An Enzyme Cascade Reaction for the Recovery of Hydroxytyrosol Dervatives from Olive Mill Wastewater

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The valorization of olive mill wastewaters (OMWW), a by-product of the olive milling, is getting rising attention. Lipophilization of the main phenolic compound 3-hydroxytyrosol (HT) could facilitate its extraction. An immobilized variant of the promiscuous hydrolase/acyltransferase from *Pyrobaculum calidifontis* VA1 (PestE) was used to perform acetylation in water using ethyl acetate as acyl donor. PestE was used in a segmented flow setting to allow continuous operation. Additionally, HT precursors were made accessible by pretreatment with almond β-glucosidase and the hydrolytic activity of PestE_I208A_L209F_N288A.

Keywords: Cascade reaction, Flow catalysis, Hydroxytyrosol, Olive mill wastewaters valorization, Promiscuous acyltransferase

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1 Introduction

Olive oil is known for its health-promoting properties, which can be attributed to the phenolic substances it contains [1]. However, only 2 % of the total olive fruit phenols are obtained in the olive oil due to the hydrophilic nature of these phenols [2]. More than half of the total phenols are disposed of with the olive mill wastewater (OMWW), which therefore has phytotoxic and antimicrobial properties and requires special treatment to degrade the organic matter [2, 3]. While various methods such as chemical oxidation, solvent extraction, membrane systems, or adsorbents have been investigated for phenol removal, open pond treatment is still widely used [4–10]. Recently, our group developed a system for the lipophilization and recovery of the main phenol 3-hydroxytyrosol (HT) in a biphasic ethyl acetate/OMWW system using an engineered variant of the promiscuous hydrolase/acyltransferase from the hyperthermophilic archaeum *Pyrobaculum calidifontis* VA1 (PestE) [11]. In contrast to commonly used lipases, promiscuous hydrolases/acyltransferases such as PestE can perform efficient transesterification reactions in bulk water [12, 13]. Moreover, PestE is a hyperthermostable enzyme that shows no decrease in activity at 100 °C [14] and can even be used in pure organic solvent [15]. Using the PestE variant optimized for HT, PestE_I208A_L209A_N288A, which was immobilized on EziG^2 beads, 265 mg L_{OMWW}^{-1} HT and hydroxytyrosol acetate (HTA) were recovered from untreated OMWW [11]. It was possible to reuse the immobilized PestE_I208A_L209A_N288A for at least ten rection cycles without loss of activity, emphasizing the high process stability of the promiscuous hydrolase/acyltransferase PestE in this system [11]. The recovered HT derivatives from 1 L OMWW would be sufficient to fulfill the EU health claim for 1 kg of high-priced health-promoting olive oil, would it be added to any olive oil. However, the reaction was carried out in batch mode and with an incubation time of 24 h, which would require many large reaction vessels to apply this system to the huge volumes of OMWW continuously produced during the olive harvest. In this work, the biphasic batch system was transferred to a segmented flow system to allow continuous operation. Additionally, the release of HT from the HT glycoside oleuropein, another main olive phenol, was investigated by combining the almond (*Prunus amygdalus*) β-glucosidase with the acyltransferase activity of PestE (Fig. 1). The release of oleuropein from HT also occurs naturally during olive ripening, but olives for olive oil production are mostly green or unripe, especially in the

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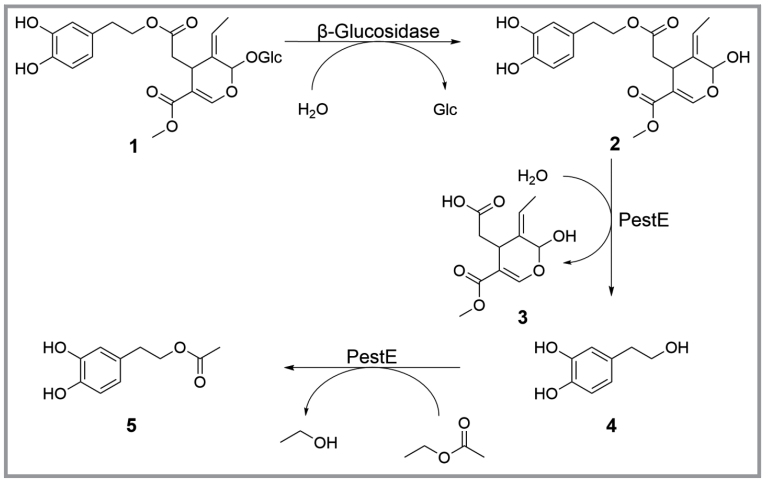

Figure 1. Reaction cascade for the conversion of oleuropein (**1**) to HTA (**5**). The almond β-glucosidase deglycosylates oleuropein (**1**) to the oleuropein aglycone (**2**) [17]. PestE_I208A_L209F_N288A is intended to release elenolic acid (**3**) and HT (**4**) by hydrolysis and to transesterify **4** to HTA (**5**) using ethyl acetate as acyl donor.

early season [16]. Degradation of oleuropein to HT would hence allow more efficient HT recovery even in the early season.

