Fungal Enzymes for the Production of Vanillin, Divanillin & Lignans

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Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig verfasst und die benutzten Hilfsmittel und Quellen sowie gegebenenfalls die zu Hilfeleistungen anderer Institutionen vollständig angegeben habe.

Die Dissertation wurde nicht schon als Masterarbeit, Diplomarbeit oder andere Prüfungsarbeit verwendet.

Hannover, den ……… Vida Esparan

Preliminary notes

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Zusammenfassung

Die vorliegende Studie konzentrierte sich auf mögliche Substrate und Reaktionsbedingungen für die Herstellung von wertvollen Aromastoffen sowie geschmacksmodifizierenden Substanzen durch enzymatische Reaktionen.

1. Die Synthese von natürlichem Vanillin - einer bevorzugten Zielkomponente biotechnologischer Verfahren zur Herstellung natürlicher Aromastoffe - aus Curcumin wurde untersucht. Vor der Spaltung des Moleküls durch pilzliche Laccasen musste eine der phenolischen Hydroxylgruppen durch Acetylierung mit einem natürlichen Acyldonator unter Verwendung einer Lipase aus *Candida antarctica* geschützt werden. Unter kontrollierten Bedingungen in einem Zweiphasensystem katalysierten geeignete Laccasen die Spaltung der aliphatischen Doppelbindung unter Bildung von Phenolresten und eine Polymerisation des Curcumins konnte vermieden werden. Dabei zeigte eine Laccase aus *Funalia trogii* unter den gewählten Reaktionsbedingungen die höchste Stabilität und Oxidationspotential. Durch Spaltung von Acetylcurcumin mit dieser Laccase konnte eine Ausbeute von 46% Acetylvanillin erzielt werden. Die Zielkomponente Vanillin wurde im letzten Schritt des dreistufigen enzymatischen Prozesses durch Deacetylierung mit Hilfe einer Esterase aus *Pleurotus* (Pe FaeA) in höherer Ausbeute im Vergleich zu anderen Esterasen freigesetzt. Vanillin konnte so mit einer molaren Ausbeute von mehr als 15% des ursprünglich eingesetzten Curcumins gewonnen werden.

2. Zur Herstellung von natürlichem Dehydrodivanillin, einem Geschmacksverstärker, der Milchprodukten und auch anderen Lebensmitteln Cremigkeit verleiht, wurde die Oxidation von Vanillin in wässriger Phase mit verschiedenen Laccasen und Peroxidasen verglichen. Ein nahezu quantitativer Umsatz wurde mit einer Laccase aus *Funalia trogii* erzielt, während die anderen pilzlichen Laccasen sowie kommerzielle Peroxidasen deutlich geringere Ausbeuten lieferten.

3. Erneut wurden mehrere Laccasen aus Basidiomyceten mit unterschiedlichen Redoxpotenzialen zur Umsetzung phenolischer Substrate verwendet. Zielprodukte waren in diesem Ansatz Lignane, (Phenoldimere) deren potenzielle geschmacksmodifizierende Eigenschaften von Interesse sind. Verglichen wurden die Umsatzraten und die Enzymstabilitäten in Gegenwart verschiedener organischer Lösungsmittel. Die Laccase aus *F. Trogii* erwies sich einmal mehr, als ein potenter Biokatalysator, der auch unter harschen experimentellen Bedingungen Aktivität zeigte, während andere Enzyme inaktiviert wurden. Die

Dimerisierungsreaktionen erfolgten in einem Zweiphasensystem mit mehr als 95% Ausbeute für Substrate, wie Ferulasäure, Sinapinsäure und Coniferylalkohol.

Stichworte: Vanillin, Dehydrodivanillin, Flavour, Laccase, Esterase, Lignans

Summary

The present study focused on possible substrates and reaction conditions for the production of valuable aroma and taste modifying compounds by enzymatic reactions.

1. The production of natural vanillin as a prime target of flavour biotechnology by the enzymatic degradation of curcumin was investigated. Curcumin was protected by an enzymatic acetylation using a lipase from *Candida antarctica* and a natural acyl donor. Under controlled conditions in a two phase system, laccase catalysed the formation of phenol radicals, so cleavage of double bound occurred, but polymerization was avoided. The laccase from *Funalia trogii* showed the highest stability and oxidative potential. Acetyl vanillin was the result of the cleavage of acetyl curcumin by the laccase with a yield of 46 %. Natural vanillin was finally released by a fungal esterase from *Pleurotus eryngii* (PeFaeA) in higher yield in comparison to other esterases. Vanillin with a molar yield of more than 15 % of the initially applied curcumin was achieved by these three consecutive steps.
2. Oxidation of vanillin in the aqueous phase using three types of laccases and two peroxidases was compared for the production of dehydrodivanillin, a taste-enhancer which imparts creaminess in dairy and other products. The reaction was catalysed by the laccase from *Funalia trogii* and delivered almost quantitative conversion to divanillin (> 95 %), while other fungal laccases and commercial peroxidases yielded much less.
3. Several laccases from basidiomycetes with different redox potential were used to direct the oxidative reaction of phenols to the production of lignan dimers with potential taste modifying properties. The rates of substrate oxidation and enzyme stability were compared in the presence of organic solvents. *F. trogii* again proved to be a potent catalyst and was effective even under harsh conditions, which strongly inhibited the activity of other enzymes. Dimerization reactions took place in a two phase system with more than 95 % yield for substrates, such as ferulic acid, sinapic acid and coniferyl alcohol.

Keywords: Vanillin, Dehydrodivanillin, Flavour, Laccase, Esterase, Lignans.

Abbreviations list

Abi	*Agaricus bisporus*
ABTS	2,2'-Azino-di-(3-ethylbenzthiazolin-6-sulfonsäure)
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
CAL	*Candida antarctica* lipase
CFR	Code of Federal Regulations
Da	Dalton
DSMZ	Deutsche Sammlung für Mikroorganismen und Zellkulturen
ESI-MS	Electrospray ionization mass spectrometry
EtOH	Ethanol
EtAc	Ethyl acetate
FAEA	Ferulic acid esterase
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
Ftr	*Funalia trogii*
GC-MS	Gas Chromatography- Mass Spectrometry
GC	Gas Chromatography
HPLC-MS	High Performance Liquid Chromatography
HRP	*Horseradish peroxidase*
Lcc C	*Trametes versicolor* laccase

LC-MS	Liquid Chromatography- Mass Spectrometry
Mgi	*Meripilus giganteus*
Min	Minute
mM	millimolar (mmol L^{-1})
MnP	Manganese peroxidase
MsP2	*Marasmius scorodonius*
MSTFA	N-methyl-N-(trimethylsilyl) trifluoroacetamide
MM	Molecular mass
m/z	Mass-to-charge ratio
NADH	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
PAGE	Polyacrylamide gel electrophoresis
PeFaeA	*Pleurotus eryngii* esterase
pI	Isoelectric point
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SNL	Standard nutrient media
TEMED	Tetramethylethylenediamine
TMCS	Trimethylchlorosilane
TLC	Thin layer chromatography

TQ	Triple Quadrupole
U	Unit (μmol min^{-1})
UmChl	*Ustilago maydis* esterase
US	United States
UV	Ultraviolet
Vis	Visible

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1 Introduction

1.1 Biotechnology

Biotechnology is the use of biological processes, living organisms or derivatives to make or modify products for specific application. One simple definition of biotechnology is commercialization of cell and molecular biology (Altman and Hasegawa, 2012). Biotechnology in food processing is using microbial and enzymatic technologies to modify properties such as the taste, aroma, shelf-life, texture and nutritional value of foods (Niar 2008). Biotechnology appears to be a new and a key technology for the 21st century, but the concept is not new. Many years ago, the use of micro-organisms and their enzymes to bring desirable changes in food was known as fermentation. Examples of this are the production of cheese, bread, wine, beer, etc. But none of these processes can be considered biotechnology in the modern sense. Recent development in molecular biology gave the new meaning and new potential to modern biotechnology (Klefenz 2002). Biotechnology is widely involved in the production and preservation of microbial cultures, enzymes, flavours, fragrances, food additives and a range of other high value-added products (Knorr and Sinskey 1985). These are increasingly produced in advanced developing countries for use in their food and non-food processing applications. Many of them are also imported by developing countries for use in their food-processing applications. Biotechnological developments have resulted in the widespread availability of low-cost rapid methods when compared with the significant cost/time requirements of traditional techniques (Maryanski 1995).

Biotechnology has applications in many different areas, such as food, medicine, agriculture and various environmental uses. To demonstrate the utility of biotechnology-based methods a series of definitions have been used for different branches of biotechnology (Niar 2008):

1- Blue biotechnology is the use of biotechnology in marine and aquatic applications. It is focused on advances in biotechnology to discover, produce or transform compounds from marine sources to be incorporated as functional and healthy ingredients in potential functional foods (Freitas 2012).
2- Green biotechnology is related to agriculture to produce more environmentally friendly farming solutions as an alternative to the traditional industrial agriculture. The agro-biotechnology is not a new science. For thousands of years, farmers have increased the desirable characters of their products with different biotechnological methods (Murray

2011). Since then, the use of biotechnology in agriculture has increased more and more through the introduction of new genes in 1960 by predicting the units of heredity or gene in plants (Niar 2008). The science of genetics was transformed by the discovery of DNA which contains the information in the cells (Murray 2011). The first transgenic crop was the "Flavr Savr ™" tomato; in this case the ripening was delayed and shelf life was increased (Zinnen and Voichik 1995; Falk 2002). One of the well-known developments in genetic modification in plants was indicated as an increase of herbicide tolerance and resistance against pathogens, such as fungi and insects, to improve the yields of crops and protect the environment. For instance, the resistance of maize (BT maize), oilseed rape and sugar beet can give the farmer much more flexibility in controlling weeds. Herbicide resistance is being used worldwide in cotton, potato, maize, soya, tobacco and wheat crops against insect pests. This is achieved by the integration of toxin forming genes from the common soil bacterium *Bacillus thuringiensis* into the genome (Maxwell *et al.* 1990). Another example of green biotechnology is designing of transgenic plants which are modified to improve flavour, for increasing the resistance to pests and diseases, or for enhancing growth in adverse weather conditions. Modifications of crop plants can be organized into two main categories: those that benefit the producer and those that benefit the consumer. Generally, modifications which involve resistance genes and are introduced into a crop with the use of genetic engineering methods will be called input trails. These resistant genes allow tolerance to herbicides or protect from fungi, pests certain insect, disease and other harmful organisms. The majority of modified crops in commercial use fit in this group. Scientists have just begun to tap the large potential of biotechnology to produce varieties of plants that confer a wide spectrum of advantages to consumers (Murray 2011).

3- Red biotechnology is most applicable in medical processes. The most well-known examples of red biotechnology are developing antibodies for the treatment of cancer, diagnosis of diseases, such as DNA chips for genetic diagnosis and tumor markers in cancer patients, or the cultivation of tissues such as cartilage, bone or skin stem cells in tissue engineering (Schmid and Urlacher 2007).

4- White biotechnology or industrial biotechnology is biotechnology applied to industrial and chemical processes. The most important and well-known application of white

biotechnology is the use of enzymes as catalysts to either produce valuable chemicals or destroy hazardous/polluting chemicals. White biotechnology processes can help to make industrial manufacturing processes more environmentally friendly. They are performed in a contained environment, and have the potential to produce high yields of specific products with low energy use and minimal waste generation (Lorenz and Zinke 2005). The current demand of industry in enzyme productivity and stability, leads to development of novel technique for increasing shelf life and stability of enzymes. These requirements are inevitable to facilitate new and economic formulation. Using isolated enzyme in the reaction and immobilization provide an excellent base for increasing availability of enzyme. The main applications of enzymes are detergents (32 %), industrial processes (20 %) and the production of food and feed (33 %). The proportion of enzymes involved in the production of fine chemicals and pharmaceuticals with 4-5 % is comparatively low (Antranikian 2006).

1.2 Flavour (Definition and history of usage)

Flavours are extremely important chemicals. They have applications in different fields, such as food, feed, chemical, pharmaceutical and cosmetic industries. Financially, 25 % of the total market of food additives belong to flavours (Berger 2009). According to the U.S. Food and Drug Administration (FDA) regulation, flavours are compounds which can be added to food to impart taste and smell (All CFR regulations 2006). Flavours usually contain volatile and nonvolatile components and may be obtained through chemical and physicochemical process. Nonvolatile flavours evoke mainly taste sensations while volatiles have the influence on both taste and aroma (Gatfield 1988). The FDA identifies that flavours can be divided to natural and artificial. Artificial flavours are defined as any substance, which is not derived from natural sources such as spice, fruit or fruit juice, vegetable or vegetable juice, edible yeast, herb, root, leaf or similar plant material, meat, fish, poultry, eggs, dairy products, or fermentation products. On the other hand, natural flavours are defined as substances which are extracted from ‘essential oil, oleoresin, hydrolyse of protein, distillate, or any product of roasting, heating or enzymolysis, which contains the flavouring constituents derived from the mentioned above sources.

The terms nature identical defines as substance, which is present in nature and may also be produced synthetically. For example, natural benzaldehyde is produced from the nature, but this

process has a rather low yield and produces waste material with the costly disposal. On the other hand, production of benzaldehyde from chemical feedstock is much more economical and is putatively the same as that produced from nature; therefore, it is nature identical. The nature identical designation is still used by the International Organisation of Flavour Industries (IOFI) but this term does no longer exist in the EU regulation. EU and US legislation has incorporated under the term 'natural flavours' those flavours produced from biological sources by living cells, including fungi or their enzymes (Cheetham 1993; Burdock 2002).

The flavour market is expected to increase at a high rate. Many flavour compounds on the market are produced from chemical synthesis or by extraction from natural sources such as plants and animals (Krings and Berger 1998). A preparation of natural flavours from the wild is the easiest method, but influence of the weather and the risk of plant diseases cause the reduction of natural resources (Bedoukian 1986). Many products are still obtained by chemical synthesis even though chemical synthesis often causes undesirable side effects on the environment, and the raising awareness of consumers about the safety of the products, especially food and beverages drives the progress in biological production of these compounds (Berger 1995; Schrader *et al.* 2004).

