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Functional investigation and applications of the acetylesterase activity of the *Citrus sinensis* (L.) Osbeck peel

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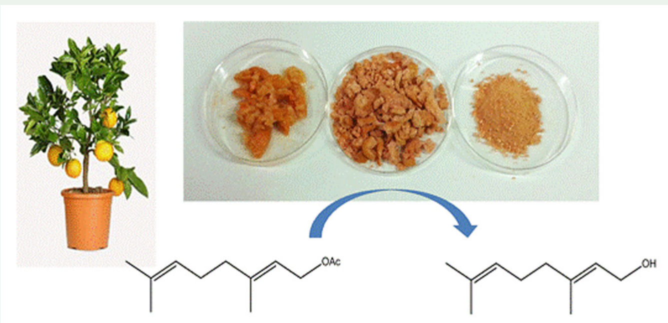
ABSTRACT

The hydrolysis of acetyl moieties on a set of commercially relevant substrates was performed by employing the whole tissue of *Citrus sinensis* (L.) Osbeck peel as an efficient biocatalyst in mild reaction conditions with high degree of regioselectivity. The reaction is done in aqueous media and the product is easily recovered. Optimal reaction conditions were deduced and two practical applications were investigated: the elaboration of acetylglicerols and the preparation of vitamin K_1 precursor. Peel waste (flavedo and albedo) from orange juice manufacturing was successfully employed as a biocatalyst.



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1. Introduction

Biocatalytic transformations are a wide class of processes in which a chemical modification of a suitable organic substrate takes place, generally with a high degree of regio- and stereoselectivity, in mild reaction conditions. The catalysts employed can include pure isolated enzymes and intact tissues, as well as cell cultures suspended

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into a suitable growth broth (Hamada et al. 1996). The use of vegetables in this context is attractive in consideration of the easy availability of a wide variety of possible biocatalysts. From the pioneering work of Stohs and Staba (1965) on the chemical modification of digitoxigenin with tissue cultures of *Digitalis*, a lot of work had been made to develop protocols for performing many chemical transformations by vegetables (Cordell et al. 2007) including reduction of alkenes, carbonyl and nitro compounds, oxidation of alcohols, ester hydrolysis. Among these enzymatic activities, the esterase-like one is particularly attractive both for its potential application in organic synthesis (Bracher and Krauss 1998) and from the economic point of view, in consideration of the many important applications of esterases in food, perfume and pharmaceutical industries (Panda and Gowrishankar 2005). The commercial purified enzyme acetylesterase from the flavedo of orange peels was employed, for example, by Waldmann and Heuser (1994) as the catalyst in the removal of acetyl protecting groups from monosaccharides.

The utilization of the whole plant preparation in order to perform the hydrolysis of the ester bond was deeply investigated by Mironowicz and Siewinski; they disclosed the possibility to hydrolyze various aromatic acetates by intact plants (Mironowicz et al. 2014) of *Spirodela punctata, Nephrolepis exaltata, Cyrtomium falcatum, Nephrolepis cardifolia* and the suspended coltures of *Helianthus tuberosus*. Further, acetates and propionates of arylic, benzylic and alyphatic (terpenoidic) alcohols were successfully hydrolyzed with the pulp of *Solanum tuberosum* and *Malus silvestris* (Mironowicz et al. 2014). The same group performed the enantioselective hydrolysis of a number of racemic chiral esters with *Spirodella oligorrhiza* (Pawlowicz and Siewinski 1987) *Malus silvestris* (Mironowicz 2014); *Solanum tuberosum* and *Helianthus tuberosus* (Mironowicz 1998); *Daucus carota*, *Apium graveolens* and *Armoracia lapatifolia* (Maczka and Mironowicz 2002). A set of 28 vegetables were tested by Vanderberghe et al. (2013) as biocatalyst in the hydrolysis/kinetic resolution of the racemic 1-phenylethyl acetate and more recently the peel of *Citrus aurantium* was employed for the same purpose (Da Silva et al. 2016).

Citrus sinensis (L.) Osbeck is an endemic plant in the Italian region of Sicily, known for its high nutraceutical value (Celano et al. 2019) and with several recently recognized bioactivities, such as antioxidant (Tomasello et al. 2019) and mosquitocidal (Badawy et al. 2018).