2 Experimental

2.1 Materials

OMWW samples were collected from different three-phase olive oil mills on Crete (Greece) and prepared as reported previously [11]. Ethyl acetate was purchased from VWR (Darmstadt, Germany) in HPLC grade (99.8+ %). The β-glucosidase from almonds was purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

2.2 Flow Settings

The ethyl acetate flow was controlled with a syringe pump (Perfusor® fm, Braun, Kronberg, Germany). The OMWW was pumped using a peristaltic pump (Minipuls 3, GILSON, Middleton, WI, USA). The ethyl acetate and OMWW phases were introduced into a packed-bed reactor of 0.5 mL volume (200 mg), which consisted of EziG^2 beads (EnginZyme, Solna, Sweden). A thin layer of sand was placed at the bottom and top of the packed bed. EziG^2 beads were loaded with 5 mg of PestE_I208A_L209A_N288A. The residence time on the column was approximately 10 min and the system was operated at room temperature. The ethyl acetate phase was separated, dried with anhydrous Na_2SO_4 and analyzed by gas chromatography, as described previously [11].

2.2.1 Acetylation/Extraction Setup

For the acetylation and extraction of HT in flow, ethyl acetate was pumped into the packed-bed reactor at 1.0 mL h^{-1} and OMWW at 1.6 mL h^{-1}. OMWW was either untreated or pretreated with 1 U mL^{-1} almond β-glucosidase for 1 h at room temperature.

2.2.2 Oleuropein Hydrolysis Setup

OMWW was pretreated with 1 U mL^{-1} almond β-glucosidase for 1 h at room temperature and then added to the packed-bed reactor at a flow rate of 1.6 mL h^{-1}.

2.3 Protein Expression und Immobilization

Protein expression was performed as reported previously [13]. The purification of PestE_I208A_L209F_N288A was performed with heat shock for 40 min at 80 °C. The denatured proteins were separated by centrifugation (30 min, 17 000 *g*, 4 °C). The supernatant was added to the packed-bed reactor and the flow-through was collected and applied two more times. PestE_I208A_L209F_N288A (5.0 mg) were immobilized on the packed-bed beads.

3 Results and Discussion

The recovery of HT derivatives by acyltransferase-catalyzed lipophilization in batch mode was described previously [11]. HT was acetylated in the aqueous phase by PestE_I208A_L209F_N288A and then extracted into the organic ethyl acetate phase, which also functions as the acyl donor. In order to allow a continuous operation of the recovery of HT from OMWW, a segmented flow setup was used. In addition, ethanol, which is a by-product of enzymatic HT transesterification with ethyl acetate, cannot accumulate in a flow system and enhance the back reaction of the resulting product HTA. The packed-bed reactor was loaded with EziG^2 beads, porous glass beads coated with a semi-hydrophobic polymer that can bind His_6-tagged proteins. EziG^2 beads were found to be suitable for the immobilization of PestE_I208A_L209F_N288A and could be used for at least ten reaction cycles without loss of activity [11].

Using the flow system described in Fig. 2a, 93 mg L_{OMWW}^{-1} HTA and 37 mg L_{OMWW}^{-1} HT could be extracted in the organic phase. However, with 130 mg L_{OMWW}^{-1} less HT derivatives were recovered compared to the batch process reported previously (265 mg L_{OMWW}^{-1}) [11], the reaction time could be reduced from 24 h to 10 min and elevated temperatures could be avoided.