The first creation roots of biotech flavours were the flavours in fermented foods, such as cheese, yoghurt, beer, wine and other products. From a technical point of view, advances in analytical techniques such as infrared (IR) spectroscopy, nuclear magnetic resonance (NMR) spectroscopy and in particular, high resolution capillary gas chromatography (HRGC) and mass spectrometry (HRMS) have been improved the knowledge about flavours. In recent decades, extensive research has been done to increase the potential and variety of flavours from biotechnological sources. Flavours produced by biotechnological methods compete with those from agricultural sources in terms of quality, variety, selectivity of the end products (Havkin-Frenkel and Belanger 2008). The production of natural flavours can be performed by two basic methods; through *de novo* synthesis or by biotransformations. *De novo* synthesis refers to the production of complex substances from simple molecules through complex metabolic pathways, while biotransformations are single reactions catalysed enzymatically to result in a product structurally similar to the substrate molecule (Bicas *et al.* 2010; Baines and Seal 2012).

1.2.1 Biotechnological approach for the production of flavour compounds

Nowadays, more than 100 commercial aroma compounds are made by biotechnology with the use of microorganisms or enzymes as biocatalyst. A wide range of microorganism, such as bacteria, fungi and yeast are well known to produce flavours from simple substrates by synthesis or by bioconverting specific substrate into the desirable compounds (Vandamme 2003). Production of sweet and fruity flavours with the aid of enzymes and microorganism was most successful in the recent decade. For instance, *Ceratocystis* species and the yeast *Kluyveromyces lactis* were able to produce a wide range of terpenes with a fruity or floral odour (Drawert and Barton 1978; Collins 1976). *Trichoderma viride* and *Trichoderma harzianum* strains produce efficiently the coconut-flavoured lactone, 6-pentyl-*α*-pyrone in a fermentation process (Palomares *et al.* 2001). In 1972, the yeast *Sporobolomyces odorus* was found to produce large amount of peach-smelling compounds *γ*-decalactone (4-decanolide) and 4-hydroxy-cis-6-dodecenoic acid flavour (Takahara 1972). In further innovations, conversion of castor oil to 4-hydroxydecanoic acid and further to 4-decanolide by alkanophilic yeasts were investigated (Farbood and Willis 1983). *Geotrichum klebahnii* produced *de novo* a broad spectrum of ethyl esters of branched carboxylic acids, generating a pleasant fruity flavour. When supplied with isoleucine, especially ethyl-2-methyl butanoate was formed (Janssens *et al.* 1989). Yeasts, such as *Candida tropicali*s or *Yarrowia lipolytica* degraded ricinoleic acid to C16, C14 and C12 acids and produced *δ*-decalactone, which exhibits fruity and oily notes important in the formulation of peach, apricot or strawberry aromas (Gatfield 1999). *Hanseniaspora guilliermondii* and *Pichia anomala* were found to produce 2-phenylethyl acetate and isoamyl acetate, respectively (Rojas *et al.* 2001). Different yeast strains such as *Hansenula anomala*, *Kluyveromyces marxianus* or *Saccharomyces cerevisiae* have shown a high potential for industrial production of aroma compounds, such as 2-phenylethanol, which is derived from L-phenylalanine by bioconversion (Stark *et al.* 2002). After a while, several yeasts were found to produce different aroma compounds (King and Dickinson 2003).

Numerous bacterial strains also are capable of producing specific single flavour molecules either *de novo* or by converting an added substrate/precursor molecule. Traditionally, all dairy flavours were produced by fermentation processes, involving many varieties of bacteria. For instance, pyrazines in cheese which are produced by strains, such as *Pseudomonas perolens* and *Corynebacterium glutamicum* or other important cheese flavours produced by Lactic acid

bacteria (Germond *et al.* 1995). Catabolising ferulic acid to vanillin is one of the most important and well known examples in terms of bacterial production of flavours (Muheim and Lerch, 1999).

Beside microbial fermentation for flavour production, enzymes as biocatalysts have vast possibilities for food flavour production. Enzymes can be used as processing aids for flavour production, and can also correct off-flavours caused by specific compounds naturally occurring or produced during processing (Armstrong and Yamazaki 1986). There are about 25,000 enzymes found in nature of which 400 have been suggested in organic synthesis and also for the biotechnological production of flavour compounds (Cheetham 1997). The majority of enzymes which have applications in flavour production include hydrolytic enzymes, transferases, oxidoreductase and lyases (Serra *et al*. 2005). There are many examples in the food industry for the production of flavours by enzymatic reactions. Enzymes involved in cheese flavour biosynthesis (Kinsella and Hwang 1976), enzymes which have effect on the flavour of citrus, for example *α*-terpineol dehydratase which catalyses the reaction of limonene. This enzyme has been isolated from *Pseudomonas gladioli* (Cadwallader *et al.* 1992). Raspberry ketone (4-(4-hydroxyphenyl)butan-2-one) which is the main aroma compound of raspberry can be produced by an enzymatic pathway involving hydrolysis of betuloside from the European white birch tree by *ß*-glucosidase and then transformation by an *Acetobacter* alcohol dehydrogenase into the ketone (Whitehead 1998). An industrial example is the production of L-menthol, the major constituent of peppermint oil. Microbial lipases have been found to hydrolyse L-menthyl esters (from the d, L-racemic mixture) into L-menthol, leaving the d-menthyl esters intact (Schrader *et al.* 2004). Benzaldeyde which is the most important molecule in cherry flavour can be produced by enzymatic biotransformation of benzyl alcohol by both whole cell of *Pichia pastoris* and cell free extract of *Saccharomyces cerevisiae* (Duff and Murray 1989; Nikolova and Ward 1992).

1.3 Biotransformation

Biotransformation is a growing field of biotechnology, which converts a compound from one form to another form and results in a product structurally similar to the substrate molecule using an appropriate biological system (Bicas *et al.* 2010). This biological system can be both enzymatic or microbial. Biotransformation has the potential to produce products more cost effective by efficient utilization of raw materials, lower investment and energy sources and leads to less hazardous products (Faber 2011; Liese *et al.* 2006). Biotransformation has been made to produce not only new ingredients, but also improved processes to produce ingredients from chemical synthesis or extraction from natural sources. Biotransformation in the organic phase is a recent approach for those reactions which are limited because of low solubility and/or instability of substrates and/or products in water (Bornscheuer and Kazlauskas 2006). Biotransformation has been found to be superior to chemical reactions because of the following features:

- Regio-specificity which means that substrate is usually attacked at a particular site, even if several groups are present.
- Reaction specificity and no side reaction as long as one catalysis will be involved in a particular biotransformation.
- Production of optically pure compound with producing one enantiomer.
- Performing under mild reaction condition and decreased activation energy of chemical reaction.

Microbial and enzymatic catalysis allow classification of the generated biotechnological compounds as natural which has increased the attention in industry (Faber 2011).

1.3.1 Basidiomycetes as fungal cultures

The kingdom fungi have been estimated at 1.5 to 5 million species, which from this amount about 5 % have been formally classified (Bruns 2006). Fungi have a worldwide distribution, and grow in a wide range of habitats, including extreme environments such as deserts or areas with high salt concentrations and deep sea (Blackwell 2011). Traditionally, higher fungi have been treated as non-green plants. The reason for grouping fungi with plants was mainly due to the presence of cell walls. From biochemical, physiological and genetic analysis fungi are today

placed closer to animals. True fungi divide into four taxonomic groups, *Chytridiomycota*, *Zygomycota*, *Basidiomycota* and *Ascomycota* (Webster and Weber 2007).

Basidiomycota are a large family of fungi with more than 30,000 species that together with the Ascomycota, constitute the subkingdom Dikarya (Figure 1.1) (Carlile and Watkinson 1994). Basidiomycota are filamentous fungi composed of hyphae (except for yeasts), and reproduce sexually via the formation of specialized club-shaped end cells called basidia that normally bear external meiospores. These specialized spores are called basidiospores. (Alexopoulos *et al.* 1996). The class of basidiomycetes contains some of the most common and familiar known fungi, including mushrooms, bracket fungi and puffballs. The majorities of this class are found in woody plant materials and degrade plant residues (Moore-Landecker 1996). Most of the Basidiomycota are scattered by wind but some of them also grow in fresh water environment (Webster and Weber 2007). There are about 12,000 species of fungi considered as mushrooms, with at least 2,000 edible. More than 200 species have been used for different purposes. About 35 species have been cultivated commercially and 20 species are cultivated on an industrial scale (Aida *et al.* 2009).

Figure1.1 Basidiomycetes *(Meripilus giganteus)*

Basidiomycete species act as lignocelluloses destroyers and include very different ecological groups such as white rot, brown rot, and leaf litter fungi (Cho *et al.* 2009). White rot basidiomycetes secrete a wide diversity of enzymes, in which oxidative enzymes represent an important and wide range of this group; for instance white-rot fungi which live on dead or living timber, have a powerful oxidative enzyme system that can degrade lignin to carbon dioxide (Siripong *et al.* 2009). White-rot fungi can degrade a vast range of toxic environmental

pollutants; this ability makes these organisms unique and attractive for the bioremediation of polluted sites (Breitenbach *et al.* 2002).

Besides many applications of fungi in food and other industries, it has been known that many fungi can generate flavours. Compared with other microorganisms, the volatile spectrum which can be obtained from living cells of fungi or enzymes thereof is closest to plant sources (Abraham and Berger 1994). The basidiomycetes represent a group of fungi for the industrial production of natural flavours. Starting from specific substrates, different reactions such as oxidations, hydrolytic reductions, dehydrations, formation of new C–C bonds and several degradation reactions can be performed by basidiomycetes in order to produce natural flavours (Lomascolo *et al.*1999).

In order to increase the role of production of natural flavours by fungi in the flavour industries, biotechnological processes involving basidiomycetes have to compete economically with traditional processes or extracted flavours from nature. Choosing more appropriate culture conditions, supplementing with cheap, natural precursors, continuously developing more-suitable bioreactors designs could open the way to high-yielding processes (Lomascolo *et al.* 1999; Ramachandra Rao and Ravishankar 2002).

1.3.2 Enzyme as biocatalyst

A broad spectrum of chemical reactions such as hydrolysis, esterification, isomerization, addition and elimination, alkylation and dealkylation, halogenation and dehalogenation, oxidation and reduction and etc. require to be catalyzed to proceed at a significant rate. Catalysts are molecules that reduce the required energy to convert a substance chemically into another one and can be referred as enzyme (Figure 1.2) (Andres 2008). Principally, enzymes are proteins, but some of them contain additional non-protein compounds, such as lipids, metals, phosphate or some other organic moiety. The whole molecule of enzyme is called holoenzyme, while the protein part is known as apoenzyme, the rest of the molecule is called cofactor (Copeland 2000). Many enzymes retain their catalytic potential after extraction from the living organism, so it did not take long to recognize and exploit the catalytic power of enzymes for commercial purposes. In other words, they are highly specific biological catalysts and are ideal to be used in different applications (Aehle 2004).

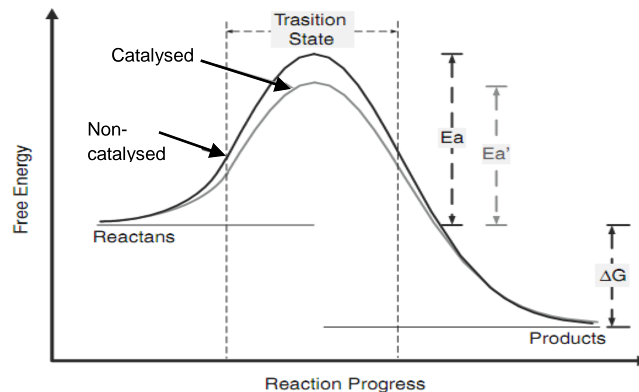

Figure 1.2 Catalyst mechanisms, Ea and Ea´ are the energies of activation of the non-catalysed and catalysed reaction. ΔG is the free energy change of the reaction.

Enzymes are capable of accelerating a wide range of biotransformation reactions. Enzymes have a number of advantages, such as high specificity, high activity under moderate conditions, high turnover number and biodegradability, but on the other hand they have disadvantages, such as high molecular complexity, denaturation at inappropriate pH and temperature. Microorganisms are attractive sources of enzymes, as they can be cultured in large quantities in a short time, hence they can provide a regular supply (Aehle 2007).

The enzyme industry as we know it today is the result of a rapid development seen primarily over the past four decades of the evolution of modern biotechnology. In fact, the historical background of using enzymes was in ancient times in food applications, such as beer, wine, vinegar, cheese and bread (Aehle 2004). Enzymes have been used unknowingly for hundreds of years. The first production of alcohol was by fermentation. In France, Anselme Payen and Jean-Francois Persoz described the isolation of an amylolytic substance from germinating barley (1833). Shortly afterwards the Swedish chemist Jons Jacob Berzelius coined the term catalysis (1835) to describe the property of certain substances to accelerate chemical reactions. In Germany, the physiologist Theodor Schwann (1836) discovered pepsin and the usage of an enzyme from the papaya fruit to tenderize meat. Dairy processes, such as the conversion of milk to cheese, were another important field of enzyme applications. In 1870's the Danish chemist Christian Hansen succeeded in obtaining pure rennet from calves' stomachs; this resulted in considerable improvements in both product quantity and quality. There are also many texts

related to the process of vinegar production, which is a kind of enzymtic conversion of alcohol to acetic acid. The leavening of bread by yeast, which results from the enzymatic production of carbon dioxide, was well known and widely used since ancient times (Bornscheuer and Buchholz 2005; Polaina and MacCabe 2007).

