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Vermicomposting of Winemaking By-Products

Jorge Domínguez*, Juan C. Sanchez-Hernandez**, Marta Lores†

*UNIVERSITY OF VIGO, VIGO, SPAIN ; **UNIVERSITY OF CASTILLA-LA MANCHA, TOLEDO, SPAIN; †UNIVERSITY OF SANTIAGO DE COMPOSTELA, SANTIAGO DE COMPOSTELA, SPAIN

3.1 Introduction

Grape is the largest fruit crop in the world and its production is increasing. The respective annual worldwide production accounts for about 78 million tonnes (FAO, 2015), ~80% of which is used to make wine. The main by-product of wine-making is grape marc, also known as grape bagasse or grape pomace, which consists of a mixture of the stalks, pressed skins, disrupted cells from grape pulp, and the seeds that remain after pressing the grapes to obtain the stum or grape juice. The grape marc represents almost 20% by weight of the total grape input. If the stalks are removed from the grapes before processing, the residue consists of 40% seeds and 60% skin and pulp. Winery waste pressed with the stalks comprises of 30% stalks, 30% seeds and 40% skin and pulp, with slight differences depending on the grape variety.

Traditionally, grape marc has been used to produce pomace brandy spirits (orujo, grappa, zivania, törkölypálinka, etc.). Nowadays a relative small fraction of the grape marc produced during the winemaking process in the wine industry is used to produce ethanol, extract organic acids and produce grape seed oil and other food ingredients (Alvarez-Casas et al., 2014; Fontana et al., 2013; Negro et al., 2003). Grape marc is easily silaged due to its high acidity, and therefore it has been used as fodder to feed livestock, although the high lignin content makes it rather indigestible (Kammerer et al., 2005).

This by-product is potentially a very valuable resource that could be used as a nutrient-rich organic soil amendment. However, overproduction at local scale in small geographic areas has led to inappropriate disposal of the material on agricultural land. Moreover, application of the untreated raw material can damage crops owing to the release of excessive amounts of phytotoxic polyphenols to soils (Inderjit, 1996). Phenolic compounds are responsible for the phytotoxic and antimicrobial activity of the grape marc, including negative effects on the physical, chemical and biological properties of the soil, phytotoxic effects on crops and potential groundwater pollution (Barbera et al., 2013).

Earthworms can partially digest polyphenols (Hättenschwiler and Vitousek, 2000); thus the agronomic problems associated with the application of the grape marc to soil can be minimized or eliminated by vermicomposting (Domínguez et al., 2010).

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On the other hand, polyphenols (secondary plant metabolites that are one of the most widely occurring groups of phytochemicals) have well known human health-promoting effects and other properties in different biological and food systems (Fontana et al., 2013; Aizpurua-Olaizola et al., 2015). The average distribution of polyphenolic compounds in grape marc is about 1% in the pulp, 5% in the skin, and a large proportion, ca. 60%, in the seeds (Yilmaz and Toledo, 2004). Indeed, an interest has been expressed to recover these polyphenols and use them as functional compounds in the pharmaceutical, cosmetics and food industries (Alvarez-Casas et al., 2014; Fontana et al., 2013; Makris et al., 2007; El Gharras, 2009; Quideau et al., 2011).

One option could be to use vermicomposting process as a pretreatment for grape marc in order to extract polyphenols and minimize agronomic problems associated with the application of grape marc to soil (Domínguez et al., 2010, 2014). The grape seeds can also be easily obtained and processed to recover polyphenols, fatty acids, seed oil, and other bioactive compounds (Patent no. ES2533501, Domínguez et al., 2015). Other final products derived from the vermicomposting process include large numbers of earthworms that can be processed as fish bait and as a source of animal feed protein. The grape marc-derived vermicompost can be used as a rich-source of enzymes to improve soil biochemical performance and to detoxify pesticide-contaminated soils (Fig. 3.1).

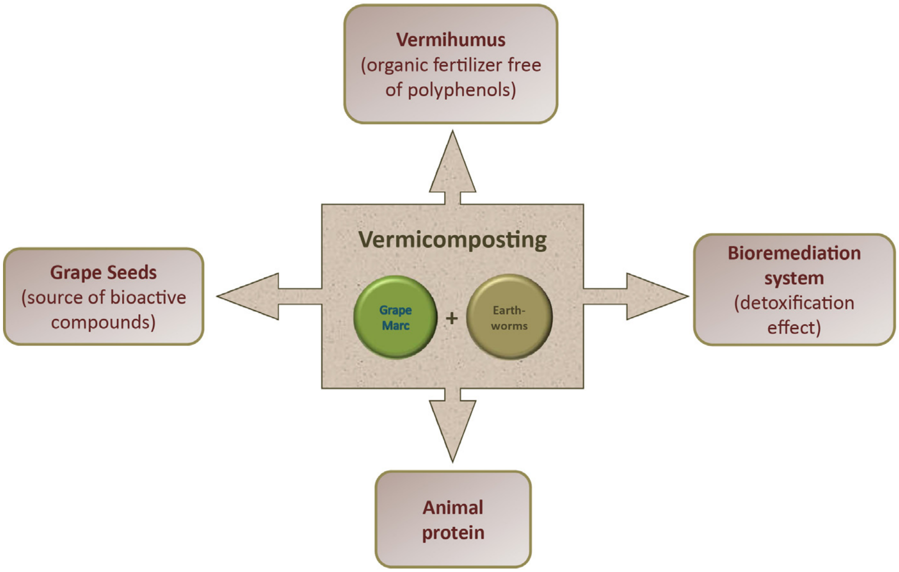

FIGURE 3.1 Vermicomposting of grape marc yields an organic fertiliser and grape seeds as a source of bioactive compounds. The process reduces by more than a half the amount of waste, and it transforms grape marc into a high quality, nutrient and microbial rich, polyphenol-free organic fertilizer. Sieving the material separates the vermicompost from grape seeds, eliminates the polyphenol-associated phytotoxicity from the vermicompost, and enables them to be easily processed to obtain different bioactive compounds, such as polyphenol-rich extracts and fatty acid-rich seed oil. These coproducts can be directly extracted for use in the pharmaceutical, cosmetics, and food industries. During the process, very large numbers of earthworms are obtained and represent a source of animal protein. The grape marc-derived vermicompost is also as a rich-source of enzymes that could be used to improve soil biochemical performance and to detoxify pesticide-contaminated soils.

3.2 Basis and Principles of Vermicomposting

The processing of organic waste by earthworms, known as vermicomposting, is a rapid technique for transforming dead organic matter into vermicompost. Vermicomposting is a biooxidative process in which detritivorous earthworms interact intensively with microorganisms, and this way affecting strongly decomposition processes, accelerating the stabilization of organic matter and greatly modifying its physical, chemical and biological properties (Domínguez, 2004; Domínguez, 2011; Domínguez and Gómez-Brandón, 2012, 2013). Although microorganisms produce the enzymes that cause the biochemical decomposition of organic matter, earthworms are the key drivers of the process. Earthworms participate in the indirect stimulation of microbial populations through fragmentation and ingestion of fresh organic matter, which yields a larger surface area available for microbial colonization and alters drastically biological activity. Earthworms modify microbial biomass and activity through stimulation, digestion and dispersion in casts, while they interact closely with other biological components of the vermicomposting system. As a result, they affect the structure of microbiome and microfaunal communities (Domínguez et al., 2003; Lores et al., 2006).