The aim of this work was to investigate the use of *C. sinensis* peel (CSP) as the biocatalyst for performing potentially useful chemical transformations, also in light of the benefits connected with the potential application of recycled waste, such as fruit peel derived from the industrial production of fruit juices and canned fruits.

Although the presence of an acetylesterase EC 3.1.1.6 enzyme in the orange peel is known since a long time (Jansen et al. 1947), the use of the fresh peel and the raw material, obtained from *C. sinensis*, as biocatalyst for the chemo- and regioselective removal of acetyl groups was not deeply elucidated and the number of applications already reported is still limited. For this reason, we decided to investigate the use of easily obtained, low cost and eco-friendly catalyst in the deacetylation reaction of some compounds, also useful in the preparation of valuable commercial products. Geranyl acetate (**1**, Figure 1) is a natural product occurring in many plants essential

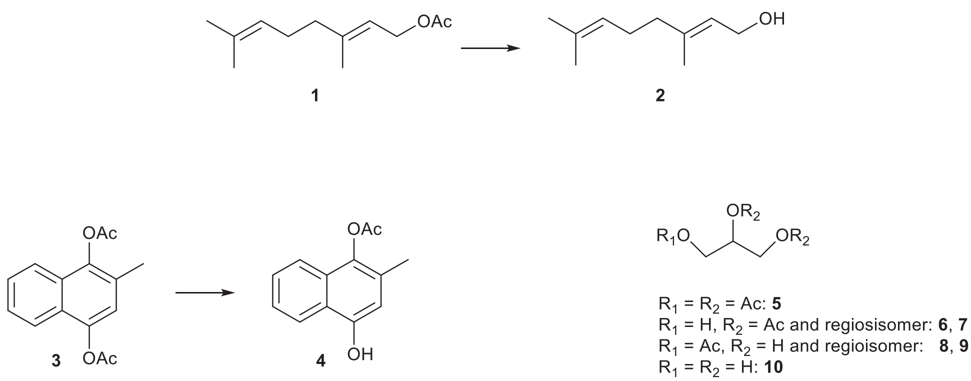

Figure 1. Biocatalytic transformations by *Citrus sinensis* peel.

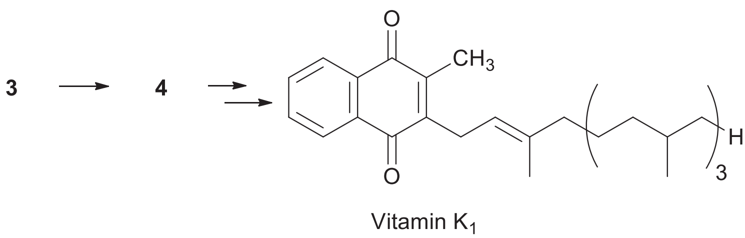

Figure 2. Synthesis of K_1 vitamin.

oils and its deacetylated derivative, the alcohol geranyol, is the constituent of many natural essences used in perfume industry and a key starting material for other terpenes. Furthermore, it possesses many biological activities (Chen and Viljoen 2010).

1-Acetoxy-4-hydroxy-2-methylnaphthalene **4** is a key intermediate involved in the preparation of the pharmacological relevant molecule vitamin K_1 (Hirshmann et al. 1954), as depicted in Figure 2. The transformation of compound **3** to **4** reported in literature involve traditional chemicals such as *t*BuNH$_2$ or MeONa in the highly toxic methanol as solvent (Tomkuljak et al. 1980; Huang et al., 2006).

Finally, triacetin (TA) **5**, diacetins (DA) **6**,**7** and monoacetins (MA) **8**,**9** are important acetyl-derivatives of glycerol **10** with many commercially relevant applications (Kong et al. 2016). MAs are used as plasticizers for vinyl polymers and cellulose-like material, DAs are used as raw material in the production of polyesters and finally, TA is used as solvent and excipient in pharmacy as well as additive in fuel mixture to modulate viscosity and knocking properties. Further, as the functional profile of *Citrus sinensis* acetylesterase was not yet fully investigated and the data available (Pasta et al. 2004, Brenda 2019) at present for this enzyme are incomplete, the initial part of this work was devoted to the identification of the most suitable reaction conditions (pH, temperature, reaction time).

