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## Biocatalytic synthesis of the non-pungent capsaicinoid olvanil from agri-food waste

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Capsaicin and simple capsaicinoids have been shown to possess multiple beneficial effects as antibacterials, anticancer agents, antioxidants or against obesity, just to name a few. Olvanil is one of the first synthetic capsaicinoid derivatives designed to activate the same receptor as natural capsaicin, without eliciting its powerful stinging or burning effects. The traditional synthetic approaches to olvanil involve the chemical conversion of vanillin into vanillylamine and a poorly atom-economic chemical amidation with an activated derivative of oleic acid. In this work, a simple biocatalytic two-step procedure has been developed and optimised to produce olvanil, starting from two biomass-derived synthons (oleic acid from vegetable oil soapstock waste and vanillin from lignin). The process employs only biobased reagents and catalysts, under mild conditions and without wasteful purifications, aligning well with the concepts of green chemistry and circular economy.

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### Sustainability spotlight

The agri-food industry, strictly linked to the needs of an ever-growing population, generates substantial amounts of waste that needs to be destroyed and/or treated. Recovering such waste to manufacture useful chemicals is instrumental to the efficient use of natural resources. The integrated implementation of waste-derived source materials and biological catalysts is an excellent strategy to improve the sustainability and environmental footprint of synthetic processes. In this project, as an example, a synthesis of the pharmaceutically relevant capsaicinoid olvanil was achieved using only biobased chemicals and catalysts, starting from waste materials and operating under mild conditions. Consequently, this work aligns well with the United Nations' SDG 12 (responsible consumption and production) and SDG 9 (industry, innovation and infrastructure).

## Introduction

### Transforming food waste: green chemistry and circular economy

Over the past few decades the attitudes of society and industry towards waste have undergone a significant transformation, evolving from waste remediation, through prevention and utilisation to finally become waste valorisation.<sup>1</sup> Green chemistry has become essential in driving sustainability in the chemical industry, representing a philosophy that seeks to minimize environmental impact across all stages of chemical production. Aligned with the green chemistry principles is the concept of circular economy, which shifts away from the traditional linear “take–make–use–dispose” model towards a circular approach that closes material loops, prevents the accumulation of harmful products, and avoids their overutilisation.<sup>2,3</sup>

Focusing specifically on the food industry, approximately 1.05 billion tonnes of food waste were generated globally in 2022, with 60% originating from households, 28% from food services, and 12% from retail. In the European Union alone, over 132 kilograms of food waste per inhabitant are generated annually.<sup>4</sup> Therefore, applying the principles of green chemistry and the circular economy mindset is increasingly critical for valorising waste and by-products generated throughout the food production chain.

### Soapstock valorisation

Edible oils, derived from the seeds, germs, and fruits of many plants, such as sesame, olive, soybean, rapeseed, sunflower, camellia, palm, and coconut, generate large quantities of by-products and wastes throughout all the production stages. Raw vegetable oil, obtained by a combination of pressing and solvent extraction, is a complex mixture comprising free fatty acids (FFAs), mono-, di-, and triglycerides, phosphatides, pigments, sterols, tocopherols, trace amounts of metals, flavonoids, tannins, and glycolipids.<sup>5</sup> Raw oils are generally unedible (or at least unpalatable) until non-triglyceride compounds are

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removed through a series of operations collectively known as refining.

Refining processes remove undesirable substances such as FFAs, and other materials, *e.g.*, oxidation products, sulphur compounds and waxes, which can impart unpleasant smells or tastes to the oil. Additionally, refining removes all the components considered unsafe for human consumption.<sup>6</sup> The primary by-products generated during the refining are shown in Fig. 1a.<sup>7</sup> With a production of almost 600 million metric tons per year of raw oil only in the US,<sup>8</sup> the economical and, above all, sustainable management of the refining wastes has become crucial: increasing the recycle and reuse of these by-products is, indeed, essential for achieving the green chemistry mission.

Soapstock, which accounts for approximately 6% of the volume of crude oil, is usually characterised by a high variability in composition, primarily influenced by the characteristics of the starting raw material. Currently, soapstock is used for the production of soap, methane in anaerobic digesters, and as an additive in animal feed. Its abundance and versatility make it one of the most promising materials for use as a primary resource.<sup>9</sup> In particular, it can be subjected to hydrolysis to afford a mixture of FFAs as a feedstock for chemical synthesis. Typically, this is achieved by treatment with either hot mineral acids or hydrolase enzymes. For example, our research group recently optimised a fully enzymatic procedure based on the engineered lipase Eversa<sup>®</sup> Transform 2.0 (Fig. 1b) to convert high-oleic sunflower oil (HOSO) soapstock into oleic acid, in high yield and purity.<sup>6</sup>

Biocatalysis, the use of enzymes or microorganisms as substitutes for conventional chemical catalysts, aligns well with the growing demand of the chemical industry for processes that promote both economic and environmental sustainability. Biocatalytic methods overcome many of the limitations generally

associated with conventional synthesis, such as the reliance on toxic heavy metals, harsh reaction conditions, and extensive purification steps. Instead, biocatalysts operate under mild reaction conditions and offer high levels of stereo- and regio-selectivity, without the need for protection and deprotection steps or additional purification stages. With a view to expanding the biocatalytic toolbox for the valorisation of soapstock-derived FFAs, this project is focused on the development of a fully enzymatic cascade strategy for the production of olvanil, a capsaicinoid derivative with significant potential in the pharmaceutical industry, incorporating the structure of oleic acid.