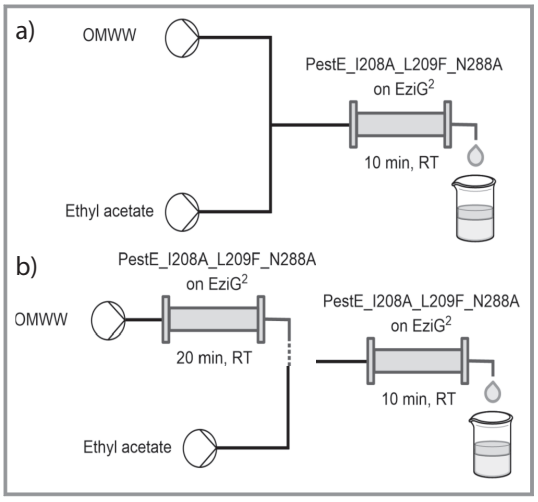

Figure 2. Schematic diagram of the flow settings. a) Acetylation/extraction setup. Ethyl acetate and OMWW were added to the packed-bed reactor at flow rates of 1.0 mL h^{-1} and 1.6 mL h^{-1}, respectively. b) Combined oleuropein hydrolysis and acetylation/extraction setup. Hydrolysis of oleuropein was performed in a separate flow-through reaction at a flow rate of 1.6 mL h^{-1}. The acetylation/extraction reaction (as described above) was performed sequentially.

The HT concentration of the OMWW was about 2 mM (300 mg L^{-1}), which is relatively low compared to literature [5, 6]. One reason for the low HT content could be the ripeness of the olives used for olive oil production. Since the olives were mostly unripe at the time of sampling in November, the HT could still be bound in the precursor oleuropein. Since β-glucosidase from almonds has already been used for the cleavage of oleuropein and has a pH optimum of about 5, as well as tolerance to potential impurities such as heavy metals [17, 18], almond β-glucosidase was used in this work. However, because limited data are available on the tolerance of almond β-glucosidase towards organic solvents in a two-phase system, pretreatment of the OMWW was performed before the OMWW was used for the flow reaction.

OMWW pretreated with almond β-glucosidase was used in the flow setting described in Fig. 2a. However, only 61 mg L_{OMWW}^{-1} HTA and 38 mg L_{OMWW}^{-1} HT could be extracted using this system, possibly due to the fact that the hydrolase activity of PestE towards the oleuropein aglycone is suppressed under acyltransferase conditions. The lower acyltransferase activity could possibly be explained by the inhibition of the acyltransferase reaction by the oleuropein aglycone. Inhibition of PestE by structurally similar phenols has been reported previously for ferulic acid [11]. Therefore, deglycosylation and hydrolysis of oleuropein and acetylation of HT must be carried out sequentially. For this purpose, pretreated OMWW was added to the packed-bed reactor without the addition of ethyl acetate (Fig. 2b). The resulting solution was again applied to the immobilized enzymes using the acetylation/extraction flow setting with ethyl acetate. With this approach, 86 mg L_{OMWW}^{-1} HTA and 69 mg L_{OMWW}^{-1} HT could be extracted. In total, 155 mg L_{OMWW}^{-1} HT derivatives could be extracted, which is more compared to the approach without β-glucosidase (130 mg L_{OMWW}^{-1}), indicating successful release of HT from oleuropein by β-glucosidase and PestE. However, the acyltransferase reaction proved to be incomplete, as this approach extracted more unreacted HT than HTA despite its more hydrophilic properties. To convert the released HT to HTA, which is easier to extract, another acetylation/extraction run was performed with the separated ethyl acetate and OMWW phases. After this flow run, 166 mg L_{OMWW}^{-1} HTA and 62 mg L_{OMWW}^{-1} HT (228 mg L_{OMWW}^{-1} HT derivatives) were recovered, representing 75 % improvement in extracted HT derivatives compared to the original flow system. Using the sequential flow approach, similar amounts of HT could be extracted as with the batch reaction [11], but without heating and with much shorter reaction times. For industrial application the system would have to be scaled up. Additionally, the sequential approach would have to be included in a flow system with two separate pack-bed reactions to allow continuous processing. However, the proof of concept of a flow system for the valorization of OMWW using β-glucosidase and both catalytic activities of promiscuous hydrolase/acyltransferase PestE_I208A_L209F_N288A was already successful as confirmed by our results. To further reduce the cost of the process, purified β-glucosidase could be co-immobilized [19] or, even cheaper, microbial pretreatment of OMWW could be applied to release HT from oleuropein [20].

4 Conclusion

Immobilized PestE_I208A_L209F_N288A was previously used for valorization of OMWW in a batch system. Herein we developed a flow system that shortens the reaction time and does not require heating. Using almond β-glucosidase and the hydrolase/acyltransferase activities of PestE_I208A_L209F_N288A, previously inaccessible HT precursors could be utilized. Using this system, 228 mg of HT derivatives could be extracted from 1 L OMWW.

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Abbreviations

HT	3-Hydroxytyrosol
HTA	Hydroxytyrosol acetate
PestE	Promiscuous hydrolase/acyltransferase from *Pyrobaculum calidifontis* VA1

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