2. Results and discussion

The efficiency of the CSP as a biocatalyst in the deacetylation reaction was evaluated on substrate **1** in order to establish the optimal reaction conditions. A range of pH from 4.4 to 12.0 was explored using citric acid/phosphate buffers at a constant substrate/catalyst ratio (0.0075 w/w) and temperature (30 °C). The reaction mixture was analyzed by GC/MS after 72 h. The higher rate of conversion is achieved at pH comprised between 8.0 and 9.0 with a conversion quite complete (97%). The same pH range was evaluated without the biocatalyst; as it can be seen from Figure S1a (supplementary material), the basic, non-enzymatic, ester hydrolysis occurred over pH 9.7 and became predominant above pH 11.5. This point can also explain the appearance of a relative minimum at pH 9.7 that is due to the decrease in the enzymatic activity after pH 8 accompanied by the increase of the chemical hydrolysis rate after pH 10.

The influence of temperature was investigated (Figure S1b). The higher conversion was obtained at 40 °C. However, the blank experiments gave a certain degree of thermal hydrolysis taking place above 35 °C.

The reaction is more sensitive to the pH variation than to the changes in temperature (45% at 30 °C and 85% at 40 °C without buffer, 97% at 30 °C and 97% at 40 °C with pH 8.5 buffer). In order to follow the time course of the geranyl acetate conversion, the substrate **1** was left to react with the usual substrate/catalyst ratio under the optimal reaction conditions previously identified (pH 8.5, 40 °C, Figure S1c). The amount of produced geranyol reaches a plateau after about 60 h.

Finally, experiments were conducted for the evaluation of the efficacy of the deacetylation reaction performed with differently prepared biomass batches, i.e., chopped fresh fruit peel, freeze-dried peel powder, residual material after industrial squeezing of the fruit for juice production. No difference was evidenced between the fresh peel and the orange industrial waste recovered directly from an industrial squeeze apparatus, dried and stored for one week at 4 °C (fresh peel vs. waste conversion efficiency: 78,0/75.5 ± 6.5%, r substrate/biocatalyst 0.0075 w/w, 40 °C, pH 8.5, 50 h) that demonstrating the possibility to employ this kind of waste as biocatalyst.

The optimized reaction conditions obtained for the compound **1** were employed for substrates **3** and **5.** After 72 h at 30 °C and buffered pH of 8.5, the CSP catalyst transforms partially (55% of yield) the 1,4-diacetyl-2-methylnaphtalene **3** in a single product (TLC analysis) identified, after CC isolation and spectroscopic comparison with a commercial sample, as the 1-monoacetylderivative **4** showing an interesting regiospecificity of the enzymatic system towards this substrate. The method showed herein can be considered as a possible valid eco-friendly, mild and safer alternative for obtaining compound **4**, as the methods already reported in literature involve the use of toxic reactants (Huang et al. 2006).

TAG **5** was incubated in a suspension of the CSP biocatalyst at the usual reaction conditions and the results are summarized in Table S1 (supplementary material).

It is relevant to note how it is possible to modulate the degree of acetylation of the acetins mixture (TAG → DAs → MAs → glycerol) by simply modulating the reaction time. Indeed, the GC chromatogram showed two isomeric peaks for both the diacetins and monoacetins due to the presence of regioisomeric mixtures, very probably arising from an acetyl shift occurred after the first removal of an acetyl group. We did not collect direct evidences on the structure of the main component in the monoacetins mixture.

In conclusion, the catalytic activity of the *Citrus sinensis* peel in the hydrolysis of acetates was investigated in relation to the pH of the medium, reaction temperature and time. The method works in very mild conditions and in aqueous solvent. A significant point of interest lies in the opportunity to exploit the use of the readily and affordably available *citrus* canned food industry wastes as a green biocatalyst in relevant industrial processes such as the production of fragrance ingredients as geraniol, vitamin K_1, mono- and diacetins.

3. Experimental

See supplementary material.

Disclosure statement

No potential conflict of interest was reported by the authors.

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