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MINI-REVIEW

The antioxidant hydroxytyrosol: biotechnological production challenges and opportunities

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Abstract Hydroxytyrosol (HT) is a highly potent antioxidant originating in nature as a second metabolite of plants, most abundantly in olives (*Olea europaea*). In the last decade, numerous research studies showed the health benefits of antioxidants in general and those of HT in particular. As olive oil is a prime constituent of the health-promoting Mediterranean diet, HT has obtained recognition for its attributes, supported by a recent health claim of the European Food Safety Authority. HT is already used as a food supplement and in cosmetic products, but it has the potential to be used as a food additive and drug, based on its anticarcinogenic, anti-inflammatory, antiapoptotic and neuroprotective activity. Nevertheless, there is a large gap between the potential of HT and its current availability in the market due to its high price tag. In this review, the challenges of producing HT using biotechnological methods are described with an emphasis on the substrate source, the biocatalyst and the process parameters, in order to narrow the gap towards an efficient bio-based industrial process.

Keywords Hydroxytyrosol · Biocatalyst · Protein engineering · Antioxidant · Olive oil

Introduction

The growing demand by society for a healthier life style and extended life expectancy poses a great challenge to the food and drug industries to find health-beneficial natural and effective sources for drugs and food additives (Kotler 2011; Szakály et al. 2012). One such chemical is hydroxytyrosol [3,4-dihydroxyphenylethanol (HT)], a phenolic compound originating from olives, which is considered as one of the strongest antioxidants in nature (De Leonardis et al. 2008; Mateos et al. 2008; Pérez-Bonilla et al. 2013; Rietjens et al. 2007). Accordingly, it is the only phenol that has been recognized by the European Food Safety Authority (EFSA) as a protector of blood lipids from oxidative damage (Visioli 2012). In order to bear the health claim, 5 mg of HT and its derivatives (e.g. oleuropein complex and tyrosol) in olive oil should be consumed daily. These amounts can be easily consumed in the context of a balanced diet (EFSA Panel on Dietetic Products Nutrition and Allergies 2011). Besides the cardioprotective effects (Cabrerizo et al. 2013; Merra et al. 2014), numerous studies, mostly in vitro assays and using animal models, have shown the potential of HT for preventing additional diseases. These include protection against metabolic diseases (Bali et al. 2014; Bulotta et al. 2014; Cao et al. 2014; Kaminaga et al. 2006), anti-carcinogenic activity (Anter et al. 2014; Burattini et al. 2013; Carrera-González et al. 2013; Sun et al. 2014; Weng and Yen 2012; Zhao et al. 2014), anti-inflammatory activity (Takeda et al. 2014), protection against digestive disorders (Sánchez-Fidalgo et al. 2012) and antimicrobial activity (Bisignano et al. 2014). Some recent examples are presented in Table 1.

The accumulating disease-preventing evidence and the fact that HT has no known toxic effects (Auñon-Calles et al. 2013) emphasize the potential of HT as a nutraceutical in functional foods, food supplements and even medicine. The benefits of the Mediterranean diet in general, and olive oil and HT in particular, have been well documented in recent reviews and are beyond the scope of this paper (Carrera-González et al. 2013; Hu et al. 2014; Liebgott et al. 2009; Rafehi et al. 2012). Though HT seems to be the perfect candidate for the

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Table 1 Examples of recent research focusing on the beneficial effects of HT