Due to the high level of use of enzymes in the nineteenth century, to answer the question of how enzymes were working and achieve substrates in the active sites, many investigations were done on the three dimensional structure of enzymes (Copeland, 2000). The characteristics of enzymes as catalysts are derived from their molecular structure. Enzymes contain a number of amino acid residues that ranges from 100 to several hundreds. These amino acids are covalently bound through the peptide bond that is formed between the carbon atom of the carboxyl group of one amino acid and the nitrogen atom of the α-amino group of the following. According to the nature of the R group, amino acids can be non-polar (hydrophobic) or polar (charged or uncharged) and their distribution along the protein molecule determines its behaviour (Lehninger 1970). According to this structure, some enzymes are so well designed for this purpose that they can accelerate the rate of chemical reactions by as much as 10^{12} times over the rate of the non-catalysed reaction (Polaina and MacCabe 2007).

Today enzymes have an important role in many food and pharmaceutical applications and as ingredients of many consumer products. Generally there are six main types/groups of enzymes classified, based on their chemical reaction mechanism.

1. Oxidoreductases: Enzymes catalyzing oxidation/reduction reactions that involve the transfer of electrons or redox equivalents between donor and acceptor molecules in the reaction (Xu 2005).

2. Transferases: Enzymes catalysing the transfer of a functional group such as methyl or glycosyl groups from one molecule which is called a donor to a suitable acceptor.

3. Hydrolases: Enzymes catalysing reactions of hydrolysis, this is an example of the cleavage of a chemical bond such as C-C, C-O, C-N, P-O and other bounds like acid anhydride bonds by the action of water.

4. Lyases: Enzymes catalysing reactions of non-hydrolytic and non-oxidative cleavage of chemical bonds such as C-C, C-O, C-N and other bonds by eliminating, adding or leaving groups to double bonds.

5. Isomerases: Enzymes catalysing reactions of conversion of a substrate into an isomer, this is a substance with the same number and types of atoms.

6. Ligases: Enzymes catalysing reactions of covalent linkage of two molecules. These are the enzymes responsible for cell anabolism and perform an essential role in the reactions of synthesis inside the cell (sometimes they are named synthetics) (Belitz *et al.* 2008).

1.3.2.1 Oxidoreductases

Oxidation reactions are essential for many industrial applications. However, they may produce non-specific or undesirable side-reactions and involve the use of environmentally hazardous chemicals. This calls for new technologies based on biological systems, such as enzymatic oxidation. Enzymatic oxidation has substrate specificity and is using biodegradable catalysts that perform under mild conditions (Burton *et al.* 2003).

Oxidoreductases are a class of enzymes which catalyse redox reactions. There is a wide range of enzymes which are distributed in microbial, plant and animal organism (Burton *et al.* 2003; Xu 2005). Oxidoreductase catalyses the transfer of electrons from one molecule to another molecule similar to the following $A^- + B \rightarrow A + B^-$ (Munro *et al.* 2000). Generally, they are divided into oxidases and dehydrogenases. Oxidases are enzymes involved when one or two oxygen molecules act as an acceptor of hydrogen or electrons. Whereas dehydrogenases are enzymes which oxidize a substrate by transferring hydride ions to an acceptor which is either NAD^+/$NADP^+$ or a flavin molecules. Other oxidoreductases include peroxidases and laccases which are large groups of enzymes and have many applications in food and flavour, pharmaceutical and textile (Burton 2003).

1.3.2.1.1 Peroxidases

Peroxidases are oxidoreductases which are produced by a number of microorganisms, plants and animals and have a molecular mass ranging from 30,000 to 150,000 Da. They are a group of oxidoreductases which catalyzes the reduction of peroxides, such as hydrogen peroxide and the oxidation of a variety of organic and inorganic substrates in the presence of peroxides (Adam *et al.* 1999).

Peroxidases are haem proteins and contain iron (III) protoporphyrin IX (ferriprotoporphyrin IX) as the prosthetic group. In the initial step the native ferric enzyme is oxidized by hydrogen peroxide to form an unstable intermediate, called compound I (Co I), which has a haem structure of FeIV = O-porphyrin π-cation radical, and at the end reduction of peroxide to water has happened. Then Co I oxidizes the electron donor substrate to give compound II (Co II), releasing a free radical. Co II is further reduced by a second substrate molecule, regenerating the iron (III) state and producing another free radical (Conesa *et al.* 2002; Hofrichter *et al.* 2010).

Reduction of peroxides at the expense of electron-donating substrates makes peroxidases useful in a number of industrial and analytical applications (Vamos-Vigyazo 1981), such as the treatment of waste water containing phenolic compounds, synthesis of various aromatic chemicals and removal of peroxide from foodstuffs and industrial wastes (Agostini *et al.* 2002). Enzymes, such as lignin peroxidase (LiP) and manganese peroxidase (MnP), both associated with lignin degradation and polymerization (Ward *et al.* 2001; Sakurai *et al.* 2003). Peroxidase from *Lepista nuda* was investigated as an enzyme for the degradation of carotene to produce flavour compounds (Zorn *et al.* 2003). More research on the degradation of *ß*-carotene was done using a peroxidase from *Marasmius scorodonius* (Scheibner *et al.* 2008).

Horseradish peroxidase (HRP) is a known enzyme with many practical applications in industry, such as degrading phenolic and other organic compounds, oxidative polymerization via free radical, degrading and precipitating azo dyes and decolorization of textile dyes (Tatsumi *et al.* 1996; Bhunia *et al.* 2002). HRP has been used to polymerize phenolic and aromatic amine compounds, while new types of aromatic polymers have been synthesized in water and in water miscible organic solvents (Oguchi *et al.* 1999). A fungal peroxidase from *Coprinus macrorhizus* was used as an alternative to HRP for the removal of aromatic compounds from waste water. Its performance was found to compare favourably to HRP, in that it could catalyse the same reactions, although it was noticeably more easily inactivated (Al-Kassim *et al.* 1994).

Peroxidases are facing some issues. The inactivation of peroxidases by peroxides through the oxidation is one of the major problems. Another one is the low water solubility of the substrates of interest. Peroxidase activity is also greatly affected by temperature, limiting its application to processes at relatively low temperature. Meanwhile, the dependency of enzymes from hydrogen peroxide is a main reason of concern (Sahare *et al.* 2014).

1.3.2.1.2 Laccases

Laccases (EC 1.10.3.2, *para*-benzenediol: dioxygen oxidoreductase) are multicopper proteins which mainly use molecular oxygen to oxidize various organic compounds, such as *para*-diphenols, aminophenols, polyphenols, polyamines and lignin by a radical mechanism (Couto and Herrera 2006). Laccases belong to the so-called blue-copper family of oxidases. They are glycoproteins and include ascorbate oxidase, ceruloplasmin and bilirubin oxidase (Hoegger *et al.* 2006). Typically, laccases are proteins with approximately 60–70 kDa and an acidic isoelectric point around pH 4.0 (Baldrian 2006). Laccase or laccase-like enzymes are widely found in plants, fungi (O'Malley *et al.* 1993), bacteria and insects (Dittmer and Kanost 2010). They have numerous biological roles that include lignification, delignification, pathogenicity, detoxification, morphogenesis, polymerization of melanin precursors (Faure *et al.* 1994), and many others (Hirai *et al.* 2004; Strong *et al.* 2011). Due to the variety of substrates, the use of available oxygen as the electron acceptor, and no requirement for cofactors, the interest of laccases has raised, especially to transform or degrade compounds which are found in nature or polluted soils and wastewaters (Majcherczyk *et al.* 1999; Strong *et al.* 2011).

Yoshida (1883) discovered laccase for the first time in plants, based on the observation of rapid hardening of the latex in the Chinese or Japanese lacquer trees (*Rhus vernicifera*) in the presence of air (Yoshida 1883). Isolation, purification and the active site of catalyst responsible for this enzyme was done by another scientist (Bertrand 1894). Consequently, a significant number of reports have been published in the past decades which have focused on new laccase enzymes in numerous other plant tissues (Bligny and Douce1983; Lehman *et al.* 1974), but because of difficulties to detect and purify them, laccases have not been extensively used or characterized (Ranocha *et al.* 1999). For many years, it was thought that the only sources of laccases are plants due to their capability of degrading lignin (Bao *et al.* 1993). Fungal counterparts have now been discovered in most basidiomycetes and ascomycetes as an alternative source for laccase in the late twentieth century (Leonowicz and Trojanowski 1978; Baldrian 2006). This finding led to the discovery of more multicopper enzyme with phenol oxidase activity in yeasts (Augustine *et al.* 2008), and some phenol oxidases laccase-like with enzymatic properties have been purified from insects (Yamazaki 1972). Recently, classes of laccases have been found widespread in bacteria (Claus 2004; Sharma *et al.* 2007). The first bacterial laccase was isolated from *Azospirillum lipoferum* (Givaudan *et al.* 1993; Diamantidis *et al.* 2000). Multicopper laccase-like enzymes

were identified in spores of *Bacillus sphaericus* (Martins *et al.* 2002), *Marinomonas mediterranea* (Sanchez-Amat *et al.* 2001) and *Streptomyces antibioticus* (Freeman *et al.* 1993). Laccases have also been isolated and characterized from *Streptomyces cyaneus* (Arias *et al.* 2003), *Streptomyces grizeus* (Endo *et al.* 2003), *Streptomyces lavendulae* (Suzuki *et al.* 2003), and *Streptomyces coelicolor* (Koschorreck *et al.* 2008).

The comparison of the molecule structures of laccases from different sources has confirmed the fact that laccases generally need four copper atoms as functional catalytic groups. There are three major types of copper in a laccase catalyst. Type 1(T1) is a blue copper with a maximum absorbance at 610 nm (ox) which corresponds to an intense blue colour. This absorption band is associated with the ligand–metal charge transfer from the sulfur atom of the cysteine ligand to the copper atom. This is the position where oxidation of the substrate takes place. The T2 centres containing conventional copper are similar to those observed for Cu (II) tetragonal complexes. The absorption spectrum of these centres frequently has a low intensity and is a non-blue copper. The T2 is connected by two histidines and one molecule of water. This position is very close to T3 and is connected by a strong hydrogen bridge. The T3 copper centre contains two copper ions bound to ligands and is called the binuclear site. The T3 sites are diamagnetic and exhibit an absorption band at 330 nm (Thurston 1994; Solomon 1996, 1998). From a mechanistic point of view, the Type 1 copper is oxidized by substrate. This electron will then be transferred from Type 1 to Type 2 and Type 3, and in the last step the oxygen molecule is reduced to water. However, in this reaction, laccase uses oxygen as the electron acceptor to remove the proton from the substrate (Gianfreda *et al.* 1999). The T1 copper is connected with two histidines and one cysteine as ligands. Many studies have claimed that this axial ligand has influences on the oxidation potential, specificity and stability of the enzyme, however the catalytic efficiency (*kcat*/*Km*) of laccase has been related to their redox potential Type 1 copper and plays a major role in the overall performance of these enzymes (Xu 1996a; Xu *et al.* 1996b) (Figure 1.3)

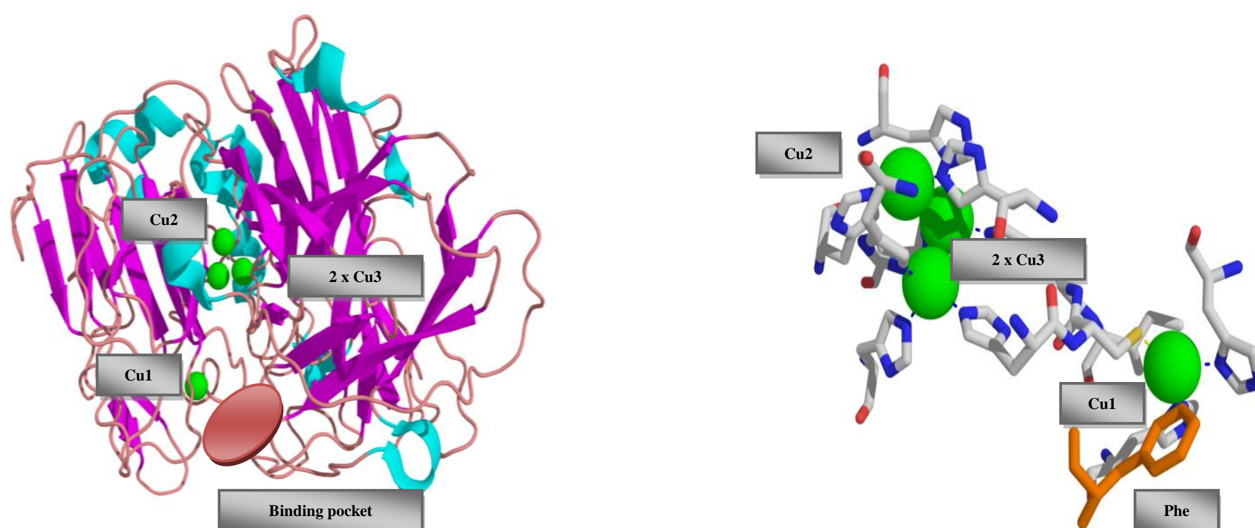

Figure 1.3 A) Ribbon model of the X-ray crystal structure from *T. versicolor* Lac C. B) Cu- atom structure which shows Phe is responsible for redox potential (adapted from Rodgers *et al.* 2003).

Generally, the redox potential is defined as the energy that is necessary to capture one electron to reduce substrate and produce a cation radical (Xu 1997; Lahtinen *et al.* 2009a). According to this definition and to compare laccase primary structures, laccases are divided in 3 major groups; laccases with higher redox potentials (730–780 mV; e.g. *Trametes versicolor*) occur when phenylalanine is the axial ligand of the enzyme (Shleev *et al.* 2005), while laccases with middle - range redox potential (470–710 mV; e.g. *Ganoderma sp.*) carry leucine in the axial position (Sharma *et al.* 2013), and lower redox potential laccases (340–490 mV; e.g. the bacterial CotA enzyme from *Bacillus subtilis*) have a methionine as the axial ligand (Rodgers *et al.* 2010; Enguita *et al.* 2013).