The vermicomposting process includes two different phases in relation to earthworm activity (Fig. 3.2):

1. An active phase during which earthworms ingest and process the organic waste, thereby modifying its physical state and microbial composition (Lores et al., 2006; Gómez-Brandón et al., 2011a; Aira et al., 2015, 2016); and

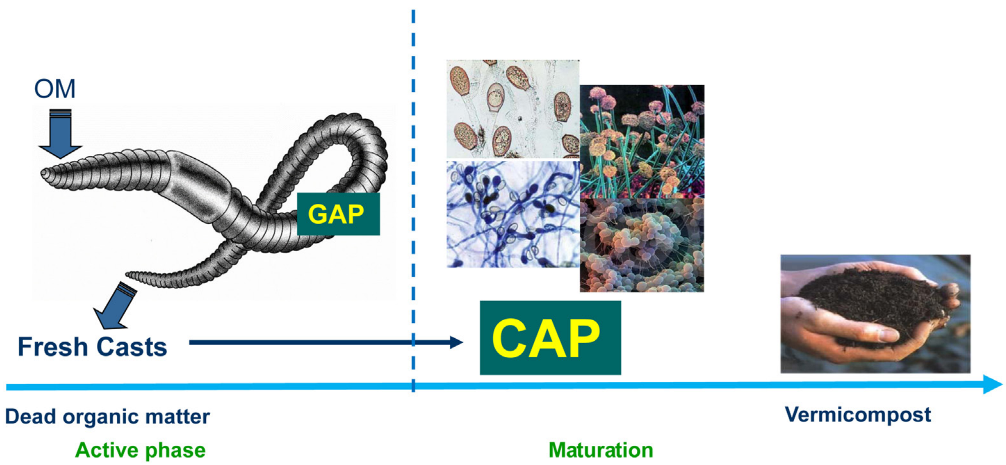

FIGURE 3.2 The vermicomposting process includes two different phases in relation to earthworm activity: (1) an active phase during which earthworms ingest and process the organic waste, through gut-associated processes *(GAPs)*, thereby modifying its physical state and microbial composition; and (2) a maturation phase, during which the microorganisms take over the decomposition of the earthworm-processed waste through cast associated processes *(CAPs)*.

2. A maturation-like phase marked by the displacement of the earthworms toward fresher layers of undigested organic waste, during which the microorganisms take over the decomposition of the earthworm-processed waste (Domínguez et al., 2010; Domínguez and Gómez-Brandón, 2012).

The length of the active phase is variable and depends on the species and density of earthworms (the main drivers of the process), as well as the rates at which they ingest and process the organic waste (Aira and Domínguez, 2008).

The effect of earthworms on the decomposition of organic waste during the vermicomposting process is, in the first instance, due to gut-associated processes (GAPs). These processes include all the modifications that the decaying organic matter and the microorganisms undergo during intestinal transit. They also include the addition of sugars, enzymes and other substances, modification of the structural and functional diversity of the microbial communities and their activity, modification of the microfaunal populations and homogenization. Finally, they include the intrinsic processes of digestion, assimilation and production of mucus and excretory substances, such as urea and ammonia, which constitute a readily-assimilable pool of nutrients for microorganisms (Fig. 3.2). Decomposition of the dead organic matter is enhanced by the action of endosymbiotic microbes that reside in the earthworms' guts and produce extracellular enzymes. The latter degrade cellulose and phenolic compounds (Aira et al., 2006; Domínguez et al., 2010) and thus enhance further the degradation of ingested material. Many of the microorganisms that pass through the earthworm gut appear to be transient. However, others react well to the anoxic, moist environment inside the earthworm gut and become active. This results in complex mutualistic relationships, in which the functional diversity of the microbial communities is modified as a result of selection for microorganisms capable of anaerobic metabolism (Drake and Horn, 2007). Members of the genus *Acidovorax* are thought to degrade proteins, allowing the host earthworm to reabsorb nitrogenous compounds that would otherwise be excreted (Schramm et al., 2003). Some earthworm gut-associated microbes, such as *Streptomyces*, are known to produce cellulases, which would help the earthworm host to degrade plant residues. Other physical modifications of the substrate caused by the digging activities of earthworms, such as aeration and homogenization of the substrate, also favour microbial activity and further enhance decomposition (Domínguez, 2004).

On completion of GAPs, the earthworm casts produced undergo cast associated processes (CAPs), which are more closely associated with aging processes (days to weeks), the action of the microbiota and microfauna present in the substrate and with the physical modification of the egested materials (Aira et al., 2008). During these processes, the earthworms exert mainly indirect effects derived from the GAPs (Fig. 3.2). In vermicomposting systems, earthworm casts are mixed with other material not ingested by the earthworms, which is largely dependent on the heterogeneity of the organic waste, and the final vermicompost consists of a mixture of the two different fractions. During this aging process, the vermicompost will reach an optimum stage in terms of biological properties promoting plant growth and suppressing plant diseases (Domínguez and Edwards, 2011a).

Vermicompost, the end product of vermicomposting, is a finely divided and porous peat-like material with a high water-holding capacity. It also contains many nutrients in forms that are readily taken up by plants (Domínguez and Edwards, 2011a). At the end of the vermicomposting process, the vermicompost can be easily separated from the remaining and more recalcitrant fractions of the material.

From the point of view of its commercial development and application, vermicomposting (like any other biological treatment of organic wastes) can be considered as a two-step processes. In the first step, the organic waste is converted into nontoxic products, thus eliminating or reducing human pathogen content and the concentrations of heavy metals and organic pollutants. In a later step, the new stable product can be converted into a valuable organic soil amendment with greatly increased microbial activities and humification of the organic material, which enhances the presence of plant growth promoters. The main criterion used in deciding on the treatment or processing strategy should be the "quality" of the organic waste. "Nonproper" residues may only need to be stabilized, to eliminate their contaminating potential, whereas "proper" residues can be transformed into a valuable organic soil amendment. Examples of nonproper waste include the organic fraction of municipal solid waste and sewage sludge, due to its heterogeneous and highly variable composition, as well as their high concentration of human pathogens and inorganic and organic contaminants. In this case, the aim of the treatment is the rapid stabilization of the material and regular composting is probably a better solution than vermicomposting. In this respect, grape marc can be considered as a "proper" waste, because it does not contain pollutants, it has a homogeneous composition and contains a good balance of nutrients. Thus, the aim of processing is to obtain a good quality organic soil amendment with biofertilizing and bioplaguicide properties, for which vermicomposting is probably the best choice.