Target treatment	HT form	Dose	Experimental procedure	The beneficial effects	Reference
High cholesterol	Pure HT (98 %), HT-acetate (HT-Ac) and HT-ethyl ether (HT-Et)	0.04 % of HT, HT-Ac and HT-Et of the daily diet resulted in a daily intake of 25 mg/kg body weight	Feeding male Wistar rats with a high-cholesterol diet with/without HT supplementation. Animal study.	HT and its lipophilic derivatives were able to reduce the metabolic imbalance induced by a high-cholesterol diet in rats (improved glucose, insulin, leptin and MDA levels and antioxidant capacity status), with HT-Ac, being the most effective phenolic compound.	(Tabernero et al. 2014)
Oxidative stress provoked heart damage	Pure HT	0.5 mg/kg, 5 days/week	Investigation whether doxorubicin-associated chronic cardiac toxicity can be ameliorated with the antioxidant hydroxytyrosol in rats with breast cancer. Animal study.	HT improved the cardiac disturbances enhanced by doxorubicin by significantly reducing the percentage of altered mitochondria and oxidative damage.	(Granados-Principal et al. 2014)
Oxidative stress	Pure HT	9.0 mg/kg body weight	Effect of HT on oxidative stress induced by cadmium injections ($CdCl_2$ 2.5 mg/kg body weight) in spleen and testes of adult male rats. Animal study.	An antioxidant effect exerted by HT on superoxide dismutase and catalase activity in cytosol of spleen from Cd-intoxicated rat, accompanied by the failure of HT to restore the enzymatic activities studied in mitochondria of both spleen and testes. HT may serve as a potential prophylactic agent against a wide range of disorders, including inflammatory and neurodegenerative diseases, blood disorders, cancer, diabetes and aging.	(Merra et al. 2014)
Diet-induced metabolic syndrome	Pure HT	10 mg/kg/day 50 mg/kg/day	Investigation of HT supplementation at two doses for 17 weeks of rats fed on a high-fat-diet (HFD). Animal study.	HT could effectively normalize obesity, diabetes, dyslipidemia, inflammation, fatty liver, and insulin resistance induced by HFD feeding in rats. The primary mechanisms of this observation involved down-regulating the SREBP-1c/FAS pathway, reducing oxidative stress, attenuating mitochondrial abnormalities and suppressing apoptosis.	(Cao et al. 2014)
Antigenotoxic, cytotoxic and proapoptotic	Pure HT and Alperujo (AL) an olive oil industry by-product	6.25 and 100 μM HT, 3.75 and 30 μL/mL AL	Genotoxic tests in the Somatic Mutation and Recombination Test (SMART) of *Drosophila melanogaster* and exerted antigenotoxic activity against DNA oxidative damage generated H_2O_2. Antiproliferative and caspase-3-dependent proapoptotic effects toward the human tumoural cell line HL60. Drosophila and in vitro study.	AL and its major phenolic compounds are safe, induced a clear antimutagenic effect in the wing spot assay and may acts as inducer of cell death in HL60 cells in different extent.	(Anter et al. 2014)
Anti-cancer effects	Pure HT	0–400 μM	Investigation of the anti-cancer effects of HT in human hepatocellular carcinoma cells (HCC) (lines HepG2, Hep3B, SK-HEP-1 and Huh-7). In vitro study.	HT potently inhibits KT and NF-κB activation, leading to the inhibition of proliferation and pro-motion of apoptosis in human HCC cells.	(Zhao et al. 2014)
Anti-apoptotic activity	Pure HT and HT laurate (laur-HT)	20 μM HT and 5 μM laur-HT	Effects of HT and laur-HT onU937 cells, a human monocytoid cell line, and in C2C12 myoblasts, a murine proliferating muscle cell model, after apoptotic death induction with H_2O_2. In vitro study.	HT and laur-HT have potential as protective agents against H_2O_2-induced apoptotic death in different cell lines.	(Burattini et al. 2013)
Anti-inflammatory and anti-atherosclerotic activity	Pure HT	1–10 μmol/L	Examine the HT effects on inflammatory markers inhuman activated monocytes, including MMP-9 and COX-2 activity	HT, at nutritionally relevant concentrations, reduced MMP-9 and COX-2 induction in activated human monocytes via PKCa and PKCb1 inhibition.	(Scoditti et al. 2014)

Table 1 (continued)

Target treatment	HT form	Dose	Experimental procedure	The beneficial effects	Reference
			and expression and explore protective mechanisms. In vitro study.		
Treatment of inflammatory diseases	Pure HT	0–12.5 μg/mL	Investigation of the HT anti-inflammatory effects on peritoneal macrophages of BALB/c mice. In vitro study.	HT suppressed nitric oxide production by decreasing iNOS gene expression through a mechanism independent of the NF-κB signalling pathway.	(Takeda et al. 2014)
Neuroprotective effect	Pure HT	1, 5 and 10 mg/kg per day	Analyse the mechanism of the neuroprotective effect of HT in an experimental model of hypoxia-reoxygenation in rat brain slices. Animal study.	HT showed antioxidant and anti-inflammatory effects in a model of hypoxia-reoxygenation in rat brain slices in vitro and after 7 days of oral administration.	(Cabrerizo et al. 2013)

functional food market, its prices are very high. For instance, the pure product can reach more than $12,500/g (Sigma-Aldrich), or $1000/g as reported by Zhang et al. (2012), while the extract from olive leaves in an un-purified form can be as low as $15/g (https://www.prohealth.com/shop/product.cfm/product__code/PH398). It is worth mentioning that most of the desired health effects have been obtained in recent works by using the purified form (Table 1). Consequently, by lowering the commercial price of pure HT, a new set of products such as foods enriched with HT could be introduced into the markets and benefit consumers that do not consume olives or olive oil on a daily basis (Larrosa et al. 2003). The high price can be attributed to the low HT concentration in its natural sources, low extraction yields and difficulty to synthesize HT chemically (Zhang et al. 2012). Therefore, development of biotechnological approaches is of increasing interest and prospects. Here, we review these potential bio-processes by looking at the key factors along the production pipeline including choice of the source, the biocatalyst and the process itself.

Source selection

Efficient and feasible production of HT must start with a suitable source or substrate, implying a chemically stable compound from an available source, of low cost and high safety (see Fig. 1). Such a source can derive from the natural HT synthetic pathway or initiate a new synthetic route while itself originating from a natural source or a synthetic one (Fig. 2). Here, these two possibilities are discussed.