It is difficult to define laccase by its reducing substrate due to the wide range of compounds oxidized and substrate variation from one laccase to the other. This can generally be attributed to the high variation of the redox potential (E^0) of various laccase enzymes (Kunamneni *et al.* 2008a). Generally, the reaction mechanism of laccase is to produce a radical from one electron reaction which is bound to four other electrons. This radical is unstable and will be converted to a quinone in second step enzymatic-catalysed reaction (Thurston 1994). This intermediate radical can undergo for more non-enzymatic reactions via cross-linking or polymerization of covalently coupled bound such as C-C, C-O and C-N. This reaction leads to the formation of non-soluble

dimers, oligomers or polymers that can precipitate in the solution mixture (Gianfreda *et al.* 1999; Mayer *et al.* 2002; Kunamneni *et al.* 2008b). Many studies have reported that laccase is involved in the degradation of polymers and also in ring cleavage of aromatic compounds. The intermediate reactive radicals result in the cleavage of covalent bonds and release the monomers from polymers in degradation mechanisms (Kawai *et al.* 1998). In some cases, enzymes cannot be directly involved in the reaction due to steric hindrance of the substrate, however small molecules which are called mediators and normally can be oxidised by enzyme, will participate in the reaction and can promote or facilitate enzyme action (Elegir *et al.* 2005; Camarero *et al.* 2008). A mediator can participate in enzyme mechanism reaction via an electron-transfer mechanism, a radical hydrogen transfer route, or ionic oxidation (Riva 2006).

Fungal laccases have been studied for many decades because of their functions in many applications. Laccase can be used in beverage (wine, fruit juice and beer) processing, ascorbic acid determination, sugar beet pectin, gelatin, baking, as a biosensor and to improve food sensory parameters (Kunamneni *et al.* 2008b). Laccase is also well known for use in flavour application. Laccase from *Coriolus versicolor* can improve the flavour and taste of cacao by removing bitterness and unpleasant taste (Takemori 1992). The flavour and colour quality of many vegetable oils can be improved or modified by laccase eliminating dissolved oxygen. Oils, especially vegetable oils (e.g., soybean oil), are present in many food items and can be deoxygenated by laccase (Petersen *et al.* 1996). Conversion of benzyl alcohol to benzaldeyde has been reported by laccase catalysed oxidation of aroma compounds (Potthast 1996). Laccase produced nootkatone, an important flavour compound of grapefruit in the presence of synthetic mediators (Huang *et al.* 2001). Various enzymatic treatments have been reported to clear and stabilise drinks, such as fruit juice, beer and wine (Alper *et al.* 2004). For apple and grape juices excessive oxidation of phenols has been considered detrimental to the organoleptic quality of the product. These beverages are typically stabilised to delay the onset of protein-polyphenol haze formation. In 2004, laccase from *Myceliophthora thermophila* was expressed in *Aspergillus oryzae* for the use of brewing beer and prevent the formation of off- taste compound such as trans-2-nonenal with scavenging the oxygen. In this reaction, oxygen reacts with fatty acid, proteins and alcohol and produces some precursors of off-flavour compounds (FAO 2001). Adding laccase to dough for baked products increased the strength of the gluten structure and has been used in industry in recent decades (Arnaut *et al.* 2006; Si 1994). Improvement of

rheological characteristics of wheat bread by laccase and tyrosinase has been investigated (Selinheimo *et al.* 2006 & 2007).

1.3.2.2 Hydrolases

Most of the enzymes which are commercialised in food and flavour industry belong to the class of hydrolases (Belitz *et al.* 2009). Hydrolases are a group of enzymes which catalyse reactions using water as a co-substrate to cleave a chemical bond. The hydrolases are classified according to the bond which can be hydrolysed. Most of the enzymes of this category are esterases, lipases, peptidases and glycosidases (Polaina and MacCabe 2007).

1.3.2.2.1 Lipases

Lipases (EC 3.1.1.3) are a group of enzymes that catalyse the hydrolysis of fatty acid esters under controlled conditions and the synthesis of esters in organic solvents (Anthonsen *et al.* 1995). Lipases are able to catalyze many reactions based on a phenomenon called interfacial active site; it means that high catalytic activity is observed only in the presence of a hydrophobic phase, a lipid droplet dispersed in water or an organic solvent. This phenomenon has been related to the presence of a hydrophobic lid or flap covering the entrance to the active site (Adlercreutz 2014). Lipases have higher activity when there is organic solvent and a small amount of water in the reaction conditions (Rueda 2005). They are greatly different as regards both origins and their properties, and can catalyse both reverse reaction to synthetise esters or to exchange acyl groups and catalyse transesterification among different acylglycerols, alcohols, esters and amines (Gupta *et al.* 2004). Because of catalytic versatility, lipases have a wide range of industrial applications and are the largest group in terms of total sales (Hasan *et al.* 2006). The catalytic versatility, chemoselectivity and regioselectivity, availability on a large scale, cofactor-independence and finally no side reactions make lipases interesting and highly applicable in industry (Jaeger and Eggert 2002).

In 1856 lipases were discovered to be produced by the pancreas and form part of the digestive process in humans (Peterson and Drabløs 1994). Initial interest in microbial lipase has increased

because of lack of pancreas and difficulties in collecting materials. Since then, the industrial demand of new sources of lipases has led to identify and isolate many different lipases from bacteria, fungi, plants, and animals (Jaeger *et al.* 1999). Lipases are provided in living organisms from bacteria to Eukarya, including animals, plants and fungi. The bacterial sources showed high stability in organic solvents (Olson *et al.* 1994). Examples of stable bacterial lipases in organic solvent are lipases from *Bacillus sphaerichus* and *Candida antarcita* which are used in many industrial applications (Escorcia *et al.* 2013).

The first 3D-structures of lipases were solved for a lipase from pancreas and a fungal lipase from *Rhizomucor miehei* (Jaeger *et al.* 1994 and 2002). After analysing structures of many lipases, it was clarified that all lipases have the same folding pattern, although they do not necessarily have a similar sequence (Fan *et al.* 2008). The X-ray structure of hydrolytic enzymes was shown for the first time by Ollis in 1992. He reported that all lipases have the α/β hydrolase folding pattern. The α/β hydrolase fold consists of eight standard β-sheets which are connected by six α-helices. The active site of lipases presents serine, aspartic or glutamic and histidine residues (Schmid and Verger 1998; Nardini and Dijkstra 1999) (Figure 1.4). In general, lipase is a polypeptide chain folded into two domains, the C-terminal domain and the N-terminal. The N-terminal domain contains the active site with a hydrophobic tunnel from the catalytic serine to the surface that can accommodate a long fatty acid chain (Petersen *et al.* 2001).

The reaction mechanism of lipases is defined as hydrolysis of ester bounds with the consumption of water. In this reaction the serine in the active site of the enzyme is attacked by the carbonyl carbon of the ester bond leading to the formation of the first intermediate product or acyl-enzyme. Then, the acyl-enzyme intermediate is hydrolysed by water and a carboxylic acid is formed as product (Adlercreutz 2013). The catalytic activity of each lipase in organic media is depended on different factors, such as pH, the concentration of water, the method of enzyme preparation and the kind of solvent (Schmitke *et al.* 1996). In addition to the reactions mentioned above, lipases catalyse the reverse hydrolysis reaction, (= esterification), transesterification (acidolysis, interesterification, alcoholysis), aminolysis, oximolysis and thiotransesterification. The equilibrium between the forward (hydrolysis) and the reverse (synthesis) reactions is controlled by the water activity of the reaction mixture (Aldercreutz 2013).

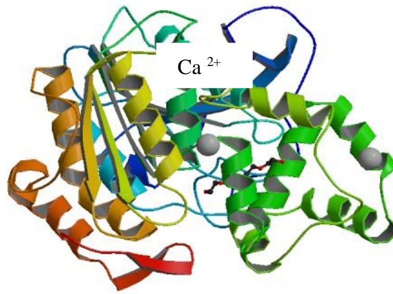

Figure 1.4 X-ray structure of *Candida antarctica* lipase A (Source: Erricson *et al.* 2008)

Since lipases have a wide range of substrate specifity and enantioselectivity for esterficiation and transesterification reactions, they are widely used in food applications and especially in the synthesis of aroma compounds (Gupta *et al.* 2004). Lipases are also used to give special flavour to food by synthesis of fatty acids and alcohols, which are accepted as flavour and fragrance compounds (Gandhi 1997). Lactones as well-known flavour compounds can be produced by transesterification of 4-hydroxy carboxylic esters with more than 80 % yield (Lutz *et al.* 1992). The first application of a lipase on industrial scale was to lipolyse milk fat. For example, *Mucor miehei* lipase has a high selectivity towards short chain fatty acids, such as butanoic, hexanoic and decanoic acid. Lipolysed milk fat can serve as cream butter flavouring agent (Schreier 1997a). Methyl benzoate has a kind of exotic fruits and berries flavour. The aroma compound was produced by esterification of benzoic acid by *Candida rugosa* (Leszczak and Trasn-Minh 1998). In the dairy industry, there are many other applications of using lipase, such as improvement of flavour in cheeses, accelerating cheese ripening and the production of cheese-like products. Addition of lipases to such products primarily releases short-chain (C4 and C6) fatty acids which have a sharp flavour, but the release of medium- chain (C12 and C14) fatty acids leads to formation of a smoother taste (Saxena *et al.* 1999). Methyl butanoate and methyl butyl esters which are essential flavour compounds in fruit flavours can be generated by lipases. Immobilised lipase from *Mucor miehei* can be used to produce fruity flavours by direct esterification of isoamyl alcohol and isovaleric acid to produce isoamyl isovalerate (Chowdary *et al.* 2000). *Stillingia* oil can be converted to ethyl (2*E*,4*Z*)-decadienoate, the impact compound of Williams pear flavour by transesterification (Gatfield *et al.* 1999). Sweet fruity banana apple

grassy flavour which is defined by (Z) -3- hexenyl acetate can be synthesised by lipase from *Candida antarctica* or using immobilised lipase of *Mucor miehei* in hexane (Schrader 2004). The alkamide *cis*-pellitorine [(2*E*,4*Z*)-*N*-isobutyldeca-2,4-dienamide] which occurs naturally in tarragon, can be produced with high yields up to 80 % by lipase-catalysed conversion of ethyl-(2*E*,4*Z*)-decadienoate which is the pear ester (Ley *et al.* 2004). Fatty acids, such as racemic 4-methyloctanoic acid in cheese from sheep and goat milk, which is responsible for the special flavour in this product, can be selectively esterified with ethanol with the help of a lipase from *Candida antarctica* (Franssen *et al.* 2005).

Modification of lipids (oils and fats) is an important process in the food industry. Some moieties can be added or deleted from triacylglycerols by lipase catalysed specific reactions. Also, several esterification and transesterification reactions can be carried out by lipases to form modified lipids. Cheap oils may be converted to nutritionally or technologically modified triacylglycerols, such as cocoa butter substitutes, triacylglycerols with low caloric content and oils containing high amounts of oleic acid (Hasan *et al.* 2006).

The use of lipases for a variety of biotechnological applications, especially flavours and fragrances, is rapidly increasing. Many novel lipase genes have been identified and enzymes with new properties were discovered. In parallel, the combination of optimised lipases with improved reaction conditions will lead to novel synthetic routes, allowing the production of high-value chemicals components (Hasan *et al.* 2006).

1.3.2.2.2 Esterases

Esterases catalyse the hydrolysis and synthesis of ester bonds. Substrate acceptance variety, high stability, enantioselectivity and the fact that they do not require cofactors make them attractive biocatalysts for organic synthesis (Bornscheuer 2002). Esterase and lipase have the same catalytic mechanism, whereas the lipases display high activity towards the aggregated state of the substrate, the esterases typically show highest activity towards the soluble state of the substrate (Fojan *et al.* 2000). The mechanism starts with the attack of the active site of the enzyme on the carbonyl group of the substrate ester. This ‘chemical operator’ can be the hydroxyl group of a serine for instance, or, in the case of porcine liver esterase, a carboxyl group of an aspartic acid. The reaction will be continued with the formation of an acyl enzyme complex, respectively.

Finally, the reaction will be ended by releasing of the acid (Greenzaid and Jenckst 1971; Fojan *et al.* 2000). The esterase mechanism is different from the lipase mainly on the basis of substrate specificity, protein structures and biological functions (Long 1971; Pleiss *et al.* 1998). It appears that the physical state of the substrate is most likely a contributing factor towards the substrate specificity. Long-chain fatty acids are typically insoluble or at least poorly soluble (emulsions). Thus the lipase has to be capable of identifying an insoluble or heavily aggregated substrate. Since lipases are active towards aggregated substrates, lipase activity is directly correlated with the total substrate area, and not with the substrate concentration. Esterase activity is found to be highest towards more water soluble substrates (Fojan *et al.* 2000).

Esterases have been isolated from plants, animals, fungi and other microorganisms. The most applicable esterases are from microbial sources because of low cost of growing and maintaining the process (Gupta *et al.* 2012). Esterase from *Fusarium oxysporum* was able to produce flavour and fragrance compounds, such as geranyl esters by direct esterification of alcohol and acid (Chaabouni *et al.* 1996). In 1996, Ostdal discovered that the flavour of fermented meat will be improved by an esterase from *Pedicoccus pentosauces* (Ostdal *et al.* 1996). An esterase from *Saccharomyces cerevisiae* synthesised isoamyl acetate which is the major and important flavour of sake from isoamyl alcohol (Fakuda *et al.* 1998). Short chain fatty acids esters are commonly used in the manufacturing of flavours and fragrances because of their fruity odour. They are provided from nature by extraction or using chemical synthesis, but they can also be produced enzymatically by inverse enzymatic hydrolysis in organic solvents. An esterase from *Bacillus licheniformis* was used for ethyl ester synthesis in *n*-heptane by direct esterification of fatty acids. The highest reaction rates and yields were with mid-chain length fatty acids (Alvarez-Macarie and Baratti 2000). A feruloyl esterase from *Aspergillus niger* catalysed the synthesis of pentylferulate which is an important compound as flavouring agent in water-in-oil microemulsion systems (Giuliani *et al.* 2001). Feruloyl esterases (FAEA) are a subclass of the carboxylic acid esterases (EC 3.1.1.1.). They hydrolyse ester bonds including hydroxycinnamates from plant derived material (Haase-Aschoff et al. 2013). Isoamyl acetate as strong banana flavouring compound occurred *via* esterification of the alcohol by esterases from *Acetobacter* spp. (Kashima *et al.*, 2000). Using esterase in dairy application is known for many years. In this application, esterase from *Bacillus casei* was used as transesterification catalyst to improve the milk fat in cheese production (Choi and Lee 2001). Esterases hydrolysed methyl

esters, such as ferulic acid, sinapic acid and caffeic acid. Feruloyl and cinnamoyl esterases of *Aspergillus niger* released hydroxy cinnamic acids from wheat bran (Ashter 2002).