3.3 Vermicomposting of Grape Bagasse: a Case Study

Different types of grape bagasse derived from white and red winemaking were processed in pilot-scale vermireactors (6 m^2) housed in a greenhouse, using the earthworm species *Eisenia andrei*, commonly known as redworm. *E. andrei* is an epigeic earthworm (Oligochaeta, Lumbricidae) with a worldwide distribution and is tolerant to a wide range of temperature and moisture conditions (Domínguez et al., 2005; Domínguez and Edwards, 2011b). It is the most common earthworm in vermicomposting facilities worldwide, and is often confused or wrongly cited as its close relative, *Eisenia fetida*. At the start of the trials, the initial earthworm population density was 214 ± 26 individuals/m^2. The grape bagasse was placed in the vermireactor in successive layers through time, for processing by the earthworms. Population density and biomass of earthworms were determined periodically. Samples of the processed grape bagasse and the vermicompost were periodically collected and analyzed to determine their chemical and biological properties (Domínguez et al., 2014).

3.3.1 Production of Earthworm Biomass

Vermicomposting typically refers to the use of epigeic earthworms to process and convert organic wastes and by-products into valuable biofertilizers, while vermiculture refers to the activity of breeding earthworms. In either case, earthworms are both the foundation and a final product of the operation. Earthworms can be commercialized as fishing bait, for vermicomposting purposes and as a source of animal protein. The market for earthworms as fishing bait is quite large, well established and is mainly supplied by traditional, relatively small earthworm farms. It mainly involves the earthworm species *E. fetida* (tiger worm) and *E. andrei* (redworm). Production of vermicomposts and earthworm protein for animal feed or other purposes may be complementary and produced in the same vermicomposting systems.

In a case study (Domínguez et al., 2014), before adding the grape bagasse, the initial earthworm biomass in the vermireactor was 58 ± 15 g fresh weight (fw)/m^2, corresponding to an earthworm population density of 214 ± 26 individuals m^{-2}, including 19 ± 3 mature earthworms/m^2, 215 ± 37 juveniles m^{-2} and 63 ± 18 cocoons m^{-2}, (Fig. 3.3). After the addition of 158 kg fw of grape marc, the density and biomass of earthworms increased continuously and significantly during 70 days, reaching a maximum earthworm density of 556 ± 47 individuals/m^2 with an earthworm biomass of 213 ± 17 g fw/m^2. After the addition of a new layer of grape marc (150 kg fw), the population density and the earthworm biomass increased further and more rapidly, reaching values of 2655 ± 80 individuals/m^2, representing an earthworm biomass of 958 ± 23 g fw/m^2, after 28 days. After one year of

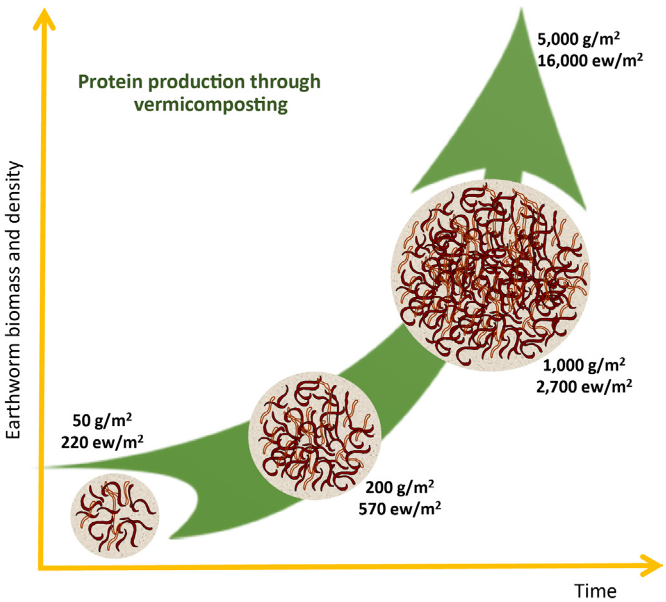

FIGURE 3.3 Production of earthworm biomass and evolution of earthworm density during vermicomposting of grape marc.

continuous functioning of the vermireactor fed with grape marc, the earthworm density reached maximum values up to almost 16000 earthworms/m^2, and earthworm biomass of almost 5 kg/m^2 (Fig. 3.3).

At this case study, the initial population of earthworms in the vermireactor was quite low, and although it increased considerably as a consequence of the input of organic matter from the grape marc, it was far from its maximum capacity. As *E. andrei* lives in environments where the same material acts as the substrate and the food, the availability of this material improves the conditions for earthworm growth and reproduction, and leads to the presence of high numbers of earthworms. When large quantities of organic matter are available, the density of epigeic earthworms can be very high, for example, up to 8,000 individuals/m^2 in cow manure and 14,600 individuals/m^2 in pig manure (Monroy et al., 2006). Therefore, grape marc appears to be an excellent substrate for feeding earthworms and provides sufficient energy to sustain very large populations (Fig. 3.3).

Sources of animal protein are becoming more and more scarce, thus new sources of protein are needed to replace the fishmeal used in poultry, fish, swine, cattle and other animal feed. The average dry weight of earthworms is about 15%–20% of the fresh weight (representing the entire carcass weight after complete dehydration). Studies describing the nutritional components of the tissues of different species of earthworms are scarce (see review in Edwards and Niederer, 2011). Zhenjun et al. (1997) studied the chemical composition of the earthworm species *E. foetida* in comparison with other common animal feeds. The closely related species *E. fetida* (Savigny, 1826) and *E. andrei* Bouché, 1972 are those most commonly used for the management of organic wastes. This is happening because they are ubiquitous and distributed worldwide, they have short life cycles, a wide temperature and moisture tolerance range and are resilient to handling (Domínguez, 2004). They were first described as different morphotypes of *E. fetida* according to differences in body pigmentation (André, 1963), and Bouché (1972) gave them sub specific status, naming them *E. foetida foetida* and *E. foetida unicolour*. Although many authors now accept *E. foetida* and *E. andrei* as different species, older literature and even abundant current literature refer to these species collectively as *E. fetida* or *E. foetida* (an erroneous emendation of the original *E. fetida*). In short, *E. foetida* is a misnomer that may refer to either *E. andrei* or *E. fetida*. However, as the tissue composition of other earthworm species is quite similar and both species are morphologically similar, the data from Zhenjun et al. (1997) can be considered a good reference for the composition of the vermicomposting earthworms. These authors reported that the protein content of the earthworm meal was 54.6%, that is, higher than Chinese fishmeal, hen eggs, and soybean meal. They also reported that the essential amino acid contents of earthworm meal compares well with those of fishmeal, hen egg, and raw cow milk, with the lysine content of earthworm meal being higher than all of the aforementioned ones. In addition, they denoted that the crude fat content of earthworm meal is lower than that of fishmeal, but higher than that of soybean meal and corn meal, and that, except for corn meal, earthworm meal has the highest metabolic energy content (2.99 kcal/g) of the foods and feeds tested.

The composition of earthworm tissues does not differ greatly from that of many invertebrate tissues. Protein accounts for the largest fraction of the dry weight (60%–70% dw) depending on the earthworm species, types, feeding, and experimental treatments. The essential amino acid profile of earthworm tissues compares well with that from other tissues currently used to produce animal feed. The mean amounts of essential amino acids were also satisfactory, particularly in terms of lysine and the combinations of methionine and cysteine, phenylalanine and tyrosine, all of which are important components of animal feeds (Sabine, 1983).