Sources from the natural pathway

Being a phenolic compound, HT can be found in fruits and vegetables, but it is mostly abundant in olive trees (*Olea europaea*) as a metabolite of oleuropein degradation (Charoenprasert and Mitchell 2012; Pérez-Bonilla et al. 2013; Purcaro et al. 2014) (Fig. 2a). The concentration of HT in olive leaf extracts is 10–40 μg/mL (Bali et al. 2014; Hayes et al. 2011) and in the oil can reach 200 μg/mL (Romero and Brenes 2012). It can also be found in grapes and wine but in lower concentrations of 1–2 μg/mL (Fernández-Mar et al. 2012; García-García et al. 2013). Consequently, olive tree derivatives are the most accessible source for HT. Indeed, the majority of the HT-production processes described in the literature, and used in the market, come from olive sources. Yet, the concentrations achieved from any of these sources are still quite low ranging from 0.5 to 1.5 g/L (Rigane et al. 2012). Purified hydroxytyrosol at a concentration of 2.3 g per 100 g of fresh olive leaves was obtained by a hydrolysis reaction of leaf extract and subsequent purification using a C-18 silica gel column (Bouaziz

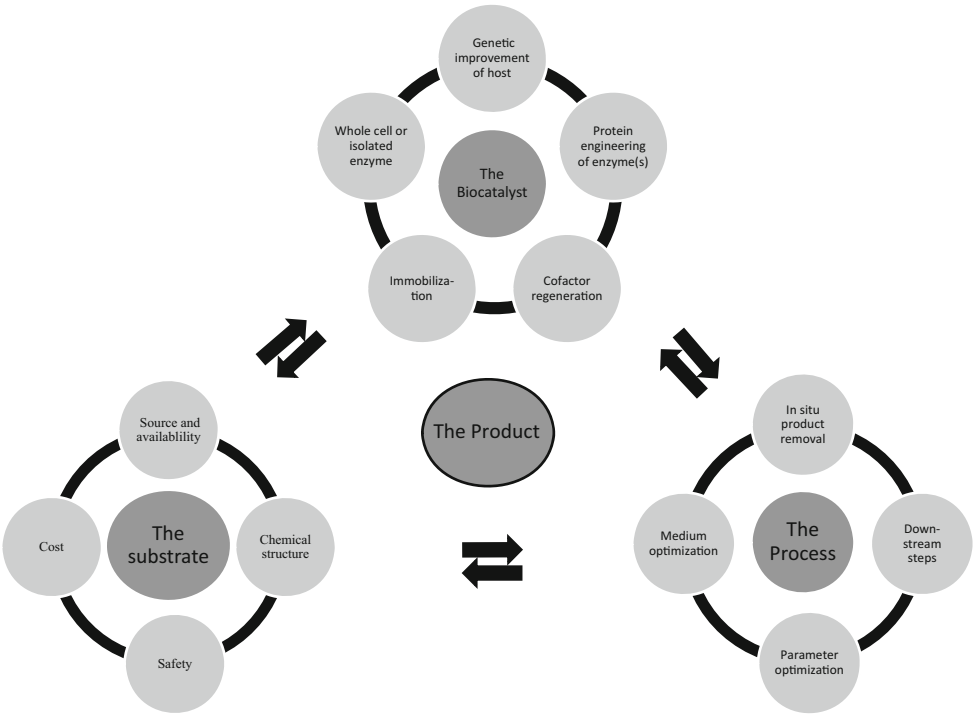

Fig. 1 A schematic diagram of the main variables and interrelationships which should be considered in the evaluation of a biotechnological process for the production of HT

and Sayadi 2005). A lower concentration of 0.2/100 g was reported by another group (De Leonardis et al. 2008). Instead, the use of the olive oil industrial waste (olive mill wastewaters (OMW)) as a source for HT is much favourable due to the fact that it originates from a by-product, and many processes use it as the substrate (Feki et al. 2006; Jerman Klen and Mozetič Vodopivec 2011; Kalogerakis et al. 2013; Oral et al. 2014). Although OMW is a good source for HT production, the process still suffers from long duration and low recovery yields as shown in Table 2. Therefore, new routes for HT synthesis are sought, as well as attempts to produce it de novo.