1.4 Target product

1.4.1 Vanillin

1.4.1.1 General features

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is the major compound of vanilla flavours which are extracted from *Vanilla planifolia* with a concentration of 1-2 % in cured vanilla pods and extensively used in food, cosmetic and pharmaceuticals industry (Berger 2007). It is a flavouring compound belonging to the C6–C1 phenolic compounds and structurally is a phenol substituted with an aldehyde and a methoxy group (Figure 1.5) (Müller *et al.* 1998; Sinha *et al.* 2008). In addition to its use as a fragrance ingredient in perfumes and cosmetics, vanillin has become an important deodorant to mask unpleasant odours of medicines, cleaning products, and many manufactured goods, such as paper products, plastics, rubber goods, etc. In several studies, antioxidant, antimicrobial, antimutagenic and anticarcinogenic activities of vanillin were published (Durant and Karran 2003). Some relevant physical properties of vanillin are shown in Table 1.1. Vanilla pods are mainly grown in south-eastern regions of America and some parts of Asia, such as Indonesia. Isolated vanillin occurs in the form of white needle-like crystalline powder with a pleasant aromatic vanilla odour and an intensively sweet taste, which are the main reasons for its widespread demand. In terms of toxicity, vanillin does not present any particular risk for humans (Clark 1999).

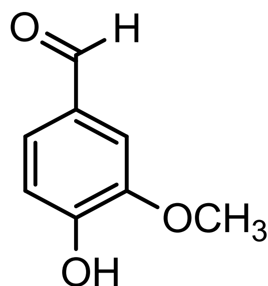

Figure 1.5 Vanilla bean and chemical structure of vanillin molecule.

Table 1.1 Physical properties of vanillin

Physical properties of vanillin	Value	Reference
Melting point	81-82 ºC	Perry, Green and Maloney, 1997
Boiling point at 760 mmHg	285 ºC	Perry, Green and Maloney, 1997
Density at 20 ºC	1.056	Perry, Green and Maloney, 1997
Water solubility at 25 ºC	10 g/l	The Merck Index, 2001
Vapour pressure at 25 ºC	2.2×10^{-3} mmHg	Perry, Green and Maloney, 1997
Heat of solution in water	-21.8 kJ mol^{-1}	Washburn, 2003
pH	4.3	http://www.chem.unep.ch

1.4.1.2 Production routes

The methods to obtain vanillin can be divided in three main classes: natural, chemical synthesis and biotechnological routes.

1.4.1.2.1 Natural route

The most relevant natural source of vanillin is the beans, or pods, of the tropical *Vanilla* orchids, principally the *Vanilla planifolia* species (Walton *et al.* 2000). The flowers of this plant have a

closed structure that makes selfpollination almost impossible. Artificial pollination is the only solution, and to obtain reasonable yields it is manually made, which is a very laborious task which discourages the cultivation of these plants on a larger scale (Rao and Ravishankar 2000). Vanillin was first isolated in 1858 by Gobley after recrystallization of vanilla extract. Less than 20 years after its initial isolation, synthetically produced vanillin was marketed (Hocking 1997). Vanillin is found in trace amount in many other plants, but just two species, *Vanilla planifolia* and *Vanilla tahitensis*, are allowed for food application (Clark 1999). The cured vanilla beans contain about 200 components, where the most abundant aromatic is vanillin with 2 % of the dry matter, followed by *p*-hydroxybenzaldehyde and vanillic acid with 0.2 % and 0.1 %, respectively (Rao and Ravishankar, 2000).

The curing process is an important stage to gradually develop flavour which requires special growing of plant and fermentation at the elevated temperature. Vanillin which is linked to glucose molecules in the green pods separates from the sugar moiety during the curing process, and the vanillin β -D-glucoside is enzymatically hydrolysed to give glucose and vanillin as free molecules (Figure 1.6) (Converti *et al.* 2010). The exact pathway of vanillin from vanilla bean is still not clear. In between, the shikimic acid pathway is the agreed pathway in all literatures. Zenk reported that vanillin was obtained by ferulate pathways and ferulic acid was more responsible for vanillin production (Zenk 1965), while Kanisawa claimed that 4-coumaric acid is the main precursor for *p*-hydroxybenzaldehyde glucoside, the central intermediate for the biosynthesis of the glucosides (Kanisawa *et al.* 1994). This hypothesis was named the benzoate pathway and was followed by hydroxylation and methoxylation of the aromatic ring. Figure 1.7 shows the possible pathway of vanillin which can be formed through harvesting and curing of vanilla beans with the benzoate hypothesis pathway. During harvesting, conversion of hydroxycinnamic acids to their coenzyme A esters with subsequent chain-shortening by a process analogous to NAD-dependent β-oxidation of fatty acids, is leading to a benzoic acid (Podstolski *et al.* 2002).

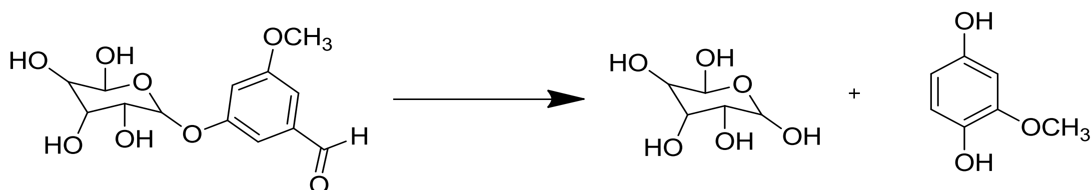

Figure 1.6 Hydrolytic liberation of vanillin during ripening (Converti *et al.* 2010)

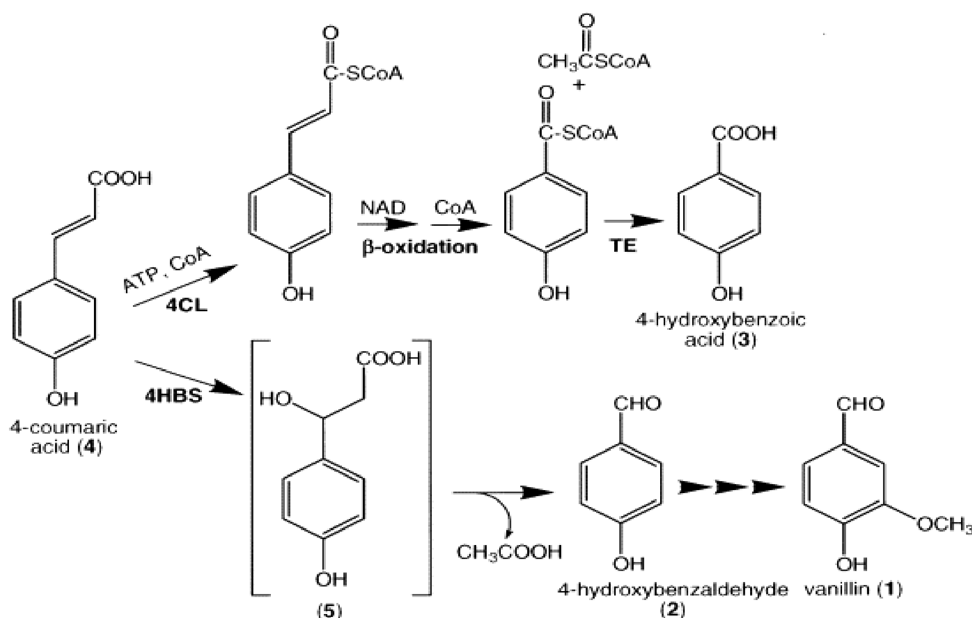

Figure 1.7 Benzoate pathway of vanillin production during harvesting the *vanilla planifolia* (Podstolski *et al.* 2002)

Less than 1% of the market (20-50 ton per year from total demand of market 15,000 ton in 2010) comes from vanilla beans as a natural source (Krings and Berger 1998; Gallage and Moller 2015). The market price of vanillin from vanilla beans is 300 times higher than that of the synthetic one (Muheim and Lerch 1999).

1.4.1.1.2 Chemical synthesis route

Limited natural sources, condition of curing process, high and variable cost of natural vanillin and large consumption in many applications were factors which encouraged the industry to find other routes, such as chemical synthesis, biotechnology and conversion of related natural products for production of vanillin. The main portion of industrial demand of vanillin is covered by chemical synthesis from lignin, the glucoside of coniferyl alcohol (4- hydroxy-3-

methoxycinnamyl alcohol), guaiacol (2-meth-oxyphenol) and eugenol (4-allyl-2-methoxyphenol) (Rao and Ravishankar 2000). One of the potential sources for the synthesis of vanillin was eugenol, but nowadays, this process has only historical interest, as synthetic vanillin is produced, on a commercial scale, from either the petrochemical guaiacol or from lignin (Van den Heuvel *et al.* 2001). In the industrial process, the condensation of the glyoxyl radical with the aromatic ring of guaiacol occurs, in alkaline media, almost entirely in the *para* position in the phenolic hydroxide group. Crude vanillin is then obtained by oxidation, acidification and simultaneous decarboxylation of vanillyl mandelic acid. The vanillin is formed with very little by-products, simplifying the subsequent separation procedure, which is an advantage for the production process. However this process is dependent on petroleum derived compounds, such as guaiacol, in opposition to the biomass based lignin oxidation process.

Alternatively to this guaiacol based process, lignin can be used as raw material to produce vanillin. Lignin is an important biomass component which finds its major industrial source as a by-product stream of pulp and paper mills, called black liquor. This vanillin synthesis route consists of treating an aqueous solution of lignin with oxidants, at very alkaline pH, and high temperatures and pressures. These oxidants can be air, oxygen, nitrobenzene or metallic oxides, with or without the help of catalysts (Mathias 1993). Lignin is degraded and oxidised, producing vanillin along with other by-products. Besides lignin fragments, typical compounds which can be observed are vanillic acid, acetovanillone, syringic aldehyde, syringic acid, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid, 5-formylvanillin, dehydrodivanillin and dehydrodivanillic acid (Bjørsvik and Liguori 2002). The presence of these contaminants with chemical structures close to vanillin requires more intensive purification procedures, as compared to the guaiacol process. These procedures have a determinant role on the economical competitiveness of the lignin-based process, and breakthroughs in this field will lead to major positive impacts to its expansion in the industrial panorama (Mathias 1993).

1.4.1.1.2 Biotechnological route

The principal biotechnological methods for production of natural vanillin consist of using microorganisms, plant cell cultures or enzyme extracts. Biotechnological approaches for

producing vanillin have been explored with plant cell cultures of *Vanilla planifolia* and *Capsicum frutescens*, but no feasible economical end product was detected. To improve the biotransformation rate, α-cyclodextrin and cell cultures immobilised in alginate were used, but still it was difficult to compare with the microbial process (Suresh *et al.* 2003). The inherent problem to produce vanillin with plant tissue culture was slow growth rate and low yields of the desired products. In the next generation, several precursors, such as isoeugenol, eugenol, phenolic substrates, lignin, ferulic acid, curcumin and aromatic amino acids were investigated for the production of vanillin (Benz and Muheim 1996; Converti *et al.* 2010). Phenylpropanoids, such as eugenol and isoeugenol, which are the main components of clove essential oil, served as potential and inexpensive substrates for the production of valuable aromatic compounds. The first biotransformation of isoeugenol to vanillin was discovered using *Aspergillus niger* in low yield (Abraham *et al.* 1988). The pathway of production of vanillin from eugenol with a strain of *Pseudomonas* is shown in Figure (1.8). The initial step of eugenol degradation was confirmed to be the double-bond-transferring hydroxylation catalysed by eugenol dehydrogenase (Rabenhorst 1996).

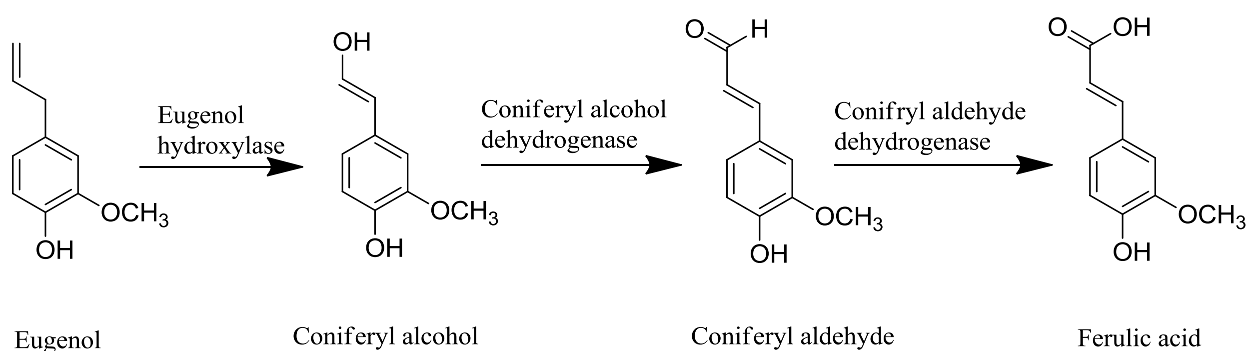

Figure 1.8 Conversion of eugenol to ferulic acid in *Pseudomonas* sp. strain HR199 (Walton *et al.* 2000)

Other microorganisms were found to transform isoeugenol to vanillin, such as *Pseudomonas* sp., *Fusarium solani* and *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas paucimobilis* (Priefert *et al.* 2001). Isoeugenol was also reported as a potential substrate for production of vanillin with isolation of a *Bacillus subtilis* with the molar yield of 12.4 % (Shimoni *et al.* 2000). The recent research in eugenol-degrading microorganisms attempted the conversion of eugenol to vanillin using cells of *Bacillus species* strain BR, with a molar conversion yield of 15.6 % (Sindhwani *et al.* 2012). Industrially applicable microorganisms which produced vanillin from eugenol and isoeugenol are summarised below. But the fact that

they are synthesised from plants and they caused toxicity and side product formation led to look for other alternative sources for the bioconversion to vanillin.