Earthworm tissues contain fats (6%–11% dw) with a preponderance of long-chain fatty acids, many of which nonruminant animals cannot synthesize. They also have an adequate mineral content, whereas they contain an excellent range of vitamins and are rich in niacin, which is a valuable component of good animal feeds. The body tissues contain carbohydrates (5%–21% dw) and minerals (2%–3% dw), too (Edwards and Niederer, 2011).

A few available studies of the economics of earthworm protein production have indicated that this process is more profitable when carried out in large vermicomposting facilities. The most important criterion is that production of earthworm meal for feed protein must be economically viable, although the value of the vermicompost produced in the vermicomposting system can also be taken into account. Currently, the main limitations of earthworm-protein production are the labour-intensive harvesting process, and the clean-up process involving emptying the gut content of the earthworms. The first remains the main economic barrier for the successful commercial earthworm-protein production destined to animal feed, although this could be resolved by developing adequate technology for separating earthworms from vermicompost. The second is not a problem when earthworms are cultured in safe plant material, such as grape marc and earthworms can be processed with their gut contents.

3.3.2 Vermihumus of Grape Marc: Biofertilizer and Bioplaguicide

Vermicomposting has been proven to be useful for the treatment of grape marc derived from red winemaking (Nogales et al., 2005; Romero et al., 2007) and white winemaking (Gómez-Brandón et al., 2010, 2011b). In the referred case study (Domínguez et al., 2014), the changes in the earthworm population and the chemical and biological parameters indicated that the vermicomposting process was optimal and rendered good quality vermicompost. The chemical and biochemical properties and the microbial activity of the final vermicompost obtained after vermicomposting of different types of grape marc are summarized in Table 3.1.

During vermicomposting of grape marc, earthworm activity reduce the abundance of bacteria and fungi and the microbial activity, and also some enzymatic activities such protease and cellulase. The changes in microbial communities are accompanied by a reduction in the labile C pool and the cellulose content. These results indicate that earthworms played a key role in the stabilization of the grape marc, via its effects on organic matter decomposition and microbial biomass and activity (Gómez-Brandón et al., 2011b).

Table 3.1 Range of Chemical Properties and Microbial Activity of Vermicompost Derived From Grape Marc.

	Grape Marc Vermicompost
pH	7.1–7.8
Electrical conductivity (mS/cm^2)	0.1–0.2
Organic matter (%)	72–90
Total carbon (g/kg dw)	350–500
Total nitrogen (g/kg dw)	12–42
C/N ratio	10–40
Total phosphorus (g/kg dw)	1–10
Total potassium (g/kg dw)	5–18
Total calcium (g/kg dw)	5–25
Total magnesium (g/kg dw)	1–5
Total sulphur (g/kg dw)	1–8
Total iron (g/kg dw)	1–10
Total manganese (g/kg dw)	0.05–2.5
Total boron (mg/kg dw)	4–40
Total molybdenum (mg/kg dw)	1–30
Basal respiration (mgO_2 kg/OMh)	30–200
Lignin (g/kg dw)	300–800
Cellulose (g/kg^1 dw)	35–200
Hemicellulose (g/kg dw)	10–150
Total polyphenols (mg/g dw)	5–25

Data correspond to mean values of vermicompost derived from different types of grape marc.

Earthworms exert beneficial physical, chemical and biological effects on soils, which can increase plant growth and crop yields in both natural and agroecosystems. These beneficial effects have been attributed to improved soil structure and soil physical properties, greater availability of mineral nutrients to plants, enhancement of mycorrhizal infection, control of plant parasitic nematode populations, increased microbial populations, and biologically active metabolites, such as plant growth regulators and humates.

Vermicompost is a nutrient-rich, microbiologically active organic amendment that results from the interactions between earthworms and microorganisms during the breakdown of organic matter. It is a stabilized, finely divided peat-like material with a low C:N ratio, high porosity and high water-holding capacity, in which most nutrients are present in forms that are readily taken up by plants (Domínguez, 2004). When added to soil or plant growth media, this complex mixture of earthworm faeces, humified organic matter, and microorganisms increases germination, growth, flowering, fruit production, and accelerates the development of a wide range of plant species. The enhanced plant growth may be attributed to various direct and indirect mechanisms, including biologically mediated mechanisms, such as the supply of plant-growth regulating substances and improvements in soil biological functions (Lazcano and Domínguez, 2011; Gómez-Brandón and Domínguez, 2014).

Vermicompost has been found to provide manifold benefits when used as a total or partial substitute for mineral fertilizer in peat-based potting media and as a soil amendment in field studies (Lazcano and Domínguez, 2011). Vermicompost significantly stimulates the growth of a wide range of plant species, including several horticultural crops, such as tomato, garlic, aubergine, strawberry, sweet corn, green gram, aromatic, and medicinal plants, cereals, such as sorghum and rice, fruit crops, such as banana and papaya, ornamentals, such as geranium, marigolds, petunia, chrysanthemum, and poinsettia, and finally trees, such as acacia, eucalyptus, and pine trees (Lazcano and Domínguez, 2011; Arancon and Edwards, 2011; Arancon et al., 2011; Jack, 2011).

Likewise, vermicomposting leachates or vermicompost water-extracts, used as substrate amendments or foliar sprays, have also been shown to promote growth of different plants. Positive effects of vermicompost include stimulation of seed germination, stimulation of vegetative growth, thus increasing shoot, leaf area and root development, and stimulation of plant flowering, thus increasing the number and biomass of the flowers and fruit produced. In addition to increasing plant growth and productivity, vermicompost may also increase the nutritional quality of vegetable crops.

The advantages of using vermicompost as a soil amendment include its potential to maintain soil organic matter, foster nutrient availability, suppress plant diseases and increase soil microbial abundance and activity. In addition to increasing microbial biomass, vermicompost amendments enhance microbial activity and promote the establishment of a specific microbial community in the rhizosphere of different plants supplemented with mineral fertilizers or other types of organic fertilizers, such as manure (Aira et al., 2010). Moreover, microbial communities in vermicompost are metabolically more diverse than those in animal manures (Aira et al., 2007b) and can be incorporated, at least in the short-term, to soils (Lores et al., 2006; Aira et al., 2010; Lazcano et al., 2012).

The activities of several soil enzymes have been shown to increase after the addition to soils of vermicompost at rates equivalent to mineral fertilizers. Soil microorganisms degrade organic matter via production of a variety of extracellular enzymes, and input of organic matter will be accompanied by higher enzymatic activity. There is also a quite large body of scientific evidence regarding the positive effects of vermicomposts on the suppression of plant diseases. Nevertheless, a more detailed understanding of the mechanisms involved and the main factors influencing the suppressive effects is required. Vermicomposts can reduce the incidence and abundance of plant–parasitic nematodes in soil. They can also decrease the incidence of arthropod pests, lower populations of these pests, and lessen damage to plants growing in the amended soils (Edwards et al., 2011).