Sources for new synthetic pathways

Several simple compounds with chemical resemblance to HT have been used as starting materials for designing new synthetic routes (Fig. 2b). These can be either natural compounds (such as tyrosol (Allouche et al. 2004) or amino acids (Satoh et al. 2012)) or synthetic compounds such as 3-nitrobenzene-ethanol (Bernath-Levin et al. 2014; Zhang et al. 2012). There are two main considerations for choosing a specific substrate for the production of HT; the first being the cost of the substrate, and the second is its compatibility with the biocatalyst. For instance, the use of tyrosol as a substrate is an obvious choice, since it is structurally related to HT. Not surprisingly, it was used by several research groups for synthesizing HT (Table 2) (Azabou et al. 2007; Bouallagui and Sayadi 2006; Espín et al. 2001; Liebgott et al. 2009; Orenes-Piñero et al. 2013). The shortcomings of using tyrosol as a substrate are the relatively high cost (~$200/g for the purified compound, Sigma-Aldrich), possible nonspecific transformation of the phenyl ring (Brouk and Fishman 2009), and substrate toxicity to the biocatalyst (Allouche et al. 2004). Brouk and co-workers used a much cheaper substrate, namely 2-phenylethanol, as the substrate for the biocatalytic reaction (~$0.15/g for a purified compound, Sigma-Aldrich) (Brouk and Fishman 2009; Brouk et al. 2010). Depending on the biocatalyst, formation of other catechols was observed (e.g. toluene *ortho*-monooxygenase produced the isomer 2,3-dihydroxyphenylethanol). A different approach is to choose the substrate from the main metabolic roots in the cell like the amino acid tyrosine, the advantage being a very cheap resource (~$0.8/g for L-tyrosine, Sigma-Aldrich) which can be found in any living organism (Satoh et al. 2012). However, such a synthetic design demands a more sophisticated biocatalytic system. The use of amino acids as a substrate gives the potential to synthesize HT from building block compounds like sugars which can be very attractive from the commercial point of view (Satoh et al. 2012). In some transformations, the substrate was chosen based on its compatibility with a specific biocatalyst. For example, Bernath-Levin et al. used the substrate 3-nitrophenethyl alcohol (3NPA) (with a price of~$100/g, Sigma-Aldrich) which is suitable for the biocatalyst nitrobenzene dioxygenase (NBDO) since 2-phenylethanol failed to give the desired conversion (Bernath-Levin et al. 2014). Another substrate that was recently used for HT synthesis by means of biocatalysis is 3,4-dihydroxyphenylacetic acid (~$15/g for a purified compound, Sigma-Aldrich) which was reduced to obtain HT. Napora-Wijata and co-workers reported a level of 29.2 mg of pure HT in 100-mL working

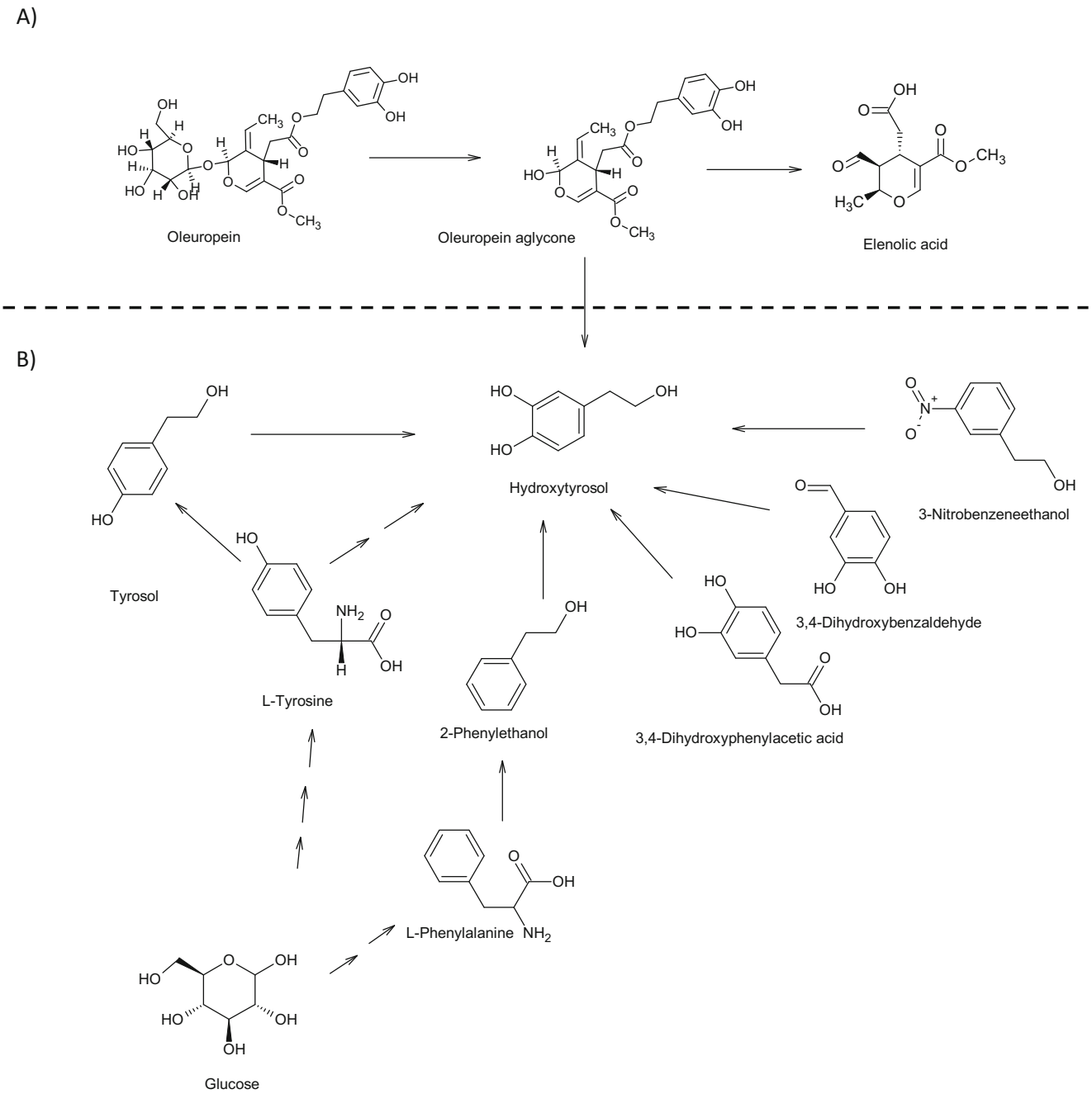

Fig. 2 Compounds used as substrates for the production of HT. **a** The natural pathway in olives and **b** nonnatural pathways