Table 1.2 Industrial microorganisms for production of natural vanillin from eugenol and isoeugenol

Substrate	Microorganism	Incubation (h)	Yield (g/l)	References
Eugenol	*Pseudomonas sp*. HR199	200	2.6	Overhage *et al.* 2000
	Amycolatopsis sp. HR167	32	>10	Overhage *et al.* 2006
Isoeugenol	*Bacillus fusiformis* SW-B9	72	32.5	Zhao *et al.* 2005
	Recombinant *E. coli* BL21 (DE3)	6	28.3	Yamada *et al.* 2007
	P. putida	24	16.1	Yamada *et al.* 2007
	Psychrobacter sp. strain CSW4	-	1.28	Ashengroph *et al.* 2012

The second and most important precursor for biosynthesis of vanillin is ferulic acid which is widely available in many cereals. In plants ferulic acid is synthesised starting with phenylalanine or tyrosine (Dewick 1989). There are several reports to explain the pathway of ferulic acid based on cell suspension cultures of *Vanilla planifolia* (Funk and Brodelius 1990), immobilised cell cultures of *Capsicum frutescens* (Ramachandra and Ravishankar 2000), and organised aerial roots of *V. planifolia* (Westcott *et al.* 1994). A two-step bioconversion of ferulic acid to produce natural vanillin was investigated. The first step was formation of vanillic acid from ferulic acid by a strain of *Aspergilus niger*, and the second step was the oxidation of vanillic acid to vanillin by a laccase from *Pycnoporus cinnabarinus* (Figure 1.9) (Lesage-Meessen *et al.* 1996). To improve the production yield of vanillin more studies have been done using culture of *Pycnoporus cinnabarinus* in glucose-phospholipid medium, and the result was the production of 760 mg L^{-1} vanillin from ferulic acid (Oddou *et al.* 1999). Two strains of *Amycolatopsis* and *Streptomyces setonii* have been identified for the production of vanillin with a yield of 11.5 g L^{-1} (Muheim and Lerch 1999). There are different approaches for the bioconversion of ferulic acid to vanillin. The easiest pathway was decribed by Priefert (2001) which consisted of a non-oxidative decarboxylation side chain reduction, coenzyme-A-independent deacetylation, and

coenzyme-A-dependent deacetylation (Priefert *et al.* 2001). Today, ferulic acid for natural vanillin is obtained from the by-product rice bran by enzymatic treatment, but the raw material for production of natural vanillin in this way is very costly. Many different microorganisms were investigated for the conversion of ferulic acid to natural vanillin, and some of them are already used as industrial sources (Table 1.3). Among all different microbial strains which were investigated for production of natural vanillin, the highest productivity (>11g per litre in 30 h) was found using an actinomycete of the *Amycolatopsis* family (Rabenhorst and Hopp 2000). Altough ferulic acid was known as a potential source for the production of natural vanillin by microbial strains, the high price of ferulic acid was a limiting factor (Priefert *et al.* 2001).

Substrate	Microorganism	Incubation (h)	Yield (g/l)	References
Ferulic acid	*Streptomyces setonii*	23	>10	Achterholt *et al.* 2000
	Streptomyces sp. V-1	55	19.2	Hua *et al.* 2007a
	Aspergillus niger and *Pycnoporus Cinnabarinus*	72	2.8	Zheng *et al.* 2007
	Recombinant *E. coli*	24	5.14	Lee *et al.* 2009
	Amycolatopsis sp. ATCC 39116		13.9 g L^{-1}	Fleige *et al.* 2013

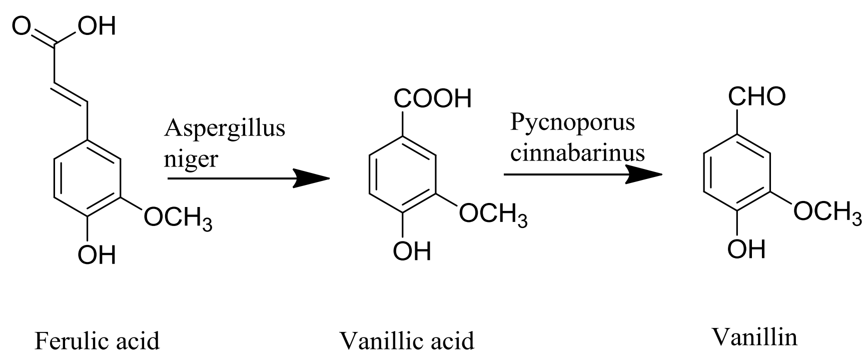

Figure 1.9 Microbial routes to vanillin. A two-step process from ferulic acid using the filamentous fungi *Aspergillus niger* and *Pycnoporus cinnabarinus* (Lesage-Meessen *et.al* 1996).

As well as for the major precusors shown above, many studies have reported the use other phenolics, such as stilbenes, siam benzoin and curcumin as potential substrates (Benz *et al.* 1996). Lignin has been investigated as one of the best natural sources for aromatic compounds

for many years, but vanillin production from lignin on an industrial scale is only possible with chemical synthesis (Kirk and Farrell 1987). Different studies have explored the degradation of lignin by biological methods with the aim of natural product formation. White-rot fungi are the most abundant tools to degrade the lignin polymer and to yield vanillin but only in trace amounts. Characterization of the enzymes resulting this pathway is still incomplete (Clark 1990; Priefert *et al.* 2001). Biotransformation of vanillic acid which is an intermediate in the conversion of aromatic amino acids, such as phenylalanine, was investigated as the starting point of flavonoids, coumarines, stilbenes, and lignin biosynthesis in plants and also synthesis of vanillin from glucose by different microbial and enzymatic methods. None of these phenylpropanoides could produce considerable amounts of vanillin on an industrial scale (Krings *et al.* 1996; Priefert *et al.* 2001). Methylguaiacol, obtained from creosote, can be converted to vanillin *via* vanillyl alcohol oxidase from *Penicillium simplicissimum* (Van den Heuvel 2001). Biotransformation of rice bran oil by *Aspergillus niger* and *Pycnoporus cinnabarinus* was reported as sucessful method for the production of natural vanillin with a high molar yield (Zheng *et al.* 2007). Bioconversion of glucose to vanillin has been reported. The mechanism was suggested to be done by soil bacteria from the *Pseudomonas* strains, although no evidence for the direct conversion of glucose to vanillin was given (Ryu *et al.* 2012). A recombinant *S. cerevisiae* was the only commercialised strain for the production of vanillin during 24-168 h with the yield of below 0.5 g L^{-1} from glucose (Hansen *et al.* 2013).

Curcumin is one of the possible precusors for vanillin production. Curcumin is a natural phytochemical which is obtained from the dried root of turmeric. It has functions as a spice, a colouring agent, a highly promising antioxidant, an anti-inflammatory agent and an anticoagolant properties (Roughley and Whiting 1973; Tønnesen and Karlsen 1985, 1986). Because of some similarity in the structure of curcumin to lignin compounds, there is high interest of the degradation of curcumin with the same microorganism for lignin degradation (Musuda *et al.*1999, 2002). In the structure of curcumin there is double bond in the molecule which can also be cleaved to form two or more smaller molecules. Cleavage is accomplished by a small number of reaction sequences, such as ozonolysis. Hydrolysis of curcumin in a water solution at appropriate temperature and pressure led to the production of vanillin (Dolfini *et al.* 1999). Degradation of curcumin was reported in the buffer solution before. The result confirmed that curcumin, under basic incubation, will degrade to many products. In this hypothesis, when the

pH is netural, proton transfer from phenolic group leads to the destruction of the molecule of curcumin, while in acidic conditions curcumin has more stability. Vanillin, vanillic acid, ferulic aldehyde and ferulic acid were the major degradation products in this assay, but the molar yield of conversion products was not reasonable (Wang *et al.* 1997). The biomimetic oxidation of curcumin confirmed the fact that vanillin and ferulic acid were the most abundant products after incubation with hydrogen peroxide catalysed by 5, 10, 15, 20-tetraarylporphyrinatoiron (III) chlorides for 24 hours with molar yields of 2.8 % for vanillin and 1.9 % for ferulic acid (Chauhan *et al.* 2003) . There are also reports which show that vanillin and ferulic acid can be produced from curcumin using UV irradiation (Khurana and Ho 1988; Tønnesen *et al.* 1986), a radical reaction (Masuda *et al.* 2002) or transformation of curcumin in 5 % (w/v) NaOH which yielded 76 % vanillin (Roughley and Whiting 1973). In all reports, the concentration of the final product vanillin was low.

1.3.3 Divanillin

Divanillin is a compound in vanilla pods which occurs naturally during the harvesting process from green vanilla pod to the fermented brown or black pod. The occurrence of divanillin in vanilla is limited to trace amounts in the range of 10 to 100 ppm dry matter according to the country of origin (Freudenberg and Renner 1965). Divanillin can be detected after the chemical or enzymatic degradation of wood (Lahtinen *et al.* 2009b). Various applications of divanillin as an antioxidant, skin lightening agent and fixative in perfume oils have been described in the literature (Ikemoto *et al.* 1995). In 2006, a patent claimed the usage of divanillin as taste enhancer in some milk and ready to eat products (Reiss *et al.* 2006).

Divanillin is commonly produced by different chemical methods. It has been synthesised by oxidative phenol coupling using iron (II) chloride ($FeCl_3$) or iron (II) sulfate ($FeSO_4$). In 1885 the first synthesis of divanillin was discovered by oxidation of the *ortho* position of phenol groups. The yield of resulting divanillin after precipitation and filtration was 57 % (Tiemann 1885). Some other chemical syntheses, such as using potassium/sodium persulfate were published. In this method, vanillin was converted to divanillin by oxidation with an iron (II) sulfate heptahydrate and sodium peroxodisulfate in hot water (Elbes and Lerch 1916). Enzymatic synthesis of divanillin with the aim of natural has been described in 1972. Divanillin was

produced after oxidation of vanillin in aqueous solution with the aid of peroxidases and hydrogen peroxide (Baumgartner and Neukom 1972). The reaction involves splitting of the H_2O_2 peroxide bond to form two hydroxyl radicals as the initiation step. The radical reaction is then propagated in various ways to form other reactive oxygen species. This is another free radical step as the free radical is essentially transferred to vanillin. The most stable location in the vanillin structure for the resonating electron to populate will be the 5-position *ortho* to the original phenol. Two vanillin free radicals will combine in a termination step (both free radicals are consumed), whereby a covalent bond is formed via both 5 positions of the vanillin molecules. Thus, 5,5'-divanillin is formed. Similar free radical chemistry exists with capsaicin (Akinchan 2003).

1.4.3 Lignan

Lignans are a large class of secondary metabolites found widely in several parts of plants, e.g. roots, stems and fruits. Many lignans occur as optically pure enantiomers or mixtures of enantiomer pairs (Saleem *et al.* 2005). The term lignan was first defined by Haworth in 1936 as a class of compounds derived from two β-β'-linked phenylpropanoid (C6C3) units. The dimerization of two or more C6C3 units can occur in a variety of ways which may or may not include the incorporation of oxygen (Umezawa 2003; Calvo-Flores *et al.* 2015). Formation of lignans in nature is achieved by the one electron-oxidation of the phenol groups. This one electron oxidation allows delocalization of the unpaired electron, giving resonance forms in which the free electron places in *ortho* and *para* positions (Suzuki and Umezawa 2007). In 1933, Erdtman discovered the formation of a dimer in the oxidative coupling reaction of phenolic compounds in isoeugenol as the model compound (Erdtman 1933). Freudenberg developed this idea by using coniferyl alcohol as the lignin precursor and the so-called dehydrogenation polymers (DHPs) were obtained by using enzymes or inorganic oxidants (Freudenberg 1968). Lignin chemistry and research have developed on many fronts including the findings of phenylpropanoid pathways and other biosynthetic routes to lignans and lignin. In natural sources, lignans typically occur conjugated with fibrous constituents in the plants. However, to use these compounds in pure form, isolation by chemical and enzymatic methods is necessary (Dewick 2002). Because of the presumed biological activity of lignans and their potential in pharmaceutical properties, syntheses of podophyllotoxin and related compounds as well as other

lignans have been studied intensively (Ward 1992). The antitumor activities of several products of the dihydrobenzofurantype lignans have also been demonstrated (Pieters *et al.* 1999). Many of these lignans used for the production of pharmaceuticals are extracted and purified from plants and modified then chemically to the end-products (Jin *et al.* 2006). Beside of the other biological activities of lignans, such as antimitotic, anti-HIV, anti-Alzheimer and antiviral in medicine and pharmaceutical application (Lee and Xiao 2004), the application of some of these dimers as taste modifiers in foods and beverages was investigated (Backes *et al.* 2014). The concentrations of lignans which are present in natural sources are not sufficient in order to have appropriate masking effects on bitter, stringent and metallic off-tastes in food stuffs. However, new biotechnical routes to the production of lignans with the use of biocatalysts may be an interesting topic (Zoia *et al.* 2008).

