCampus ComBioCat. A.S. received funding from the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (Capes) – Finance Code 001. We are very grateful for the support from Prof. Apostolos Spyros (University of Crete) and Charitomeni Aggeli (University of Crete) regarding the NMR analysis. Special thanks to the operators of the olive mills Melissourgakis, Politakis and Gaia (Crete, Greece). Open Access funding enabled and organized by Projekt DEAL.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: acyltransferases · biocatalysis · hydroxytyrosol · hydroxytyrosol acetate · olive mill wastewaters valorization

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Manuscript received: May 2, 2022
Revised manuscript received: May 17, 2022
Accepted manuscript online: May 17, 2022
Version of record online: May 31, 2022