volume bioreactor, using whole *Escherichia coli* cells over-expressing carboxylic acid reductase from *Nocardia* (Napora-Wijata et al. 2014). Contrary to using biocatalysis, chemical synthetic methods often require protection and de-protection steps. For example, Bovicelli et al. used a five-step protocol starting with tyrosol to synthesize HT (Bovicelli et al. 2007). These included monobromination with NaBr/oxone, displacement of the aryl bromide with methoxide in the presence of CuBr at 120 °C, and acetylation with acetic anhydride followed by demethylation with BBr_3 to obtain hydroxytyrosyl acetate. HT was obtained from the acetate by enzymatic or chemical hydrolysis, and the overall yield was 37 %. Zhang and colleagues also reported a five-step procedure using 2,3-dihydroxybenzaldehyde as the substrate (Zhang et al. 2012). The two free hydroxyl groups were first protected by a benzyl group. Subsequently, one carbon homologation of the protected aldehyde was achieved by employing α-(*N*-methylanilino)acetonitrile to afford α-cyanoenamine, which was readily converted to the carboxylic acid under acidic conditions. The resulting carboxylic acid was then

Table 2 Biotechnological routes for HT synthesis in comparison with OMW extraction processes

Source/substrate	Cofactor	Catalyst	Experimental procedure	Amount of product	Pros and cons	Reference
Olive mill wastewater (OMW)	–	*Olea europaea* L.	Using liquid–liquid solvent extraction in a laboratory-scale pilot unit with various organic solvents such as: ethyl acetate, diethyl ether and a mixture of chloroform/isopropyl alcohol.	Treatment of 1 m^3 OMW with ethylacetate could provide 0.247 kg hydroxytyrosol	Pros: by-product as substrate. Cons: mixture of phenols, use of organic solvents.	(Kalogerakis et al. 2013)
Olive mill wastewater (OMW)	–	*Olea europaea* L.	Using ultrasonication for improving conventional solvent extractions of HT and other phenols from OMW.	0.65 mM	Pros: by-product as substrate. Cons: low titers, mixture of phenols, use of organic solvents	(Jerman Klen and Mozetič Vodopivec 2011)
Olive mill wastewater (OMW)	–	*Olea europaea* L.	Counter current extraction of OMW stored for 5 months with 5 % or 10 % ethanol	20–22 mM	Pros: by-product as substrate and high concentrations. Cons: very long duration, use of organic solvents	(Feki et al. 2006)
Olive mill wastewater (OMW)		*Oleaeuropaea L*	Use of culture broth of *Aspergillus niger*, *Trichoderma atroviride* and *Trametes trogii* for enzymatic hydrolysis OMW to release HT	0.5–1.1 g/L	Pros: by-product as substrate and high concentration Cons: oxidation due to laccase activity.	(Hamza et al. 2012)
Olive leaves		*Olea europaea* L.	Use of strongly-acid aqueous steam, generated from 10 % HCl (*v*/*v*) at 100 °C, to directly hydrolyse the native complex phenols from integral olive leaves, and HT recovery by liquid–liquid extraction with ethyl acetate.	0.2/100 g of fresh olive leaves.	Pros: by-product as substrate Cons: high energy consuming method. Use of organic solvents.	(De Leonardis et al. 2008)
Oleuropein	–	Six different lactic acid bacteria strains	Hydrolysis reaction of oleuropein to HT is performed by lactic acid bacteria under aerobic and anaerobic conditions.	0.23 g/L from 0.8 g/L within 10 days by *L. plantarum 6907*	Pros: potential use of OMW, a by-product as substrate. Cons: long duration and unstable system with low concentration.	(Santos et al. 2012)
Tyrosol	–	*Pseudomonas putida* F6 cells	Cell extracts of *P. putida* F6 expressing tyrosinase activity and immobilized in calcium alginate beads for biotransformation in a buffer. Addition of ascorbic acid in 1:1 mol ratio with the substrate.	0.8 mM from 1 mM substrate in the presence of 1 mM ascorbic acid	Pros: easily handled and reusable system Cons: relative high cost substrate and low product titers. Need to remove oxidized ascorbic acid.	(Brooks et al. 2006)
Tyrosol	NADH and FAD	*E. coli* Rosetta cells with cloned phenol hydroxylase from *Geobacillus thermoglucosidasius*	Whole cells harbouring the cloned phenol hydroxylase in LB-kanamycin medium with ascorbic acid supplementation.	5 mM from 5 mM substrate coupled with 10 mM ascorbic acid	Pros: relative high product concentration. Cons: relative high cost substrate	(Orenes-Piñero et al. 2013)
Tyrosol glycosidic derivative	–	Marine α-glucosidase from *Aplysia fasciata*, and a commercial tyrosinase from mushroom	Enzymatic two-step procedure: (1) tyrosol α-glycosidic derivatives were produced by direct glucosylation; (2) these molecules were regioselectively oxidized by a commercial mushroom tyrosinase	9.35 and 10.8 g/L of HT mono- and disaccharide derivatives	Pros: considerably inexpensive biocatalyst Cons: relative high cost substrate. Only 20 % yield.	(Trincone et al. 2012)
Tyrosol	–	*Halomonas sp.* strain HTB24	Using *Halomonas sp.* strain HTB24 for bioconversion performed by 4-hydroxyphenylacetic acid 3-hydroxylase (HPAH)	3.5 mM	Pros: considerably fast and cheap assay. Cons: relative high cost substrate and low product titers. Further oxidation products by the cell.	(Liebgott et al. 2009)