1.5 The aim of the work

The aim of this study:

- Screening suitable enzymes from fungi for enzymatic production of vanillin from curcumin

  - To overcome cross-linking of curcumin degradation products, by enzymatic acetylation of curcumin
  - To determine laccases from selected basidiomycetes with different redox potential for the cleavage of the double bound in the molecule of curcumin with the aim of higher production yield
  - To determine suitable esterase from basidiomycetes with the aim of vanillin production

- Oxidative coupling reaction of phenolic substrates with the aim of production of divanillin and lignans as taste modifier

  - To determine the effect of catalysts. Three different laccases and two peroxidases were compared with the aim of increasing the yield of products

  - To identify structure of dimers and trimers which are produced in the oxidative coupling reaction of phenolic substrates

  - To increase the yield of end products by working on number of factors, such as redox potential of laccases and substrates, incubation temperature, the presence of organic solvent in the reaction and the pH optimum for the reaction

2. A Three-Enzyme-System to Degrade Curcumin to Natural Vanillin

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2.1 Abstract:

The symmetrical structure of curcumin includes two 4-hydroxy-3-methoxyphenyl substructures. Laccase catalyzed formation of a phenol radical, radical migration and oxygen insertion at the benzylic positions can result in the formation of vanillin. As vanillin itself is a preferred phenolic substrate of laccases, the formation of vanillin oligomers and polymers is inevitable, once vanillin becomes liberated. To decelerate the oligomerization, one of the phenolic hydroxyl groups was protected via acetylation. Monoacetyl curcumin with an approximate molar yield of 49 % was the major acetylation product, when a lipase from *Candida antarctica* (CAL) was used. In the second step, monoacetyl curcumin was incubated with purified laccases of various basidiomycete fungi in a biphasic system (diethyl ether/aqueous buffer). A laccase from *Funalia trogii* (LccFtr) resulted in a high conversion (46 % molar yield of curcumin monoacetate) to vanillin acetate. The non-protected vanillin moiety reacted to a mixture of higher molecular products. In the third step, the protecting group was removed from vanillin acetate using a feruloyl esterase from *Pleurotus eryngii* (PeFaeA) (68 % molar yield). Alignment of the amino acid sequences indicated that high potential laccases performed better in this mediator and cofactor-free reaction.

Keywords: vanillin; curcumin; lipase; laccase; esterase

2.2 Introduction

Flavours and fragrances originate from traditional extraction or distillation of plant and animal sources or from chemosynthesis, but the quality and the stability of the natural supplies are sometimes limited. Effective law in Europe (EG 1334/2008) and in the U.S. (Code of Federal Regulation, Title 21) defines flavours with the preferred label 'natural' as compounds obtained by physical, enzymatic or microbiological processes. As this disqualifies chemical synthesis, biotechnological approaches have moved into focus (Berger 2015). Biocatalysis represents an economic alternative using either intact cells or isolated enzymes, such as laccases (Mayer and Staples 2002), often resulting in the formation of products difficult to obtain by conventional chemical means. Enzymes possess a long history of safe use in producing fermented foods. They accelerate just one reaction without the ballast of an ongoing metabolism of a whole cell. Technically well manageable, many technical enzymes have become amenable through recombinant hosts expressing the target enzyme in good yield and purity (Copeland 2000). With an annual consumption of an estimated 15,000 tons, vanillin (4-hydroxy-3-methoxybenzaldehyde) is one of the most widely-used flavour compounds in baked goods, chocolates, dairy products, perfumes and even pharmaceuticals. Only 0.2 % of the total demand is provided from vanilla beans, while the rest is supplied by chemical synthesis and a ferulic acid-based bioprocess. Natural vanilla flavor is a complex of many components, but the aroma is largely determined by vanillin. Because of the scarcity and high cost of natural vanilla extract, there has been a continuing interest in its biotechnological production. There are different possibilities for the production of natural vanillin, such as biotransformation of caffeic acid and veratryl aldehyde, or the fermentation of natural substrates, such as ferulic acid, eugenol, isoeugenol, coniferyl alcohol, vanillin alcohol and stilbene, by bacteria and fungi, such as *Pseudomonas fluorescens*, *Escherichia coli*, *Amycolatopsis* sp., *Streptomyces setonii*, *Pycnoporus cinnabarinus* or *Aspergillus niger* (Negishi *et al.* 2009; Sindhwani *et al.* 2012; Walton *et al.* 2012; Korthou and Verpoorte 2007). Ferulic acid is available in abundance in plant cell walls and has become the most popular precursor substrate. The increasing price of ferulic acid has stimulated the search for other natural precursor molecules to obtain vanillin naturally. Curcumin occurs in turmeric (*Curcuma longa*) rhizome powder, a common ingredient of curry spice, in concentrations of up to 3 %. It is a food colorant (E 100) and was claimed to exhibit

numerous wide biological functions, although the bioavailability of curcumin is low (Kumar Singh *et al.* 2014). The two phenolic rings at the molecule ends are connected by two *α,β*-unsaturated carbonyl moieties. A hypothetical cleavage at the benzylic position would yield two moles of vanillin from one curcumin molecule. Because of physico-chemical and structural features similar to lignin-related compounds, it was supposed that lignin-degrading microorganism may also be able to degrade curcumin. Previously, *Rhodococcus* strains have been reported as promising candidates, which degraded curcumin to (*E*)-6-(4'-hydroxy-3'-methoxyphenyl)-2,4-dioxo-5-hexenal, feruloylmethane, ferulic acid and vanillin (Bharti *et al.* 2011). The aim of the present study was to develop an enzyme-based route starting with curcumin and resulting in vanillin as the most abundant reaction product.

2.3 Results and Discussion

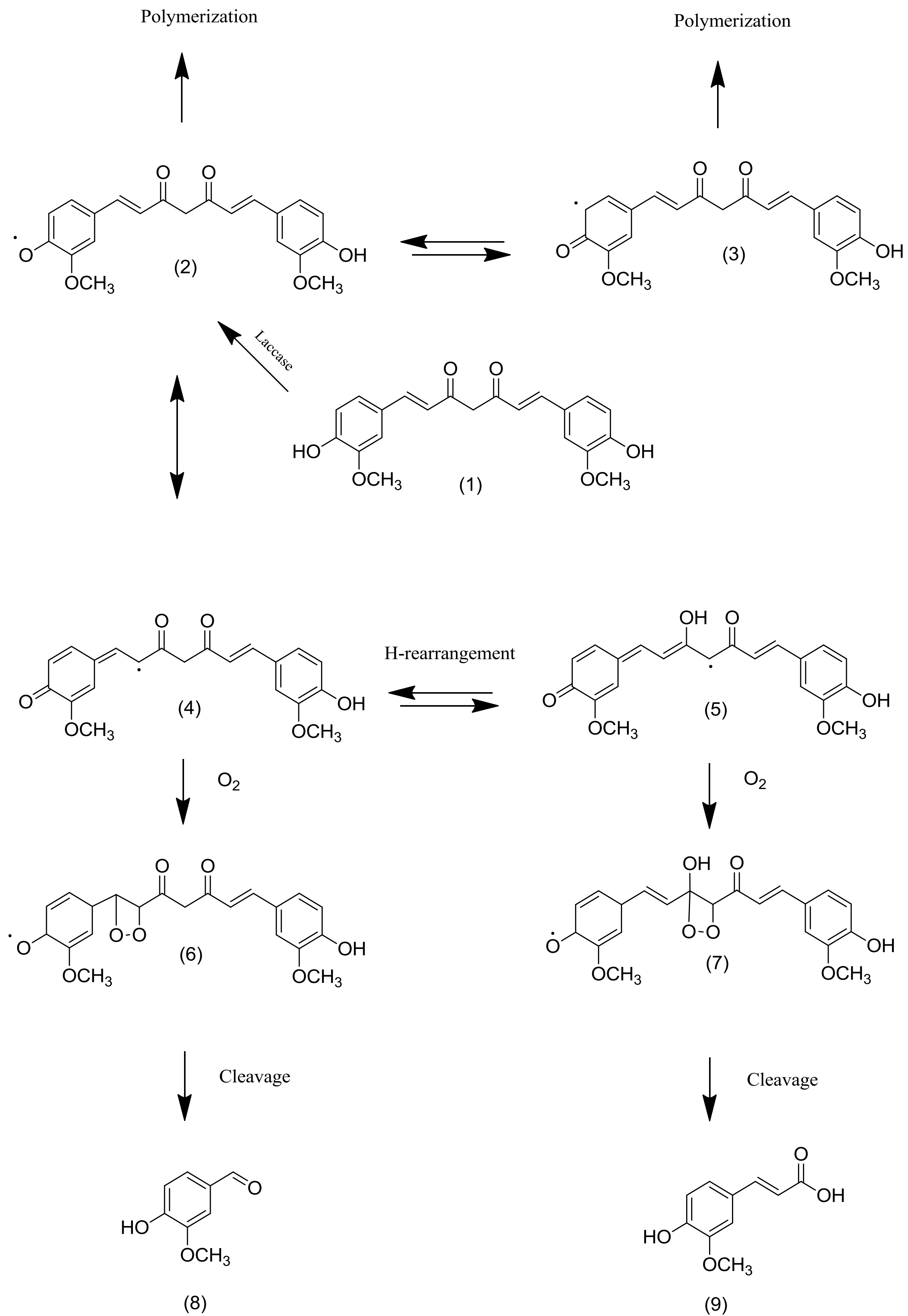
For 20 years, the degradation of natural ferulic acid to vanillin using an optimised bacterial strain (*Amycolatopsis* family) has been one of a few successful large-scale processes using whole cell cultures for the production of a natural flavour compound (Priefert *et al.* 2001). Alternative precursors and routes to natural vanillin have been intensively researched, including the symmetric cleavage of curcumin. Its autoxidative degradation at physiological conditions led to the incorporation of oxygen into a curcumin radical resulting in a bi-substituted bicyclopentadione structure, while vanillin, ferulic acid and feruloylmethane occurred as minor degradation products (Gordon and Schneider 2012). A more concerted enzymatic cleavage at both benzylic positions using either a whole cell system or an oxidoreductase could be envisaged. One curcumin molecule would result in the formation of two molecules of vanillin, and a cofactor-independent enzyme would be most preferred. Abstraction of a hydrogen from a phenol with subsequent oxidation of the substrate is the domain of fungal laccases. These multi-copper oxidases (E.C.1.10.3.2) form resonance-stabilized phenol radicals directly or by the aid of mediators, such as caffeic acid, vanillin (natural) or 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS, non-natural), and reduce molecular oxygen to water at the same time (Diaz-Godinez *et al.* 2013). The cofactor and mediator-free direct incubation of curcumin with various laccases of different redox potentials resulted in an immediate degradation of curcumin (as measured by HPLC) and visible formation of a buffer-insoluble precipitate. However, the targeted degradation products, mainly vanillin (and ferulic acid), were found in traces only.

Being phenols themselves, they were preferred substrates for the laccases, resulting in oligo-/polymerization of the intermediate monomers. To arrive at the intended benzylic cleavage, a less reactive substrate was required. A hypothetical mechanism would imply the delocalization of the unpaired electron of the phenoxy radical into the side chain and, after tautomerization and insertion of molecular oxygen, the generation of respective 1,2-endoperoxides; these, in turn, are well known to decay into two carbonyl moieties (Figure 2.1) (Kruegener *et al.* 2009).

2.3.1 Acetylation of Curcumin

2.3.1.1 By a Chemical Route

To reduce the suitability of curcumin as a laccase substrate, it was aspired to achieve the acetylation of at least one of the phenolic hydroxyl groups of the molecule. Chemical formation of acetyl curcumins yielded two pairs of peaks with identical molecular masses, corresponding to monoacetyl, *m/z* 409, ESI (−) with 50 %, and diacetyl curcumins, *m/z* 451, ESI (−) 50 % by mass of total reaction products. The respective major peak of each pair was assigned to the phenolic acetyl/diacetyl ester with 93 % by mass, whereas the minor peaks (7 % by mass) were assigned to the acetylated hydroxyl group of the tautomeric form of curcumin. The reaction mixture was partially purified by means of preparative TLC. A curcumin-free mixture after purification, which was composed of around 90 % monoacetyl curcumin and 10 % of diacetyl curcumin, was used as the substrate for the following cleavage by three fungal laccases possessing different redox potentials.



Polymerization
Polymerization
O
O
O
OH
OCH3
OCH3
(2)
O
O
O
OH
OCH3
OCH3
(3)
Laccase
O
O
HO
OH
OCH3
OCH3
(1)
O
O
O
OH
OCH3
OCH3
(4)
H-rearrangement
OH
O
O
OH
OCH3
OCH3
(5)
O2
O2
O
O
O
O-O
OH
OCH3
OCH3
(6)
OH
O
O
O-O
OH
OCH3
OCH3
(7)
Cleavage
Cleavage
O
HO
OCH3
(8)
O
OH
HO
OCH3
(9)

Figure 2.1 Hypothetical pathway of laccase-catalysed biotransformation of curcumin. (1) Curcumin; (2) O-centered curcumin radical; (3) C-centered curcumin radical; (4,5) C-centered radicals in the alkenyl chain of curcumin; (6,7) intermediate 1,2-endoperoxides of the curcumin radical; (8) Vanillin; (9) Ferulic acid.

2.3.1.2 By an Enzymatic Route

For the production of 'natural' vanillin, an enzyme-based formation of acetyl curcumin is mandatory. Therefore, reverse hydrolysis was adapted for the acetylation of curcumin in organic medium. A number of commercial lipases are available for acetate synthesis. CAL, a lipase from *Candida antarctica*, was frequently used, and vinyl acetate served as the acyl donor, thus forcing the equilibrium to the product side by tautomerization of the liberated vinyl alcohol. Yields were semi-quantified by LC-MS, but remained unsatisfactory. Reaction solvent, temperature, time and the molar ratio of the reactants were varied. A maximum yield of approximately 49 % was eventually achieved using geranyl acetate as the acyl donor and a molar ratio of 1:50 of curcumin to acetyl donor. The yield of monoacetyl curcumin increased continuously over time until day three and decreased again thereafter. Even after a long time of incubation, only traces of diacetyl curcumin were detected. It may be speculated that the large and inflexible curcumin molecule does not fit well into the deep substrate-binding site of this lipase. The larger monoacetyl curcumin fitted even less well, thereby preventing diacetylation.