### Capsaicinoid-based pharmaceuticals

Capsaicinoids (Fig. 2a) are a class of alkaloids, primarily represented by capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide), which are extracted from the fruits of *Capsicum* plants and are responsible for their characteristic pungent taste. These compounds are generally recognised as members of the vanilloid family, alongside other notable compounds such as vanillin, zingerone and eugenol. The applications of capsaicinoids span various fields, including medicine, either human or veterinary, agriculture, as well as the food and cosmetic industries. Due to their broad spectrum of pharmacological effects, largely attributed to the presence of the vanillyl group, capsaicinoids and their derivatives have raised the interest of the scientific community. To date, several studies have confirmed the diverse pharmaceutical potential of these molecules, including their antioxidant, antimicrobial, anti-inflammatory, obesity preventing, tumour preventing,

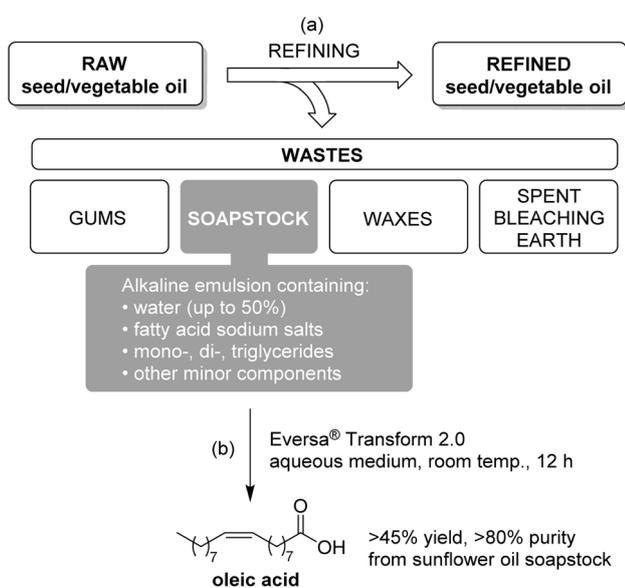


Fig. 1 Overview of the main by-products of vegetable/seed oil refining (a) and representative example of valorisation of soapstock by enzymatic hydrolysis (b).

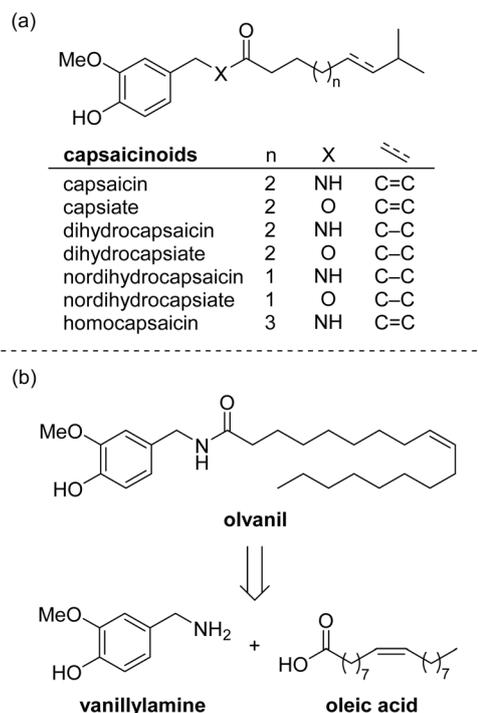


Fig. 2 Structures of the most important capsaicinoids (a) and olvanil (b), with its simplest retrosynthetic analysis.



cardioprotective, gastroprotective, and metabolic modulating properties.<sup>10–12</sup>

Capsaicin is a well-studied activator of TRPV1 (transient receptor potential vanilloid type 1), located mainly on the central endings of nociceptive neurons in the spinal cord, and in different brain regions.<sup>13</sup> When capsaicinoids bind to TRPV1, they activate the receptor, eliciting a variety of responses ranging from pain and heat sensations to the modulation of metabolic and inflammatory processes. After activation, the receptors undergo prolonged desensitisation, making capsaicin particularly promising for managing chronic pain syndromes, as well as inflammation, hyperplasia, and inflammatory skin diseases.<sup>14–16</sup> However, despite its valuable therapeutic potential, the intense pungency and burning sensation caused by capsaicin often limit its use, particularly in long-term treatments, as it can lead to significant discomfort for patients. This challenge led to the development of non-pungent capsaicin analogues that retain the pharmacological benefits of capsaicin while minimising the discomfort. Sensory studies of pure capsaicinoids revealed that the vanillyl group and the length of the alkyl chain, with suitable C=C bonds or branched methyl groups, are essential molecular features contributing to the emergence of the pungent character.<sup>17</sup> From this knowledge, in the 1980s, Procter & Gamble researchers synthesised olvanil (NE-19550, Fig. 2b), the first synthetic non-pungent capsaicin alternative, specifically designed to activate the TRPV1 receptor without the adverse stinging or burning effects of capsaicin.<sup>18</sup>

### Biocatalytic strategies for capsaicinoid production

The synthetic routes for producing capsaicin analogues, olvanil specifically, have evolved to maximise productivity while reducing the solvent usage. Particularly, the exploitation of enzymatic strategies, which aligns with green chemistry principles, has become the preferred approach for sustainable pharmaceutical production.