Table 2 (continued)

Source/substrate	Cofactor	Catalyst	Experimental procedure	Amount of product	Pros and cons	Reference
Tyrosol	–	*Pseudomonas aeruginosa*	Bioconversion of tyrosol into hydroxytyrosol was achieved *via* the immobilization of *Pseudomonas aeruginosa* resting cells in calcium alginate beads.	8.5 mM	Pros: very simple reaction. Cons: relative high cost substrate, mixture of products.	(Bouallagui and Sayadi 2006)
Tyrosol	–	Mushroom tyrosinase and ascorbic acid	Oxidation of tyrosol into hydroquinone with concomitant reduction by ascorbic acid to hydroxytyrosol	16 mM	Pros: short reaction time, full conversion Cons: excess amount of ascorbic acid which must be removed in the downstream processing stage	(Espín et al. 2001)
2-Phenylethanol	NADH	*E. coli* TG1 cells with a cloned enzyme from *Pseudomonas*	*E. coli* harbouring an improved mutant of toluene-4-monooxygenase (T4MO) obtained by directed evolution and statistical models. A whole cell biotransformation system in a buffer with cell recycling and solid phase extraction by boric acid gel beads.	0.86 mM, from 2 mM substrate, 0.244 mmol/L/h	Pros: low-cost starting material, complete substrate consumption, improved by the solid phase extraction. Cons: low product titers.	(Brouk and Fishman 2009; Brouk and Fishman 2012; Brouk et al. 2010)
3-Nitrophenethyl alcohol (3NPA)	NADH	*E. coli* BL21 with cloned nitrobenzene dioxygenase (NBDO)	Using several protein engineering approaches (neutral drift libraries, random libraries, two types of focused libraries, and family shuffling) to engineer NBDO for the selective production of HT in a whole cell system	0.78 mM	Pros: a very quick and simple one-step reaction. Cons: relative high cost substrate.	(Bernath-Levin et al. 2014)
Tyrosine, dopamine, L-DOPA, glucose	Tetrahydromonapterin (MH4)	Engineered *E. coli* whole cell harbouring an artificial pathway	*E. coli* harbouring the following enzymes: tyrosine hydroxylase (TH), carbinolamine dehydratase (PCD), L-DOPA decarboxylase (DDC), tyramine oxidase (TYO), dihydropteridine reductase (DHPR). Was used in a whole cell biotransformation system in an M9 media.	0.19 mM from 1 mM tyrosine, 0.74 mM from L-DOPA	Pros: low cost substrates, endogenous cofactor, and reduction of unfavourable over-oxidation. Cons: low product titers, high dependency on the cell ability to express the different proteins	(Satoh et al. 2012)
3,4-Dihydroxyphenylacetic acid	ATP and NAD(P)H	*Escherichia coli* BL21 (DE3) cells overexpressing carboxylic acid reductase from *Nocardia*	*E. coli* harbouring the following enzymes: carboxylic acid reductase from *Nocardia* and phosphopantetheinyl transferase from *E. coli*. Bioconversions were performed as batch reactions in 100 mL volumes	1.2 mM from 30 mM substrat within 20 h. Overall productivity of 2 mg/L/h for purified HT.	Pros: endogenous cofactor, 99 % purity of the extracted product Cons: relative high cost substrate.	(Napora-Wijata et al. 2014)

transformed to the corresponding alcohol with sodium boron hydride, and HT was finally obtained by hydrogenolysis under Pd-C/H_2 conditions. The overall yield was 27 %. These reports illustrate the advantages of biocatalysts which carry out reactions selectively and efficiently under mild reaction conditions without the need for protection steps.