3.3.3 Polyphenols and Fatty Acids From Grape Seeds

Splitting the grape seeds at some time during the vermicomposting process eliminates the polyphenol-associated phytotoxicity from the vermicompost and also leaves the seeds ready to be processed in order to extract the polyphenols along with other bioactive compounds of interest.

Grape seeds account for on average of 17% fresh weight and 45% dry weight of the grape marc (Maier et al., 2009; Fernandes et al., 2013). Considering an estimated reduction close to 60% of the initial biomass associated with the raw material (whole grape marc) due to the vermicomposting process, the initial seed density increases by a factor of 2.5, from about 170 g of seeds to 450 g of seeds per kilogram of fresh material. The remaining biomass is the excellent vermihumus produced in the bioassisted process. The earthworms continue the biotransformation process and represent a useful source of animal protein, as mentioned earlier.

The composition of grape seeds (w/w) is 40% fibre, 16% oil, 11% protein, 7% polyphenolic compounds, and other substances like sugars and minerals (Garcia-Jares et al., 2015). Polyphenols and fatty acids are the bioactive compounds of greatest interest due to their well-known associated health effects.

The polyphenolic profile of a hydromethanolic extract of white grape seeds, obtained with Pressurized Liquid Extraction (PLE) and analyzed by Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS) is shown in Fig. 3.4. Flavanol monomers (catechin, epicatechin, galocatechin, and epigallocatechin) are usually the most abundant compounds, followed by procyanidins (dimers, trimers, and more highly polimerized

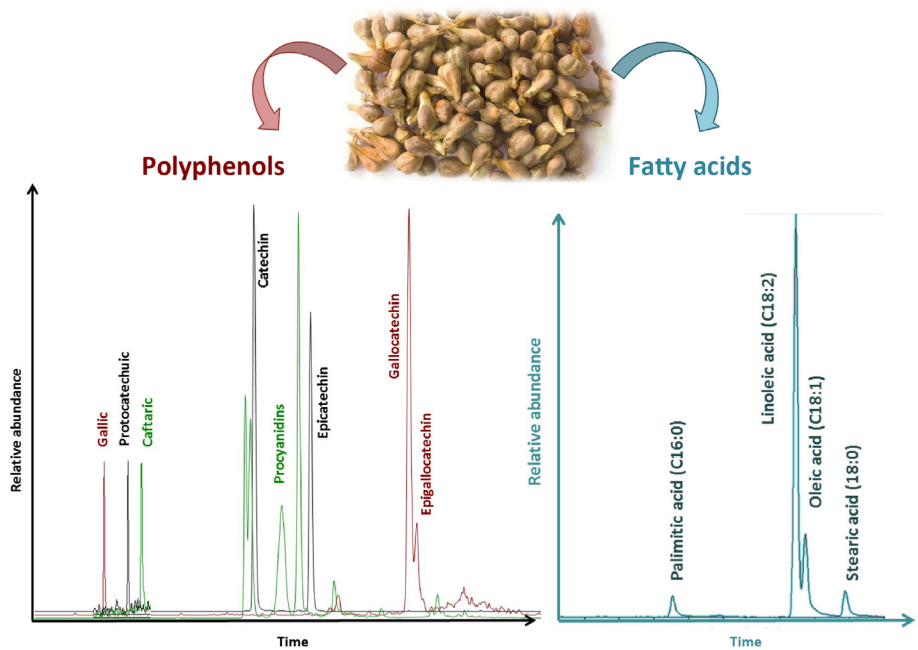

FIGURE 3.4 Bioactive compounds extracted from grape seeds. Left: Polyphenolic profile by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) of a hydromethanolic extract obtained by Pressurized Liquid Extraction (PLE) of grape seeds. Right: Fatty acids profile by gas chromatography-mass spectrometry (GC–MS) of an ethyl acetate extract obtained by Matrix Solid Phase Dispersion (MSPD) of grape seeds.

procyanidins). Simple phenolic acids (such as gallic, protocatechuic, and caftaric) are present to a much lower extent (Fig. 3.4), and esters of gallic acid and the flavan-3-ol residues are sometimes found, too. Lack of stilbenes and minimum concentrations of flavonols (quercetin and its derivatives) are other typical compositional features of grape seeds extracts (Di Lecce et al., 2014; Garcia-Jares et al., 2015). Apart from the obvious difference between seeds from white and red grapes (the absence of anthocyanins in the former); systematic approaches to the characterization of polyphenols from seeds according to the grape variety are scarce (Rockenbach et al., 2012; Cheng, 2011), particularly regarding seeds from white grapes (Di Lecce et al., 2014; Garcia-Jares et al., 2015; Lachman et al., 2013).

Some studies have shown varietal differences in the phenolic composition of grape seed extracts, which would constitute a starting point for a selective exploitation of the seeds obtained from monovarietal winemaking wastes. However, the varietal differences are slight enough to allow, when desired, extraction of polyphenols from this winemaking coproduct, independently of the grape variety. This is an interesting option from an industrial point of view. The polyphenolic content of grape marc and seeds from the same varieties of white grapes have also been compared (Garcia-Jares et al., 2015; Alvarez-Casas et al., 2016), showing that the concentration of polyphenols in seeds is about 5-9 higher than in grape marc.

The oil content of grape seeds varies from 8% to 20% on a dry matter basis. In grapeseed oil, linoleic acid (18:2) is the most abundant fatty acid, followed by oleic acid (18:1), stearic acid (18:0), and palmitic acid (C16:0) (Fig. 3.4). Fig. 3.4 shows a grape seeds extract obtained by Matrix Solid Phase Dispersion (MSPD), eluted with ethyl acetate, and analyzed by Gas Chromatography–Mass Spectrometry (GC–MS). The fatty acid profile is quite constant regardless of the grape variety (Lutterodt et al., 2011; Prado et al., 2012; Sabir et al., 2012; Fernandes et al., 2013), although it can be affected by the ripeness of the grape and peaks prior to veraison (the onset of ripening) and remains constant until harvest (Rubio et al., 2009). Linoleic acid, a polyunsaturated fatty acid (PUFA) and an essential fatty acid belonging to the omega-6 group, is important in the development and maintenance of the nervous system and physiological functions in humans. Oleic acid is a monounsaturated omega-9 fatty acid (MUFA) that is essential in human nutrition and helps to reduce levels of LDL-cholesterol, total cholesterol and the glycemic index (Rubio et al., 2009). Stearic and palmitic are saturated fatty acids that can be used to produce detergents, soaps and other cosmetic products.

The grape seeds obtained as a coproduct of the vermicomposting of winemaking by-products can therefore be processed to produce extracts containing large amounts of unsaturated fatty acids and polyphenols, along with tocopherols (Göktürk Baydar et al., 2007). Such extracts can be used to obtain natural antioxidants and edible vegetable oil. The beneficial effects of these products are due to the large amounts of unsaturated fatty acids, as well as to the antioxidants that they contain (tocopherols and polyphenolic compounds), which may serve as dietary sources of natural antioxidants to prevent diseases and promote human health.