Kobata and co-workers, starting in 1996, were among the first to attempt a chemo-enzymatic approach to synthesise capsaicin analogues from vanillylamine.<sup>19</sup> They exploited chicken liver acetone powder as an enzyme catalyst, condensing vanillylamine with methyl oleate in a biphasic system, where the organic phase was composed of the fatty acid methyl ester itself. Subsequent work by the same group managed to afford slightly better yields (16–20%) by replacing the enzyme catalyst with the commercial lipase Novozyme 435, performing the

amidation of vanillylamine.<sup>20,21</sup> In 1999, the same group proposed a different system, based on the transacylation of capsaicin with triolein using a commercial lipase at 70 °C over 144 h, reaching a final 85% yield.<sup>22</sup>

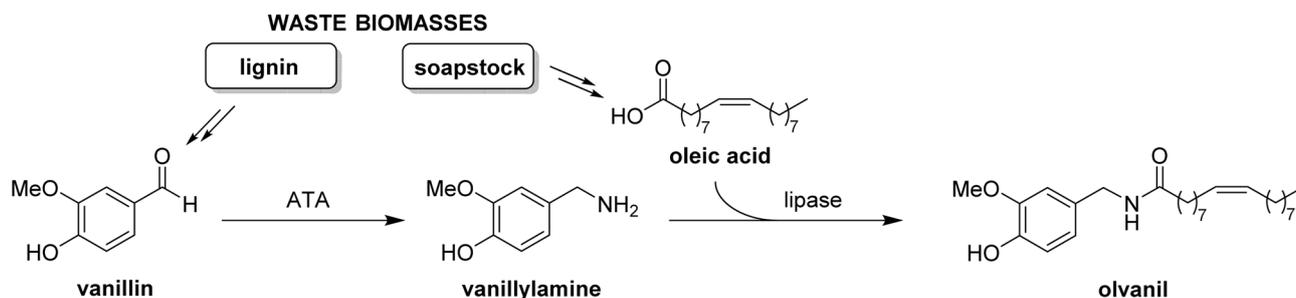
These early attempts to synthesise capsaicinoid analogues from vanillylamine and fatty acid derivatives often resulted in low conversion rates due to competing hydrolysis, as lipases catalysed both amide formation and breakdown in the same system.<sup>23</sup> Later studies employing organic solvents and optimising reaction conditions showed significant improvements in yield, demonstrating the potential of lipases to enhance the efficiency of the chemoenzymatic synthesis.<sup>24</sup> Immobilised enzyme strategies have also been implemented to minimise solvent content, resulting in low to moderate yields.<sup>25</sup> More recent work by the Berglund group developed a total synthesis of capsaicin analogues from lignin-derived vanillin, exploiting the combined catalytic activities of *Chromobacterium violaceum* transaminase (Cv-ATA) and a commercial lipase from Novozyme. This sustainable approach, reducing the need for heavy metals and supporting eco-friendly production, could also be extended to other lignin-derived substrates such as vanillyl alcohol.<sup>26</sup>

This work specifically aims at optimising olvanil production with an efficient enzymatic cascade process starting from biobased renewable materials, including lignin-derived vanillin and soapstock-derived oleic acid (Scheme 1). The process in particular comprises two key steps: first, transamination of vanillin to vanillylamine using L-alanine as the amine donor, catalysed by an amino transaminase, followed by amidation of oleic acid with vanillylamine, catalysed by a lipase.

## Results and discussion

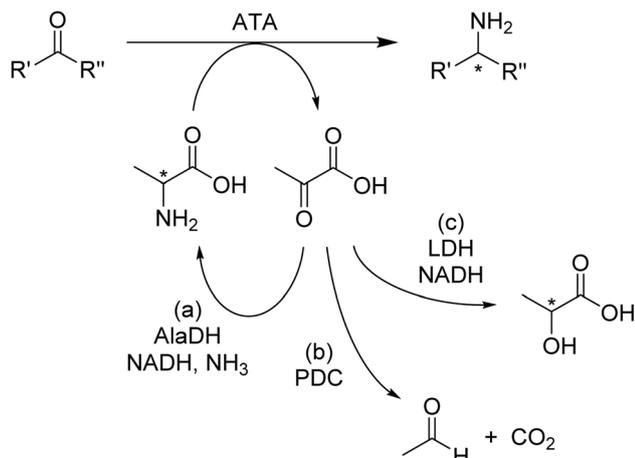
### Transamination of vanillin

Transaminases, also known as aminotransferases (ATAs, EC 2.6.1.X), are pyridoxal-5'-phosphate (PLP)-dependent enzymes that catalyse the transamination of carbonyl compounds into amines, typically using alanine as the amino donor (Scheme 2). The reaction takes place under mild conditions, with water serving as the reaction solvent.<sup>27,28</sup> However, transaminase-catalysed reactions are thermodynamically limited and readily reversible, depending on which reactant is present in excess. As a result, strategies to shift the reaction equilibrium toward the desired product ought to be implemented to improve yields.



Scheme 1 General synthetic approach to olvanil from waste biomass proposed in this work.





Scheme 2 General strategies for transaminase reactions.

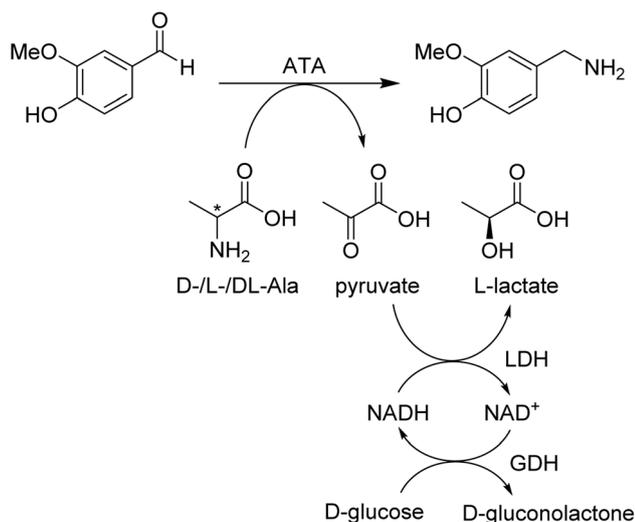
Besides the addition of an excess of the amine donor, which is not always economically viable, or the use of “smart” donors,<sup>29,30</sup> which are rarely biobased, the removal of the co-product *in situ* is the most practical method to displace the equilibrium. Pyruvate can be recycled back to the alanine donor using an alanine dehydrogenase, at the expense of  $\text{NH}_3$  and NADH (Scheme 2, route a), decarboxylated irreversibly with pyruvate decarboxylase (PDC) to yield  $\text{CO}_2$  and acetaldehyde (Scheme 2, route b), or reduced to lactic acid by lactate dehydrogenase (LDH) at the expense of NADH (Scheme 2, route c). In order to maximise the sustainability of the process, it was decided to exploit the latter method (Scheme 3), involving only biobased reagents and catalysts. Since the activity of LDH relies on the cofactor NADH, a glucose dehydrogenase (GDH) is included in the catalytic cascade to regenerate NADH from catalytic  $\text{NAD}^+$  at the expense of glucose.