Biocatalyst selection and improvements

Catalyst selection does not necessarily come after selection of the substrate. On the contrary, it is often chosen beforehand or in parallel (Fig. 1). The biocatalyst may be used in its natural form, e.g. whole cells such as lactic acid bacteria (Santos et al. 2012) or *Pseudomonas putida* (Brooks et al. 2006), or it can be an engineered cell with an artificial pathway (Satoh et al. 2012) or a specific cloned enzyme (Brouk and Fishman 2009) (Table 2). Advanced molecular tools are now available for improving natural strains or obtaining better and more efficient enzymes. Furthermore, immobilization is a powerful tool for stabilizing the biocatalyst and offering its successive use and cost reduction (Bouallagui and Sayadi 2006; Brooks et al. 2006) (Fig. 1).

The enzymatic biotransformation for the production of HT is usually conducted in a whole cell system providing in situ cofactor regeneration (see examples in Table 2). Isolated enzymes were reported in few instances in which tyrosinase was used (Espín et al. 2001; Trincone et al. 2012). This enzyme not only hydroxylates the phenolic substrate to HT but also oxidizes the catechol to the respective quinone, and therefore a reducing agent such as ascorbic acid is required to maintain HT. β-Galactosidase from various sources has been shown to effectively hydrolyse oleuropein into oleuropein aglycon by removing the glucose moiety. Subsequent chemical rearrangement can transform the aglycon into hydroxytyrosol (Mazzei et al. 2012; Mazzei et al. 2006).

Processes based on natural bacterial strains frequently use natural and easily transformed substrates, oleuropein or tyrosol (Bouallagui and Sayadi 2006; Santos et al. 2012). Far more interesting are the studies that were done with heterologous genes. The advantage of using heterologous genes as mentioned above is the possibility to improve the biocatalyst. One of the common biocatalyst improvement techniques is the use of protein engineering tools (Adhami et al. 2015). For producing HT, oxidizing enzymes such as monooxygenases and dioxygenases are good candidates (Dror and Fishman 2012). Brouk et al. used several techniques to improve toluene 4-monooxygenase (T4MO) for HT production. A combination of directed evolution, rational design and statistical methods enabled a 190-fold improvement in the transformation of 2-phenylethanol to HT by two successive oxidation reactions (Brouk and Fishman 2009; Brouk et al. 2010). Bernath-Levin et al. used additional molecular techniques such as neutral drift libraries and family shuffling, as well as rational design techniques, to improve by 375-fold nitrobenzene dioxygenase (NBDO) for HT production from 3-NPA (Bernath-Levin et al. 2014). Other researchers used heterologous genes that are capable of producing HT from a defined substrate, but without applying protein engineering techniques (Liebgott et al. 2009; Orenes-Piñero et al. 2013; Satoh et al. 2012). In these works, genes from various organisms were cloned into *E. coli* cells. For instance, Satoh et al. cloned tyrosine hydroxylase (TH) from mouse, pterin-4-alpha-carbinolamine dehydratase (PCD) and dihydropteridine reductase (DHPR) from human, and L-DOPA decarboxylase (DDC) from pig and optimized them for codon usage in *E. coli*. Additionally, they cloned the tyramine oxidase (TYO) gene from the *Micrococcus luteus* genome for creating a new metabolic pathway for the production of HT from glucose based on endogenous tyrosine (Satoh et al. 2012). Orenes-Piñero et al. cloned phenol hydroxylase from the thermophilic bacterium *Geobacillus thermoglucosidasiu* for the conversion of tyrosol to HT (Orenes-Piñero et al. 2013). These efforts to create HT-producing *E. coli* can be facilitated in the future by combining novel bioinformatic techniques with metabolic engineering and protein engineering to improve the specificity and the kinetic parameters of known and unknown enzymes as reviewed elsewhere (Bornscheuer et al. 2012; Shin et al. 2013). As an example, the possibility to produce HT from L-phenylalanine can be materialized by using the Ehrlich pathway in yeast (Hazelwood et al. 2008) or a similar pathway in engineered *E. coli* (Achmon et al. 2014) to produce 2-phenylethanol that was already used as a substrate for HT production (Brouk and Fishman 2009; Brouk and Fishman 2012; Brouk et al. 2010). The use of metabolic engineering was proved to be very efficient for the production of phenolic compounds with a similarity to HT (Atsumi et al. 2008; Nozzi et al. 2014). The only research to date that used a metabolic engineering approach for producing HT was that of Satoh et al. that identified an endogenous aromatic aldehyde oxidase and knocked it down to prevent the formation of a side product (Satoh et al. 2012).

Still, the natural biocatalyst for producing HT is the olive tree (*O. europaea*) or other HT-producing plants. According to our knowledge, experiments to improve HT production in olives by advanced molecular engineering tools or breeding have not been done yet, allowing for considerable research and development in this direction.

Process optimization and product separation and purification

Process considerations for HT production are highly affected by the selection of substrate/source and catalyst (Fig. 1). The

process can be generally divided between utilization of natural sources (OMW streams) and utilization of nonnatural sources.