3.3.4 Grape Marc-Derived Vermicompost: a Rich-Source of Enzymes to Improve Soil Biochemical Performance

The European Union has identified biodiversity decline, compaction, contamination, erosion, landslides, organic matter decline, salinization, and sealing as key environmental threats leading to soil deterioration (Jones et al., 2012). Among these, contamination is probably of most concern to human health, as humans are important vectors of soil contamination and, in turn, biological receptors of soil toxicants. In agroecosystems, inputs of pesticides and fertilizers represent the main hazard to soil quality, jeopardizing its capacity for crop production and maintenance of healthy ecosystem. Soil is an important sink for agricultural pesticides. The environmental fate and toxicity of the pesticides are determined by a wide variety of soil physicochemical and biological processes. The scientific literature provides many examples demonstrating the negative impact of agrochemicals on nontarget organisms inhabiting both belowground and aboveground ecosystems (Newman et al., 2006; Desneux et al., 2007). Furthermore, diffuse contamination represents a serious hazard to natural resources of critical concern to public health, such as groundwater and surface waters (Malaj et al., 2014). Despite the environmental risks associated with pesticides, their use is required and generalized to reduce crop damage by pests. Indeed, global pesticide consumption has increased gradually since 2007, despite the introduction of integrated pest management strategies and genetically modified crops (Peshin and Zhang, 2014). On the other hand, expansion of biofuel crops in tropical areas threatens not only the ecosystem structure, but also soil biodiversity due to excessive use of agrochemicals (Schiesari and Grillitsch, 2011).

Many biotechnological approaches have been developed to reduce soil degradation by agrochemicals (Megharaj et al., 2011). However, attention is now turning toward the use of environmentally friendly and low-cost methodologies that guarantee long-term maintenance of soil quality and reduce diffuse contamination. In this context, vermicompost provides a unique opportunity to develop a sustainable approach for improving the (bio) chemical quality of contaminated soils. Vermicompost is a finely divided peat-like material with a low C:N ratio and high content of humic substances. This biologically active material is produced from organic waste in a mesophilic biooxidative process in which microorganisms and earthworms play a cooperative role as decomposers (Domínguez, 2011). Historically, vermicompost has been used as an excellent natural fertilizer with a high moisture-holding capacity able to increase soil porosity, aeration and drainage, and which ultimately improves soil quality and plant growth. In the last decade, there has been a growing interest in the use of vermicompost for the bioremediation of contaminated soils. For instance, Romero et al. (2010) observed that the herbicide diuron degraded more rapidly in soils treated with grape marc-derived vermicompost. In addition to the stimulatory effect of vermicompost on pesticide microbial degraders, physicochemical properties also govern the partitioning of pesticides between vermicompost and soil. A soil column experiment showed that grape marc-derived vermicompost added to topsoil significantly reduced the vertical movement of diuron, imidacloprid and their metabolites in soil (Fernández-Bayo et al., 2015). These authors attributed the reduced leaching capacity to the high adsorption of pesticides to organic matter of vermicompost. Indeed, addition of this type of vermicompost to soils with a low organic matter content increased the half-life of diuron (Romero et al., 2010).

Vermicompost is also a rich-source of active enzymes with a high potential to recover soil biogeochemical processes. Pesticides are known to strongly affect many extracellular soil enzyme activities involved in C-, N-, S-, and P-cycling (Gianfreda and Rao, 2008). This impact is mainly the result of a direct interaction between the pesticide and microbes, leading to changes in microbial activity and biomass. Although pesticides may cause an increase of certain enzyme activities, most of them lead to a decrease in microbial activity with a concomitant reduction of extracellular enzyme activities (Riah et al., 2014). This is the case with organophosphorus pesticides, which are one of the main types of agrochemicals used in agriculture worldwide. Organophosphorus pesticides inhibit microbial activity, as indicated by the reduced dehydrogenase activity (Riah et al., 2014). Although some studies have shown that addition of organic wastes to soil stimulates microbial activity and biomass, this approach has some limitations, such as the environmental risks of certain organic wastes, such as grape mark residues (highly phytotoxic polyphenolic compounds) or a selective stimulatory effect on extracellular enzyme activities.

Vermicomposting dramatically changes microbial communities relative to the original raw material (Aira et al., 2007a; Gómez-Brandón et al., 2010; Zhang et al., 2000). However, little is known about the distribution, stability and reactivity of vermicompost-specific enzymes, and the key question that arises is whether vermicompost addition to soil leads to an extra loading of active extracellular enzyme activities to the original soil enzymatic burden. Although some studies have demonstrated a significant increase in soil enzyme activities in vermicompost-amended soils, probably as a result of proliferation of native microbial communities, it is not clear whether the enzyme cocktail from vermicompost makes a significant contribution to soil biochemical performance. To address this question, our research group has recently focused on the enzymatic characterization of fresh vermicomposts obtained from two raw materials: grape marc and horse manure.

Traditionally, measurement of soil extracellular enzyme activities often involves two main approaches: extraction of enzymes using appropriate buffers and preparation of soil–water suspensions (Sinsabaugh et al., 2000; Deng et al., 2013; Nannipieri et al., 2002). Although both methods may be valid, depending on the initial question or hypothesis of the study, soil–water suspensions provide more representative samples of the different forms in which enzymes occur in the soil system. Soil biochemists agree that enzymes may be dispersed in soil in three main forms: intracellularly in living and resting cells, free in soil solution, and extracellularly associated with soil organo–mineral complexes (Nannipieri et al., 2002). Therefore, use of soil–water suspension together with the appropriate substrates (e.g., lipid soluble surrogates) provides information about the total enzyme activity in the soil sample. This methodological approach has been reproduced with vermicompost samples and the procedure used to obtain water-vermicompost suspensions in order to determine a series of enzyme activities using a spectrophotometric microplate format (Sanchez-Hernandez et al., 2015) is depicted in Fig. 3.5. In this study, the variations