In this work, seven wild-type transaminase enzymes produced recombinantly in *E. coli* were screened to find the

most suitable candidate for the reaction: *Chromobacterium violaceum* (Cv-ATA), *Arthrobacter* sp. (ATA-117), *Pseudomonas fluorescens* (Pf-ATA), *Neosartorya fischeri* (Nf-ATA), *Vibrio fluvialis* (Vf-ATA), *Aspergillus terreus* (At-ATA) and a transaminase isolated from the metagenome of an Icelandic hot spring (Is3-ATA). All enzymes used were employed as crude lysates (CFE) with standardised cell wet weight (CWW) loading (except LDH, which was relatively inexpensive from commercial sources), in order to minimise the overall cost of the process.

Cv-ATA has been reported as the standard enzyme applied to the transamination of vanillin.<sup>31,32</sup> To the best of our knowledge, a comprehensive screening of ATAs for this transformation was not found in the literature, so we employed our in-house enzyme library. As a standard analytical-scale protocol for the screening, in 100 mM sodium phosphate buffer at pH 8.0, 1.0 equiv. of vanillin (50 mM loading), 2.5 equiv. of DL-Ala, 2.0 equiv. of glucose, 0.05 equiv. PLP and 0.01 equiv. of  $\text{NAD}^+$  were employed, with the addition of 6% *v/v* ATA CFE (200  $\text{mg}_{\text{CWW}} \text{mL}^{-1}$ ) and 3% *v/v* GDH CFE (200  $\text{mg}_{\text{CWW}} \text{mL}^{-1}$ ) and 0.9  $\text{mg} \text{mL}^{-1}$  LDH, in a total volume of 1 mL. Reactions were incubated at 37 °C for 24 h and analysed by reverse-phase HPLC on a non-chiral stationary phase to determine the conversions. The results are reported in Fig. 3.

Interestingly, four enzymes (Cv-ATA, Pf-ATA, Vf-ATA, Is3-ATA) achieved conversions >95% while the remaining three enzymes (ATA-117, Nf-ATA, At-ATA) afforded only a very low amount of product. These differences can be attributed to the substrate specificity of the transaminases themselves, linked in turn to the 3D structure of the active site. It is worth mentioning that the enantioselectivity for D- or L-alanine as the amino donor is generally very strong, and, in some cases, the opposite enantiomer of the donor may even have inhibitory effects. However, we set as a target to employ only readily available and inexpensive racemic alanine as a donor, in view of minimising the cost of the process. Presumably, the enzymes that performed well were those with a sufficiently broad substrate scope to accommodate vanillin in the active site, as well as not to be inhibited significantly by the other enantiomer of alanine. Among the four active ATAs, a second screening conducted using doubled substrate



Scheme 3 Screening of ATAs for the transamination of vanillin (with the selected pyruvate removal strategy employed in this work).

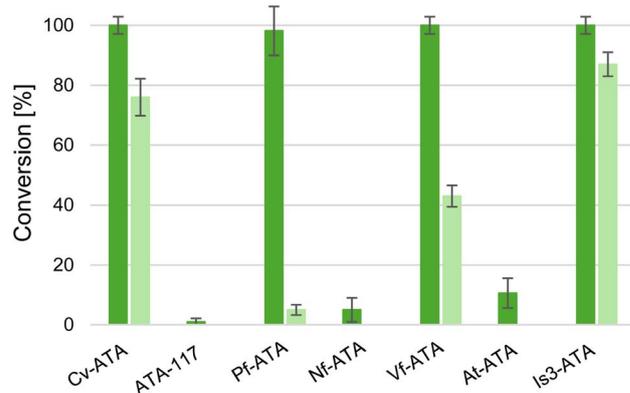


Fig. 3 Screening of ATAs for the transamination of vanillin under standard reaction conditions. Substrate loading: 50 mM (dark green bars) and 100 mM (light green bars).



**Table 1** Optimisation of the transamination of vanillin mediated by Is3-ATA. Conversions were determined by HPLC. Entries 1 and 7 correspond to the standard conditions employed in the preliminary screening (Fig. 3).