Processes based on natural resources

The main advantage of utilizing a natural resource comes from its relatively low environmental impact, since the raw material is a by-product of the olive oil industry (Roig et al. 2006). Yet, in any process designed to extract HT from OMW, a life cycle assessment should be done in order to evaluate the ecological impact especially with regard to the organic solvents used for extraction (Kalogerakis et al. 2013). The recovery of HT from OMW usually requires the following stages: (1) collection of the HT source itself (residual wastewaters out of the mill); (2) waste treatment (e.g. second oil extraction, combustion, gasification, anaerobic digestion, composting or solid fermentation (Roig et al. 2006); and (3) extraction and purification of phenols either by physical means, or using enzyme treatment (Feki et al. 2006; Hamza et al. 2012; Jerman Klen and Mozetič Vodopivec 2011; Kalogerakis et al. 2013). Any stage along the HT recovery process can be modified and optimized; however, it is very difficult to have control over the quality of the substrate (OMW) and on the waste treatment stages, due to many unknown variances. Thus, most of the reported work concentrates on the last stage, namely, the extraction and purification of HT (Jerman Klen and Mozetič Vodopivec 2011; Kalogerakis et al. 2013; Oral et al. 2014). Some researchers used olive leaves as the production source (which has high HT content (De Leonardis et al. 2008; Quirantes-Piné et al. 2013)), but leaves collection is much more difficult than OMW collection. As indicated in Table 2, the concentrations of HT extracted from OMW can be rather high (De Leonardis et al. 2008; Feki et al. 2006; Kalogerakis et al. 2013). Nonetheless, it is a seasonal process that depends on the olive agricultural industry and needs the goodwill of the waste treatment facilities to invest in advanced separation and purification abilities such as column separation and ultrasonic extraction (Adhami et al. 2015; Jerman Klen and Mozetič Vodopivec 2011; Oral et al. 2014).

Processes based on nonnatural resources

The production of HT from of a nonnatural resource usually involves biotransformation by whole cells (mostly *E. coli*). Such processes do not require co-factor regeneration, and the cells can be easily separated from the medium (Brouk and Fishman 2012). Unlike extraction from OMW in which an array of phenols and other chemicals accompany HT, when producing from a defined substrate, the separation and purification steps are easier and a purer product may be obtained, depending on the selectivity of the biocatalyst. To date, the main problem with HT production by biocatalysis is the low titers or the cost of the substrate (Table 2). Brouk et al. had succeeded in using a relatively low-cost substrate (2-phenylethanol), but the titers were still rather low (0.8 mM) (Brouk and Fishman 2012). Satoh et al. have created an *E. coli* strain that is capable of producing HT out of sugars. This is an extremely cost-effective catalyst when considering the substrate, but the titers are very low (0.08 mM) (Satoh et al. 2012). The widely used substrate tyrosol has given good conversion yields, but the drawback is the substrate price (Bouallagui and Sayadi 2006; Brooks et al. 2006; Orenes-Piñero et al. 2013).

Another important aspect of the biotechnological process is product auto-oxidation and potential toxicity to whole cells. Several techniques were applied in order to protect HT from further oxidation into the quinone. In situ product removal (ISPR) techniques are one of the practical solutions to protect and separate HT from the biotransformation process (Woodley et al. 2008). Brouk and Fishman used beads conjugated with phenylboronic acid residues for adsorbing HT from the bioreactor during the biotransformation process (Brouk and Fishman 2012). That process gave a 2-fold increase in recovery yield and purity, resulting in 84 % purity with 70 % recovery yield. Orenes-Piñero et al. (2013) used hydrophobic polystyrene resin (Amberlite XAD-4) for the purpose of collecting the HT from the media after the transformation. Ascorbic acid was used to reduce any oxidized HT by researchers using tyrosinase (Espín et al. 2001; Trincone et al. 2012). There is need for further development of effective measures to conduct ISPR of HT.

Conclusions and future perspectives

A holistic view of the three major contributors to a biotechnological process was used to assess the challenges and opportunities for biocatalysed synthesis of HT. From the source perspective, inexpensive and catalyst-specific compounds are required. Substrates that are common in nature such as amino acids and simple sugars are most likely the preferable direction. As for the biocatalyst, the construction of new metabolic pathways, based on improved and optimized enzymes, is of great potential. Employing novel molecular tools such as Gibson assembly (Gibson et al. 2009) or Golden Gate DNA Assembly (Engler et al. 2009) can be used to develop artificial and optimized pathways for HT production. Another unexplored direction is the design of cell-free synthetic enzymatic pathways by employing synthetic biology tools (Hodgman and Jewett 2012). Finally, advancement is needed in improving the direct purification and stabilization of HT from the process itself. For example, screening of additional potential resins or specifically tailored materials can be applied for recovering highly pure products (Achmon et al. 2011; Gao and Daugulis 2009).

In conclusion, HT production by means of biocatalysts appears as the optimal direction for a cost-effective production process that will enable reasonably priced HT to be used as a dietary supplement and as a common food additive. From our point of view, the production of HT from OMW or olive leaves by extraction will remain a good option with added value for waste treatment (Kalogerakis et al. 2013; Yangui et al. 2009). Yet, this process will not fulfil the market requirement for a lower cost pure HT, and henceforth other solutions are needed.

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