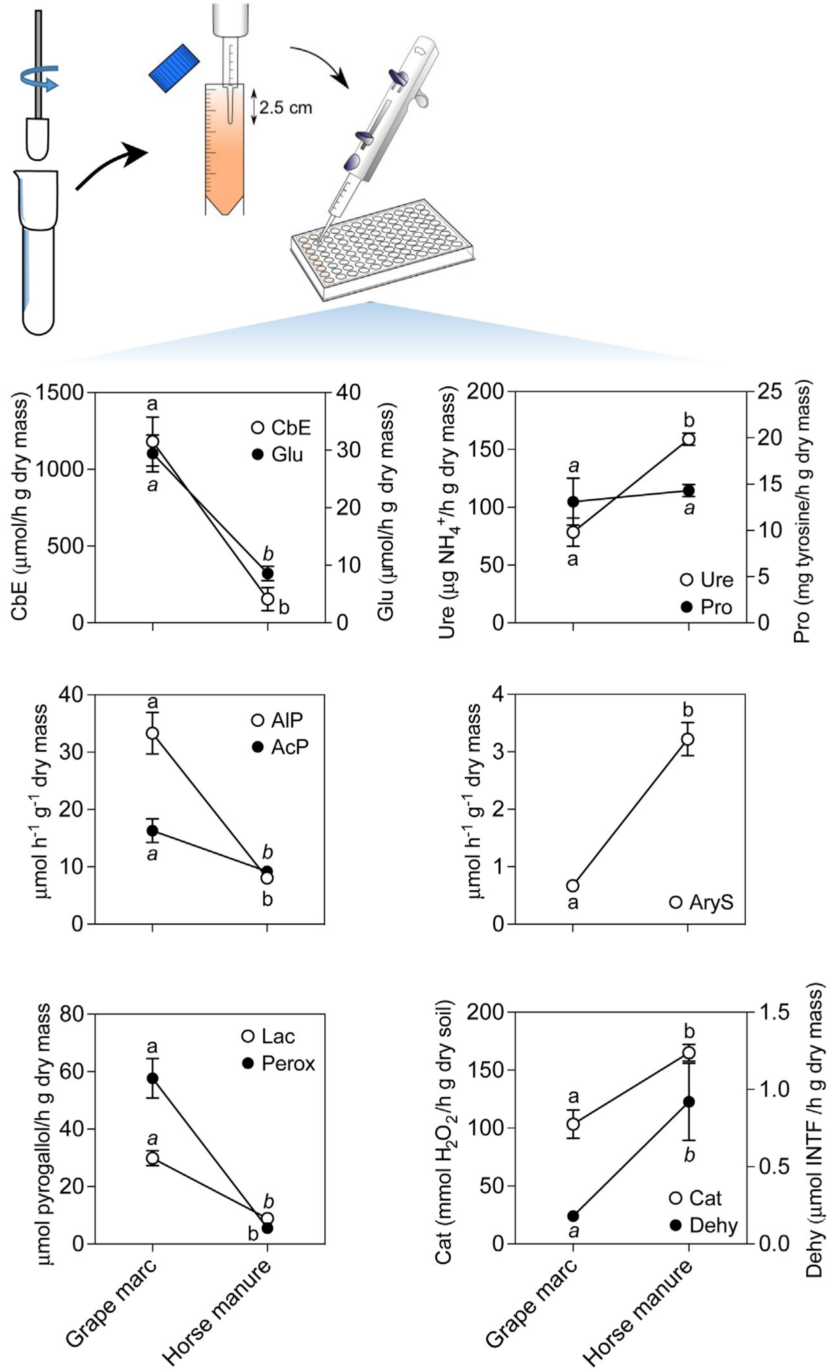

FIGURE 3.5 Preparation of vermicompost:water suspensions (3% w/v) using a Potter-Elvehjem tissue grinder. Aliquots of the extract were poured in 96-wells flat microplate for colorimetric measuring of multiple enzyme activities. Graphs show the mean (±SE, $n = 6$) activity of C-, P- and N-cycling enzyme activities in grape marc- and horse manure-derived vermicomposts. Peroxidase and laccase activities were determined as indexes of oxidative potential of vermicomposts, whereas dehydrogenase and catalase activities were determined as indicators of vermicompost microbial activity. Abbreviations: CbE, carboxylesterase; Glu, β-glucosidase; AcP, acid phosphatase; AlP, alkaline phosphatase; Lac, laccase; Perox, peroxidase; Ure, urease; Prot, protease; ArylS, arylsulfatase; Cat, catalase; and Dehy, dehydrogenase. Different letters between types of vermicompost denotes significant difference ($P < 0.05$, Wilcoxon test).

in enzyme activities were highly dependent on the type of vermicompost. C- and P-cycling enzyme activities were significantly higher in the grape marc-derived vermicompost than those in the horse manure-derived vermicompost. Conversely, urease, arylsulfatase, and the intracellular enzyme activities dehydrogenase and catalase, which are common indicators of microbial activity, were higher in the horse manure-derived vermicompost (Fig. 3.5). Although more evidence is required before solid conclusions can be reached about this marked vermicompost-specific variation in enzyme activity, the nature of the initial raw material may play a key role. Horse manure is the result of mechanical, chemical, and enzymatical treatment of a plant-derived material in the digestive tract of an herbivorous. This microbial-rich material was further modified by the joint action of earthworms and microorganisms during vermicomposting. Therefore, this double digestive process could explain the higher microbial activity compared with the grape marc-derived vermicompost, where enzymatic processes took place only during vermicomposting. Digestion of proteins and S-containing compounds by the horse gastrointestinal tract would form simpler molecules, thus accounting for the higher N-cycling enzyme and arylsulfatase activities in the vermicompost obtained from this manure, probably due to enhancement of these extracellular enzymes of microbial origin. The presence of materials (e.g., cellulose and lignin) resistant to digestion, in the grape marc would explain the higher activity of both peroxidases and laccases in the vermicompost compared with those observed in the horse manure-derived vermicompost.

The levels of enzyme activities in fresh grape marc-derived vermicompost are somewhat higher than those usually detected in soils. For example, the activities (mean ± SD) of β-glucosidase, urease, acid phosphatase, and catalase measured in agricultural Andisols were 2.5 ± 0.4 μmol h^{-1} g^{-1} dry mass, 26.2 ± 13.8 μmol NH_4^+ h^{-1} g^{-1} dry mass, 5.4 ± 0.9 μmol h^{-1} g^{-1} dry mass and 7.5 ± 1.5 mmol H_2O_2 h^{-1} g^{-1} dry mass (personal comm., Sanchez-Hernandez, J.C.), whereas the activities (mean ± SE) of these enzymes measured in grape marc-derived vermicompost were 29.4 ± 3.2, 78.5 ± 12.2, 16.3 ± 2.0 and 103.4 ± 12.2 (Fig. 3.5). The marked difference between both types of samples may be mainly due to the organic matter content in the vermicompost that binds extracellular enzymes, thereby preventing their degradation. Interestingly, the dehydrogenase activity was higher in the agricultural Andisols (0.80 ± 0.11 μmol INTF h^{-1} g^{-1} dry mass) than in grape marc-derived vermicompost (0.18 ± 0.02 μmol INTF h^{-1} g^{-1} dry mass). The aerobic microbiota must therefore contribute more significantly to extracellular enzyme activities observed in vermicompost, which is supported by a higher catalase activity. These interesting data encourage further research on the impact of the vermicompost enzymes on the biochemical performance of pesticide-contaminated soils, in addition to its known microbial stimulatory effect.