Entry	Substrate [mM]	PLP [mM]	DL-Ala [mM]	Glucose [mM]	NAD <sup>+</sup> [mM]	Is3-ATA CFE [μL]	GDH CFE [μL]	LDH [mg mL <sup>-1</sup> ]	Conv. [%]
1	50	0.5	250	100	1	60	30	0.9	>99
2	50	0.5	250	100	0.25	60	30	0.9	>99
3	50	0.5	250	100	1	30	30	0.9	>99
4	50	0.5	250	100	0.25	30	30	0.3	>99
5	50	0	250	100	1	60	30	0.9	6
6	50	0.5	150	100	1	30	30	0.9	31
7	100	0.5	500	200	1	60	30	0.9	87
8	100	0.5	500	200	0.25	30	30	0.3	89
9	100	0.5	500	100	0.25	30	30	0.3	80
10	100	0.5	200	100	0.25	30	30	0.3	19
11	100	0.25	500	100	0.25	30	30	0.3	43
12	100	0.5	500	100	1	30	30	0.3	82
13	100	0.5	500	100	0.25	60	30	0.3	83
14	100	0.5	500	100	0.25	15	30	0.3	25
15	100	0.5	500	100	0.25	30	30	0.15	42
16	100	0.5	500	300	1	30	30	0.3	>99

concentration (100 mM, Fig. 3) highlighted Cv-ATA and Is3-ATA as the most active catalysts, the latter being slightly better at higher substrate loadings. Therefore, Is3-ATA was selected for further optimisation (Table 1).

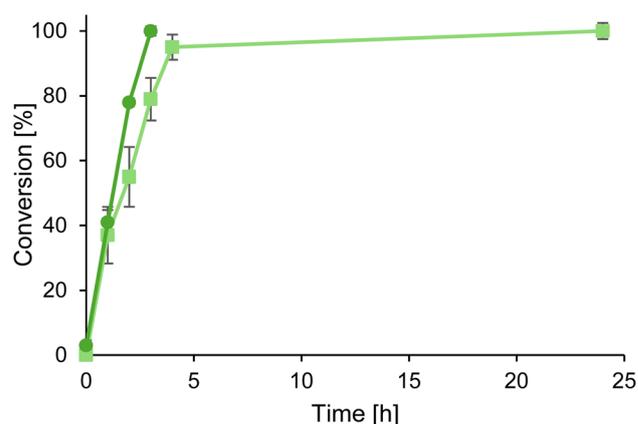
Starting from the conditions used in the preliminary screening (entries 1 and 7), multiple variables were changed to identify the most convenient setup. Firstly, replacing phosphate buffer with HEPES did not seem to affect the conversion significantly; thus, the optimisation was carried out using phosphate due to its considerably lower cost. Reducing the loading of NAD<sup>+</sup> cofactor from 0.02 to 0.005 equiv. did not negatively impact the performance of the system (entry 2 *vs.* entry 1), suggesting a very efficient enzymatic recycling. Similarly, halving the ATA CFE concentration (entry 3) and lowering the LDH amount to 0.3 mg mL<sup>-1</sup> (entry 4) still afforded quantitative conversion. On the other hand, the inclusion of PLP in the reaction medium proved essential (entry 5 *vs.* entry 1); indeed, PLP may be present bound to the apoprotein and/or in the cell lysate but not in sufficient quantities to support the reaction effectively.<sup>33</sup> Also, decreasing the excess of DL-Ala from 2.5 equiv. to 1.5 equiv. (calculated on the correct enantiomer of the donor) showed a markedly lower conversion (entry 6 *vs.* entry 3), suggesting that the pyruvate removal system on its own is insufficient to shift the equilibrium to the products.

In order to improve the productivity of the process, an increased substrate loading (100 mM) was considered next (entry 7). Employing the same ratios tested in the most optimised 50 mM reaction, which still showed quantitative conversion, only 89% conversion was achieved at 100 mM (entry 8 *vs.* entry 4). To minimise the cost of the process individual quantities were varied one at a time, illustrating clearly a marked detrimental effect of a decrease in the quantity of DL-Ala (entry 10), PLP (entry 11) and LDH (entry 15), while only minor effects were observed lowering glucose (entry 9) and increasing NAD<sup>+</sup> (entry 12). Additionally, a higher loading of ATA CFE affected the conversion only marginally (entry 13), while a lower loading showed a significantly negative effect

(entry 14). Pleasingly, the effect of combining the higher loading of NAD<sup>+</sup> (1 mM) with a slightly increased excess of glucose (3 equiv.) afforded again >99% conversion (entry 16). Those conditions were considered to be a suitable trade-off between productivity and overall cost for further scale-up.

Two preparative reactions were conducted starting from 100 mg of vanillin, testing both substrate loadings (50–100 mM), under the best conditions identified in the optimisation trials (entries 4 and 16, respectively). The progress curves of the reactions are reported in Fig. 4. As expected, both reactions were complete within 24 hours, although the time courses highlighted a much shorter reaction time needed to achieve complete conversion (3–5 h), with no detectable formation of unwanted byproducts (also confirmed by NMR analysis of the isolated product).

The recovery of the product at the end of the biotransformation proved less straightforward than expected. Vanillylamine contains both amino and hydroxy groups, with reported



**Fig. 4** Progress curves of the preparative scale transamination of vanillin mediated by Is3-ATA. Substrate loading: 50 mM (dark green circles) and 100 mM (light green squares).



$pK_a$  values of 8.5 and 10.9, respectively, at 25 °C.<sup>34</sup> The narrow  $pK_a$  range and the relatively high solubility in water make it difficult to optimise the extraction conditions reliably. After a few preliminary tests, it was found most effective to adjust the pH to 10.0 (with aqueous NaOH) before the extraction, done with EtOAc, which performed better than MTBE and *n*-hexane. Vanillylamine could be recovered by evaporation under reduced pressure and used directly for the following step without further purification. The isolated yields are in the range of 40–50%, which is in line with the reported values in the literature.<sup>26,35</sup>

On the other hand, considering the electron-rich nature of the product and its tendency to undergo oxidation and/or polymerisation relatively easily, when temporary storage was necessary before performing the subsequent step, we found it convenient to precipitate the vanillylamine in the form of its stable hydrochloride salt (41% isolated yield). NMR characterisation data are provided for both the free base and its HCl salt (see SI).

### Amidation of vanillylamine with oleic acid

High-oleic sunflower oil (HOSO) soapstock was kindly provided by Oleificio Zucchi (Cremona, Italy). Following our reported protocol,<sup>6</sup> the HOSO soapstock was submitted to enzymatic hydrolysis using the engineered lipase Eversa<sup>®</sup> Transform 2.0 from *Thermomyces lanuginosus* (1% w/w) in water at room temperature for 12 h. The hydrolysed product was isolated by acidification with phosphoric acid to pH 4.5–5.0 followed by centrifugation, yielding a mixture of FFAs mostly constituted of oleic acid (>85% by <sup>1</sup>H NMR and by GC-MS of the corresponding methyl esters) in 47% isolated yield. Crude oleic acid obtained from this hydrolysis and the raw vanillylamine obtained from transamination of vanillin were both employed for the amidation step (Scheme 4) without further purification.

Starting from the conditions optimised previously for the synthesis of oleylethanolamide,<sup>36</sup> the efficiency of the lipase-catalysed amidation was evaluated with a higher vanillylamine concentration (0.8 M) and 1.25 equiv. of oleic acid (1.0 M) using (*R*)-limonene as the solvent. Limonene was selected because it is a low-cost, low-toxicity and biodegradable terpene, abundant in agricultural waste derived from citrus peels, and commonly

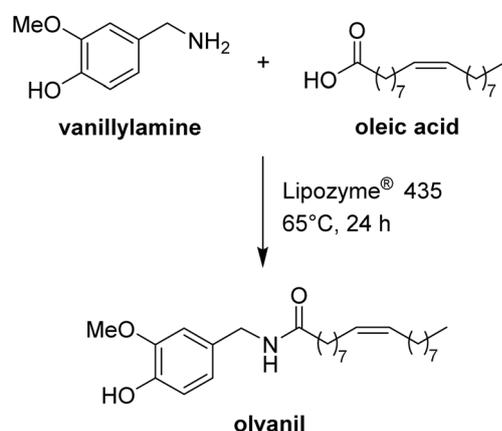
used as a viable alternative to hydrocarbon solvents.<sup>37</sup> The enzyme employed was immobilised *Candida antarctica* lipase B (Lipozyme<sup>®</sup> 435 from Novozymes), used at 30 mg mL<sup>-1</sup> loading, a lower amount compared to literature conditions in order to reduce costs. However, under these conditions, after 24 h at 65 °C under magnetic stirring, the reaction afforded only moderate conversion (42% calculated by NMR analysis). The only subproducts detected in very minor amounts were the vanillyl amides of other fatty acids contained in the hydrolysed FFAs from soapstock, while no evidence of esterification of the free phenol of vanillylamine was observed, in line with previous experiments also in the synthesis of oleylethanolamide.<sup>36</sup> However, the separation of the olvanil product required column chromatography due to the residual amount of oleic acid.

Although the chromatographic separation is relatively easy due to the very large difference in retention factors by TLC, it would be more convenient not to have to deal with an excess of oleic acid and isolate the product simply by extraction without the need for any purification. Thus, an excess of vanillylamine (1.25 equiv.) was tested instead with the same ratio, employing slightly lower concentrations of both reactants (0.3 M amine, 0.24 M oleic acid) in order to increase conversions at the same time. Three reactions were set up using different solvent systems, *i.e.*, (*R*)-limonene, *n*-heptane and solvent-free, again with 30 mg mL<sup>-1</sup> enzyme at 65 °C for 24 hours. Under these conditions, more promising results were achieved with heptane and limonene, which yielded 79% and 77% of oleic acid, respectively. In contrast, the solvent-free reaction resulted in a very poor conversion, likely due to extremely high viscosity and inefficient mixing.

Finally, a preparative scale reaction was set up using 350 mg vanillylamine (1.25 equiv., 0.23 M final concentration) and 608 mg FFA mixture from HOSO soapstock (corresponding to 517 mg oleic acid, 85% purity, 1.0 equiv., final concentration 0.18 M) in 10 mL (*R*)-limonene. After 24 h, complete conversion of oleic acid could not be achieved; therefore, 100 mg of vanillylamine (0.35 equiv.) were added and the reaction afforded complete conversion after another 24 h. Extraction with EtOAc and brine allowed removal of the excess amine efficiently, due to its high solubility in the aqueous layer. Evaporation of the organic layer under reduced pressure afforded olvanil in good yields (482 mg, 63%) and purity, as characterised by NMR and GC-MS.

## Conclusions

The convergence of biocatalysis, biomass utilisation, and green chemistry marks a transformative era for the chemical industry. By employing biobased catalysts and solvents, industry can significantly reduce its environmental footprint while maintaining economic competitiveness. Such a shift not only supports the present sustainability goals but also fosters innovation to address future ecological challenges. Biocatalysis exemplifies how nature-inspired solutions can revolutionise traditional industries, providing a blueprint for economic growth that is inherently sustainable and environmentally responsible.



Scheme 4 Amidation of vanillylamine with oleic acid.