3.3.5 Pesticide Detoxifying Enzymes in Grape Marc-Derived Vermicompost

In the last decade, there has been a growing interest in using vermicompost as a low-cost and environment friendly technology to improve soil quality in contaminated land. The

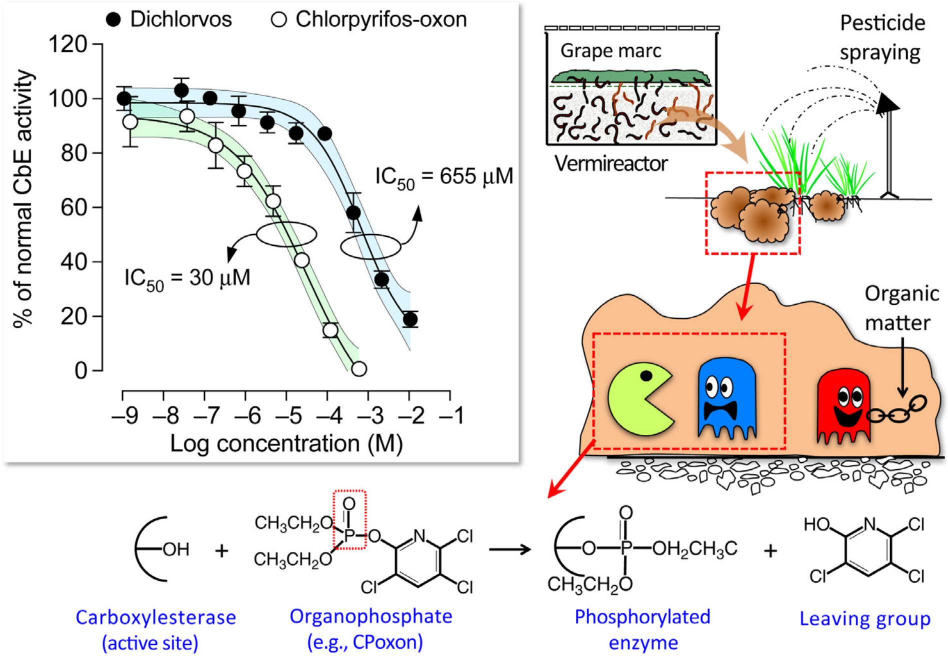

FIGURE 3.6 Proposed model illustrating enzymatic bioremediation of organophosphorus pesticides by vermicompost carboxylesterase (CbE) activity. Organophosphorus pesticides may be bound to either organic matter present in vermicompost or to the active site of CbEs, which are extracellular enzymes in vermicompost. Plots show in vitro inhibition kinetics of CbE activity from grape marc-derived vermicompost with chlorpyrifos-oxon (CPoxon) and dichlorvos. Median inhibition concentrations (IC_{50}) for both pesticides after 30 min incubation were in the range of micromolar. Symbols represent the mean and standard deviation of three independent determinations.

physicochemical and biological properties of vermicompost are compatible with bioremediation because it provides a cocktail of potentially contaminating degraders (microbes and extracellular enzymes), as well as a high organic matter content is able to bind both organic and inorganic pollutants (Fernández-Bayo et al., 2015; Romero et al., 2010). However, our understanding of how vermicompost enzymes may degrade organic contaminants, such as pesticides remains limited.

In the bioremediation of contaminated soils, extracellular enzymes belonging to oxidoreductase and hydrolase groups can potentially break down a wide range of xenobiotics, such as polycyclic aromatic hydrocarbons and phenolic compounds (Rao et al., 2014). Due to the high levels of activity of these enzymes found in vermicompost (Fig. 3.5), a comparable detoxification capacity is expected with these organic amendments. The use of carboxylesterase activity (CbE, EC 3.1.1.1) as an enzymatic system to remove organophosphorus pesticides from soil has been proposed, showing that earthworms play a key role in this bioremediation system (Sanchez-Hernandez et al., 2014, 2015). Indeed, these esterases play a pivotal role in the detoxification of organophosphorus, carbamates, and synthetic pyrethroids in animals (Sogorb and Vilanova, 2002; Wheelock et al., 2008). In the case of organophosphorus (OP) pesticides, the highly toxic "oxon" (oxygen analogs) metabolites bind stoichiometrically to the active site of CbEs. In this phosphorylated state, the enzyme is no longer active while at the same time, the OP is not bioavailable to other biological targets, such as microorganisms or other extracellular enzymes. The activity of both endogeic (*Aporrectodea caliginosa*) and anecic (*Lumbricus terrestris*) earthworm species enhanced soil CbE activity, which is very sensitive to inhibition by OP pesticides (Sanchez-Hernandez et al., 2014, 2015). Grape marc-derived vermicompost (i.e., the product from casting activity of epigeic earthworm species) is also a rich source of CbEs (Fig. 3.5), and the question therefore arises as to whether vermicompost CbE activity is a detoxifying system that could remove toxicologically active OP pesticides from soil. Fig. 3.6 shows a conceptual model whereby CbE activity in grape marc-derived vermicompost may act as a molecular scavenger contributing to inactivate OP pesticides in soil.

Vermicompost organic matter is a source of binding sites for OP pesticides and their hydrophobic metabolites. Some studies with hydrophobic pesticides, such as diuron or imidacloprid, have shown that their persistence in soil increased with the addition of vermicompost (Fernández-Bayo et al., 2015). In soil, and probably also in vermicompost, OP pesticides are degraded to their toxic "oxon" metabolites by chemical hydrolysis and microbial activity (Racke, 1993). Accordingly, a wide variety of bacteria and fungi species are able to break down OP pesticides (Singh, 2008). In this conceptual model, extracellular CbE activity, which can be released from microorganisms and earthworm gastrointestinal epithelium, inactivates OP toxicity in a way similar to that occurring in animals (Maxwell, 1992). Carboxylesterases would therefore act as exogenous bioscavengers for these pesticides. Indeed, in vitro inhibition assays of vermicompost CbE activity in the presence of both chlorpyrifos-oxon and dichlorvos revealed that CbE activity was sensitive to inhibition by both OPs, displaying median inhibition concentration (IC_{50}) in the range of μM (Fig. 3.6). However, inhibition kinetic curves suggest that this noncatalytic detoxification system is highly dependent on the type of OP. Physicochemical features of OPs, such as partitive properties (e.g., log K_{OC}) could be determinant in inhibiting CbE activity, because those OPs with high log K_{OC} are expected to bind strongly to organic matter rather than being available to inhibit CbE activity. Pending further studies, the addition of vermicompost to OP-contaminated soils, or agricultural soils receiving periodic OP treatments is expected to reduce the toxicity of these agrochemicals by the action of chemical (organic matter) and biochemical (CbEs) factors in vermicompost.

3.4 Conclusions

Vermicomposting of grape marc has proven to be a very useful procedure that yields an organic fertiliser and grape seeds as a source of bioactive compounds. The process reduces the biomass of the initial raw grape marc by more than a half, it transforms the most labile parts of the grape marc into a high quality, nutrient and microbial rich, polyphenol-free organic fertilizer. Sieving the material separates the fertilizer (vermicompost) from a residue that mainly contains grape seeds, and eliminates the polyphenol-associated phytotoxicity from the vermicompost and enables them to be easily processed to obtain different bioactive compounds, such as polyphenol-rich extracts and also fatty acid-rich seed oil. These coproducts can be directly extracted for use in the pharmaceutical, cosmetics, and food industries. During vermicomposting of grape marc very large numbers of earthworms are obtained and they have applications as fish bait, as a source of animal feed protein, as soil pollutant accumulators, and as a source of vermiceuticals (i.e., earthworm-derived pharmaceutically active compounds) and even human food. The grape marc-derived vermicompost is also as a rich-source of enzymes that could be used to improve soil biochemical performance and to detoxify pesticide-contaminated soils. The vermicomposting procedure described herein is effective, simple, environment friendly and sustainable, while it can be easily scaled up for industrial applications, yielding a variety of value added products from the grape marc.

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