In this work, an integrated strategy for the sustainable synthesis of olvanil, a vanilloid-based derivative with significant potential in the pharmaceutical industry was proposed, exploiting an efficient fully enzymatic cascade process starting from lignin-derived vanillin and soapstock-derived oleic acid, under mild reaction conditions. In a representative preparative synthesis, starting from 0.93 g of vanillin and 1.29 g of soapstock, it was possible to isolate 0.482 g of the final product in satisfactory purity, avoiding the use of harmful solvents and chromatographic separations. While a thorough economic evaluation would be important to demonstrate the industrial applicability of the proposed catalytic system, a comprehensive and meaningful cost-benefit analysis would require a full life cycle assessment (LCA), which could be the focus of future endeavours. This process holds promise from an economic standpoint, particularly because it relies on recombinant non-purified enzymes, which can now be produced at low cost using established expression systems, and also because the significant added value of the product for pharmaceutical application is likely to compensate for the slightly higher costs associated with the enzymes compared to traditional chemical catalysis. Furthermore, the process has been demonstrated using only waste-derived starting materials, highlighting its sustainability and economic attractiveness in the long term.

## Experimental section

### General methods

Analytical grade reagents and solvents were obtained from Sigma-Aldrich, Fluorochem or Fisher Scientific and used without further purification, unless stated otherwise. Vanillin produced from lignin was supplied by Sacmar s.r.l. (Settimo Milanese, Italy). HOSO soapstock was provided by Oleificio Zucchi (Cremona, Italy).

$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded on a Bruker Avance II 400 spectrometer (400 MHz). Chemical shifts are reported as  $\delta$  in parts per million (ppm) and are calibrated against residual solvent signal ( $^1\text{H}$  NMR: chloroform-*d* = 7.26 ppm, DMSO-*d*<sub>6</sub> = 2.50 ppm;  $^{13}\text{C}$  NMR: chloroform-*d* = 77.0 ppm, DMSO-*d*<sub>6</sub> = 39.0 ppm).

GC-MS analyses were carried out on an Agilent 7890A gas chromatograph with an Agilent 5975C MS detector, using an HP-5MS column (30 m  $\times$  0.25 mm  $\times$  0.25  $\mu\text{m}$ ). The following temperature program was employed: 50  $^\circ\text{C}$  (0 min)/10  $^\circ\text{C}$  min<sup>-1</sup>/250  $^\circ\text{C}$  (5 min)/50  $^\circ\text{C}$  min<sup>-1</sup>/300  $^\circ\text{C}$  (10 min).

HPLC analyses for the determination of the conversion of vanillin were carried out on a non-chiral reverse-phase Luna C-18 extend column (150  $\times$  4.60 mm, 5  $\mu\text{m}$ , phenomenex). Mobile phase aq. 0.1% trifluoroacetic acid (TFA)/MeCN 90 : 10, flow rate 0.8 mL min<sup>-1</sup>, temperature 25  $^\circ\text{C}$ , detection wavelength 280 nm.  $t_{\text{R}}$ (vanillylamine) = 3.7 min,  $t_{\text{R}}$ (vanillin) = 22.2 min. Response factor of vanillin vs. vanillylamine (determined by injection of mixtures with known concentration) = 4.6.

### Analytical scale screening of ATAs for the transamination of vanillin

Stock solutions of vanillin (1 M in DMSO), DL-Ala (1 M in 100 mM NaP<sub>i</sub> buffer pH 8.0), PLP (10 mM in water), D-glucose

(1 M in 100 mM NaP<sub>i</sub> buffer pH 8.0), NAD<sup>+</sup> (10 mM in NaP<sub>i</sub> buffer pH 8.0) were prepared separately and stored at -20  $^\circ\text{C}$ . Raw lysates of ATAs and GDH were prepared as described in the SI, with a normalised cell wet loading of 200 mg<sub>C<sub>WW</sub></sub> mL<sup>-1</sup>, frozen at -80  $^\circ\text{C}$  and stored at -20  $^\circ\text{C}$ . LDH (supplied by Roche Diagnostics) was suspended in 50 mM NaP<sub>i</sub> buffer pH 8.0 to a final concentration of 30 mg mL<sup>-1</sup> and used immediately. Appropriate volumes of the stocks were added to a 2 mL tube containing the suitable buffer (NaP<sub>i</sub> or HEPES, 500 mM, pH 8.0) and water to a final volume of 1.0 mL and final buffer concentration of 100 mM.

The tube was covered with aluminium foil to prevent exposure to direct light and incubated at 37  $^\circ\text{C}$ , 200 rpm for 12–48 h. After the desired incubation time, the reactions were quenched by adding 1.0 mL of MeOH and centrifuged to remove any precipitate. The resulting clear supernatant was appropriately diluted and analysed by HPLC (representative chromatograms are shown in the SI). Conversions were determined by integration of the peaks of vanillylamine and vanillin, taking into account the corresponding response factor.

### Preparative scale transamination of vanillin

DL-Ala (2.93 g, 5 equiv.), glucose (2.37 g, 2 equiv.), NAD<sup>+</sup> (15 mg, 0.0025 equiv., 0.25 mM final conc.) and PLP (10 mg, 0.01 equiv., 0.5 mM final conc.) were weighed and added to a clean 100 mL screw cap glass bottle. NaP<sub>i</sub> buffer (56 mL, 100 mM pH 8.0) was added, followed by Is3-ATA CFE (1.95 mL), GDH CFE (1.95 mL) and LDH solution (1.95 mL). The mixture was stirred briefly, then a solution of vanillin (1.00 g, 1 equiv., final conc. 100 mM) dissolved in 3.5 mL DMSO (final conc. 5% v/v) was added, reaching a final volume of 65 mL. The bottle was wrapped in aluminium foil and incubated in an orbital shaker at 37  $^\circ\text{C}$ , 200 rpm, monitoring the conversion periodically (for each time point, 50  $\mu\text{L}$  of the reaction mixture was quenched with 50  $\mu\text{L}$  MeOH, centrifuged and analysed by HPLC). When complete conversion was reached, the mixture was centrifuged to remove insoluble components, then adjusted to pH 10.0–11.0 by addition of aq. NaOH (4 M), and extracted with EtOAc (6  $\times$  30 mL). The combined organic phases were washed with brine (50 mL) and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. Evaporation under reduced pressure at 30  $^\circ\text{C}$  afforded crude vanillylamine (481 mg, 48% isol. yield), which was characterised by NMR analysis and used in the following step without further purification.

### Characterisation of vanillylamine

$^1\text{H}$  NMR (400 MHz, MeOD)  $\delta$  6.98 (d, *J* = 1.5 Hz, 1H), 6.84–6.75 (m, 2H), 3.91 (s, 3H), 3.74 (s, 2H).  $^{13}\text{C}$  NMR (101 MHz, MeOD)  $\delta$  149.1, 146.8, 135.0, 121.1, 116.2, 112.3, 56.4, 46.53.

### Isolation of vanillylamine hydrochloride

Vanillylamine (100 mg) was dissolved in Et<sub>2</sub>O (3 mL), and a slight excess of saturated solution of HCl in Et<sub>2</sub>O was added dropwise. After each droplet, the solution became cloudy and a white precipitate appeared. The resulting solid was filtered, dried and weighed (50.8 mg, 41% isolated yield).



In order to employ vanillylamine hydrochloride as the starting material for the subsequent amidation step, the free base was prepared as follows. Vanillylamine·HCl (50 mg) was added to water (300  $\mu$ L), and the mixture was stirred and heated to 40 °C in order to dissolve the salt completely. Aqueous NaOH (86  $\mu$ L, 4 M) was then added, causing an off-white solid to precipitate. The solid was then filtered, washed with ice-cold water, and dried under a stream of nitrogen before using it in the amidation reaction.

### Characterisation of vanillylamine hydrochloride

$^1\text{H}$  NMR (400 MHz, MeOD)  $\delta$  7.00 (d,  $J$  = 1.8 Hz, 1H), 6.88–6.72 (m, 2H), 3.88 (s, 2H), 3.86 (s, 3H).  $^{13}\text{C}$  NMR (101 MHz, MeOD)  $\delta$  149.4, 148.6, 125.5, 123.1, 116.6, 113.7, 56.5, 44.4.

### Preparative scale amidation of vanillylamine and oleic acid

Oleic acid (85% purity) was prepared from HOSO soapstock according to our previously published procedure.<sup>6</sup> Vanillylamine (350 mg, 1.25 equiv., 0.23 M final conc.) and soapstock-derived oleic acid (608 mg of FFA mixture composed of 85% oleic acid, obtained from 1.29 g soapstock, 1 equiv., 0.18 M final conc.) were added to a clean glass vial, followed by 10 mL of (*R*)-limonene as the solvent. Subsequently, lipozyme 435 (100 mg, 10 mg mL<sup>-1</sup> loading) was added. The reaction mixture was magnetically stirred and kept at 65 °C for 24 h. The conversion was monitored by TLC (Hex/EtOAc 1 : 1). After 24 h, an additional aliquot of vanillylamine (100 mg, 0.35 equiv.) was added and stirring was continued at 65 °C until completion. The reaction mixture was then diluted with brine (3 mL) and extracted with EtOAc (3  $\times$  3 mL). The combined organic phases were washed with brine (5 mL) and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. Evaporation under reduced pressure afforded crude olvanil (482 mg, 63% isolated yield). The conversion was determined by NMR analysis (details on the signals selected for integration are reported in the SI). The purity of the final compound was assessed by GC-MS analysis, after derivatisation with CH<sub>2</sub>N<sub>2</sub>, integrating the peaks corresponding to the product against all residual signals.

### Characterisation of olvanil

$^1\text{H}$  NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  6.84 (d,  $J$  = 8.0 Hz, 1H), 6.78 (d,  $J$  = 1.6 Hz, 1H), 6.73 (dd,  $J$  = 8.0, 1.6 Hz, 1H), 5.81–5.90 (m, 2H), 5.28–5.36 (m, 2H), 4.32 (d,  $J$  = 5.5 Hz, 2H), 3.84 (s, 3H), 2.18 (t,  $J$  = 7.7 Hz, 2H), 1.96–2.05 (m, 4H), 1.58–1.68 (m, 2H), 1.22–1.36 (m, 20H), 0.84–0.90 (m, 3H).  $^{13}\text{C}$  NMR (101 MHz, CDCl<sub>3</sub>):  $\delta$  173.1, 146.9, 145.3, 130.4, 130.1, 129.8, 120.8, 114.5, 110.9, 56.0, 43.6, 36.9, 32.0, 29.9, 29.8, 29.6, 29.4 (4C), 29.2, 27.3, 27.2, 25.9, 22.8, 14.2. GC-MS:  $t_{\text{R}}$  = 27.3 min,  $m/z$  (%) = 417 (M<sup>+</sup>, trace), 417 (13), 294 (5), 195 (7), 152 (13), 137 (100). GC-MS (after derivatisation with CH<sub>2</sub>N<sub>2</sub> to give the methyl ether):  $t_{\text{R}}$  = 31.8 min,  $m/z$  (%) = 431 (M<sup>+</sup>, trace), 417 (11), 294 (7), 195 (9), 152 (15), 137 (100).

## Conflicts of interest

There are no conflicts to declare.

## Data availability

The data supporting this article have been included as part of the SI.

Supplementary information is available: Methods for the production of the enzymes, representative HPLC traces, copies of the  $^1\text{H}$  and  $^{13}\text{C}$  NMR and MS spectra. See DOI: <https://doi.org/10.1039/d5su00241a>.

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