2.3.2 Transformation of Acetyl Curcumin by Laccases

The commercial laccase LccAbi of *A. bisporus* and two laccases, recovered and purified from supernatants of cultivated fungal strains, LccMgi (*M. giganteus*) and LccFtr (*F. trogii*), were compared (Table 2.1) (Strong and Claus 2011). Iso-active (1.19 U mL^{-1} adjusted against ABTS as a substrate) laccase preparations in buffered aqueous solution were added to acetyl curcumins in different solvent systems; these were monophasic organic solvents, monophasic water/water miscible organic solvents and biphasic systems composed of water/water immiscible organic solvents.

Table 2.1 Characteristics of laccases used for the degradation of acetyl curcumin.

Laccase	Origin	Redox Potential [a]	pI	pH Optimum [b]	Temperature Optimum [b] (°C)
LccAbi	*A. bisporus*	Middle (0.47–0.71 V)	3.5	4.5–5	30–40
LccMgi	*M. giganteus*	High (0.73–0.78 V)	3.1	5–5.5	30–40
LccFtr	*F. trogii*	High (0.73–0.78 V)	3.8	4.5–5	30–40

[a] According to the literature; [b] according to 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) enzyme activity.

The best reaction conditions were found to be a biphasic system consisting of water/diethyl ether. Samples were taken after 20 h and analyzed by LC-MS for substrate transformation and possible polymerization products and GC-MS for volatile degradation products. In all incubations in the presence of a laccase, as well as in the controls, diacetyl curcumin remained stable and did not change concentration over time. Monoacetyl curcumin concentration, in contrast, declined with time depending on the laccase added. For LccAbi, no distinct degradation occurred, whereas for LccMgi, a 15 % and for LccFtr 46 % declines of the monoacetyl curcumin concentration were observed. During the reaction, the yellow bright reaction solution did not show visible alteration with LccAbi, but turned into yellow/brownish with little precipitation with LccMgi and became cloudy with LccFtr. Several molecular masses in the *m/z* range >600 Da were detected over a broad retention time window in the LC-MS chromatograms of these samples, indicating the formation of oligomer phenols, but the structural elucidation of these was no the aim of this study. GC-MS analysis of the volatile reaction products showed just one major product, acetyl vanillin. This was expected, because other possible degradation products, such as vanillin or ferulic acid, were good substrates for the laccases and polymerized *in situ*, as discussed above. The highest concentration of acetyl vanillin was analyzed for LccFtr, which agreed with the rapid degradation of monoacetyl curcumin (Table 2.2).

Table 2.2 Yield of vanillin acetate after cleavage of monoacetyl curcumin in a biphasic system: 2 mL of 0.5 mM monoacetyl curcumin in diethyl ether and 2 mL aqueous buffer of laccases (each set to 1.19 U mL^{-Y}) under continuous mixing for 20 h at 20 °C.

Laccase	Vanillin Acetate (mg L^{-1})	Vanillin Acetate (mM)	Molar Product Yield (%) *
LccAbi	6.4	0.032	6.4
LccMgi	15.1	0.078	15.6
LccFtr	45.02	0.23	46

* Calculated according to the concentration of the actual precursor, monoacetyl curcumin.

2.3.3 Alignment of the Laccases

Different amino acid substituents near the substrate binding site and copper T1 coordination of laccases result in different potentials of the redox centers, thus affecting the catalytic properties of bacterial (Gunne *et al.* 2014) and fungal laccases (Uzan *et al.* 2010). To better explain the observed differences in reactivity, the respective parts of amino acid sequences were aligned (Figure 2.2). According to previous studies, the T1 copper shows a trigonal bipyramidal coordination with three highly-conserved trigonal ligands (H,C,H) and two weakly coordinated ligands in axial position, of which one is invariable Ile, whereas the second is variable. There is a modest correlation between this axial ligand and the redox potential of T1 copper, with Phe consistently producing high, Leu middle and Met low potentials. An adjacent tripeptide (LEA in terms of high potential laccases), which is part of the T1 pocket, also serving as part of the substrate-binding pocket, is indicative of the respective redox potential of laccases, as well (Uzan *et al.*2010; Rodgers *et al.* 2009). The comparison of the sequences of the laccases from Ftr, Mgi and Abi with literature data showed that with Phe, the axial ligand located in position 460 for Laccase Ftr and 480 for laccase Mgi; both had to be classified as high redox potential-type enzymes and LccAbi, having leucine in the axial position 485, as a midrange potential enzyme. The different oxidation rates of the laccases with monoacetyl curcumin as the substrate may be explained by these differences in the amino acid sequences and, consequently, redox potential. High potential laccases appear to be more suitable for the cleavage of C = C bonds in the side chain of curcumin.

```
Ftr ..WFLHCHIDFHLEAGFAVVMAEDIPEVAATNP.. 476
Mgi ..WFLHCHIDWHLEAGFAIVFAEDVPNVSSTTS.. 496
Abi ..WIFHCHIDFHLRDGLAIVFAEAPEETDAANP.. 501
```

Figure 2.2 Partial amino acid alignment of LccFtr (*Funalia trogii*), LccMgi (*Meripilus giganteus*) and LccAbi (*Agaricus bisporus*). Bold letters show three out of four invariable T1 copper ligands, bold and italic letters the variable axial ligand and letters highlighted in grey a characteristic tripeptide of the binding site of T1 copper indicative of the redox potential of the respective laccases.

2.3.4 Enzymatic Deacetylation of Acetyl Vanillin

Vanillin acetate was obtained as the major volatile compound of the laccase-catalyzed degradation of monoacetyl curcumin. To achieve the enzyme-catalyzed deacetylation of acetyl vanillin, three different esterases were compared (Table 2.3). At an optimum reaction temperature of 37 °C, the esterase PeFaeA deacetylated 68 % of acetyl vanillin to vanillin after five hours of incubation, as calculated by external standard-based GC-flame ionization detection (FID) and GC-MS analyses.

Table 2.3 Yield of vanillin after deacetylation of vanillin acetate in a biphasic system: 2 mL of 1 mM vanillin acetate in diethyl ether and hexane (5:95) and 2 mL aqueous buffer of esterases (each set to 1 U mL^{-2}) under continuous mixing for 5 h at 37 °C.

Esterase	Vanillin (mg L^{-1}) *	Vanillin (mM)	Molar Product Yield (%)
UmChlE	0	0	0
Porcine liver	75.5	0.50	50
PeFaeA	103	0.68	68

* Calculated according to the external standard (3,4-dimethoxybenzaldehyde).

In summary, many different possibilities for the biotechnological production of vanillin have been investigated in the past. Most processes were primarily affected by the high chemical reactivity and toxicity of vanillin. Thus, three-step enzymatic reactions are a novel approach to produce natural vanillin from curcumin (Figure 2.3).

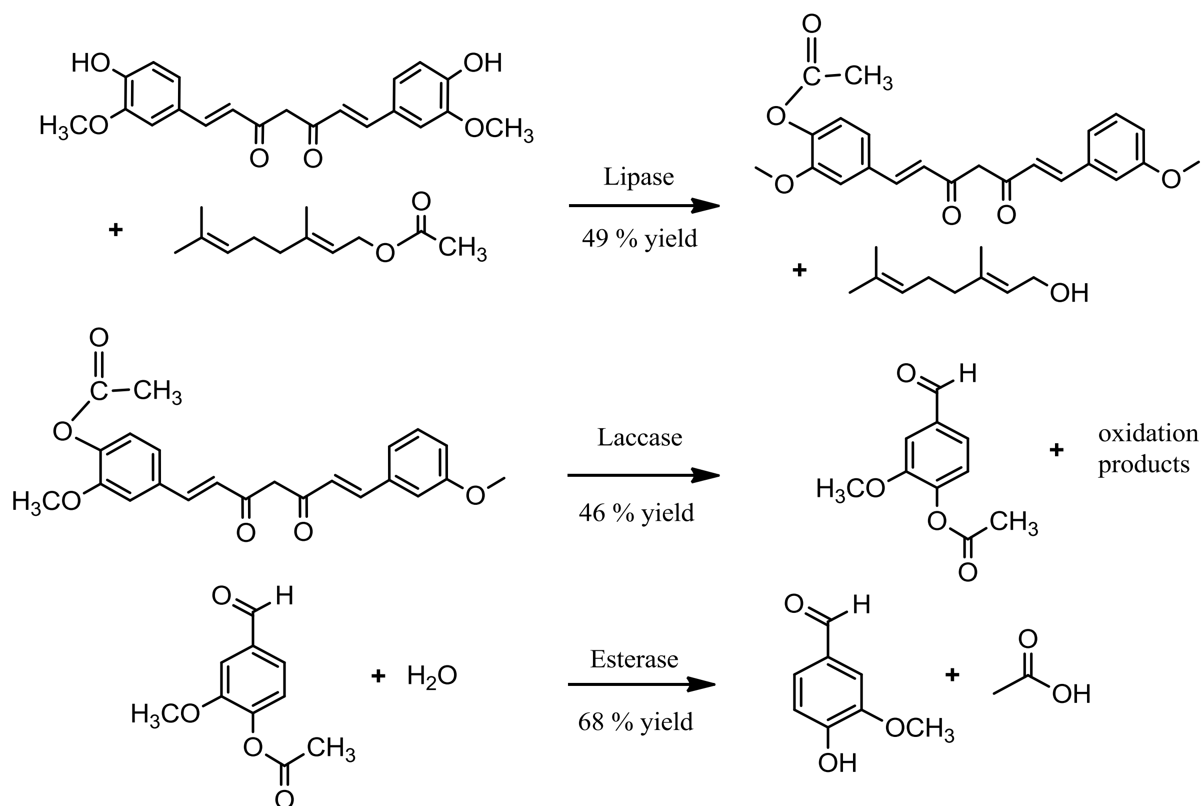


Figure 2.3 Three-step enzymatic bioconversion of curcumin to natural vanillin.

2.4 Experimental Section

2.4.1 Materials

All chemicals were analytical grade. Curcumin (>90%, natural) was purchased by Roth (Karlsruhe, Germany). 3,4-dimethoxybenzaldehyde and geranyl acetate from Sigma-Aldrich (Taufkirchen, Germany). 2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) and *p*-nitrophenyl butanoate were obtained from ICN Biochemicals (Muenchen, Germany). Diethyl ether, ethyl acetate, toluene and n-pentane were from Karl Roth (Karlsruhe, Germany), and solvents (all MS grade) used for HPLC-MS were from Carlo Erba Reactifs (Peypin, France).

2.4.2 Enzymes

Immobilized lipase (triacylglycerol hydrolase, EC 3.1.1.3 (Novozyme 435, 5000 $U \cdot g^{-1}$) from

Candida antarctica and laccase from *Agaricus bisporus* (6.8 U·mg^{-1}) were from Sigma-Aldrich (Taufkirchen, Germany), and esterase from porcine liver (lyophilisate, 15 U·mg^{-1}) was from Sigma Aldrich (Taufkirchen, Germany). Recombinant feruloyl esterase from *Pleurotus eryngii* (PeFaeA) and chlorogenic acid esterase from *Ustilago maydis* (UmChlE) were selected from our own stocks. Two further laccases were isolated from fungal culture supernatants, as described below. The strains were purchased from the Centraalbureau voor Schimmelcultures (*Meripilus giganteus* CBS 561.86) and from the German Collection of Microorganisms and Cell Cultures (*Funalia trogii*, DSMZ), respectively.

2.4.3 Cultivation of Fungi

The culture supernatant of *M. giganteus* was provided according to the paper of Schmidt *et al.* (Schmidt *et al.* 2012).Submerged pre-culture of *F. trogii* was inoculated with the same structure of *M. giganteus*, except that for the main cultures, the expression of laccases was induced either by the addition of three grams per100 mL^{-1} wheat bran and $CuSO_4$ (300 µM final concentration) to the culture medium of *F. trogii* or 300 µM $CuSO_4$ solely in the case of *M. giganteus*. At the time of maximum laccase activity (ABTS activity, pH 3.0), cultivation was stopped and the culture supernatant harvested and stored at −20 °C, unless used immediately for laccase isolation and purification.

2.4.4 Laccase Isolation and Purification

The laccase from *M. giganteus* was isolated according to the protocol of Schmidt *et al.* (Schmidt *et al.*2012). In brief, the supernatant was frozen at −20 °C, thawed and centrifuged at 25,000× *g*. After filtration using a 0.45-µM polyester filter (CHROMAFIL PET-45/25, Macherey-Nagel, Dueren, Germany) and concentration using an ultra-filtration module (30-kDa cut-off, PES, Sartorius, Goettingen, Germany), the laccase was purified using fast protein liquid chromatography (Biologic Duoflow TM, Bio-Rad, Hercules, CA, USA) at 4 °C. First, a weak anion exchange column was applied (HiPrep 16/10 DEAE,16 × 100 mm fast flow, GE Healthcare, Munich, Germany). Concentrated laccase fractions were submitted to a second purification using size exclusion chromatography (Superdex 75 10/300 GL column, GE Healthcare, Munich, Germany). Active fractions were pooled and adjusted to the activity required.

Laccase from *F. trogii* was purified as follows: The culture supernatant was frozen at −20 °C, thawed and centrifuged at 5000× *g* at 4 °C for 15 min. The supernatant was filtered (0.45 µM, Chromafil Pet-45/25, Dueren, Germany), concentrated using an ultrafiltration module (30-kDa cut-off, PES, Sartorius, Goettingen, Germany) and subjected to fast protein liquid chromatography (Biologic Duoflow TM, Bio-Rad, Hercules, United States) at 4 °C. Twenty five milliliters of concentrated solution were purified on a HiPrep 16/10 DEAE, 16 × 100 mm fast flow column with a flow rate of 3 $mL \cdot min^{-1}$ (GE Healthcare, Munich, Germany) with 20 mL running Buffer A (50 mM, potassium phosphate, pH 6.5) and eluted with 5% Buffer B (50 mM potassium phosphate, pH 6.5 + 1 M NaCl). Purification was controlled using SDS-PAGE electrophoresis. SDS-PAGE was performed using 12 % (w/v) polyacrylamide gels. Samples were diluted in native loading buffer (0.05 M Tris/HCl pH 6.8, 0.1 % bromophenol blue, 10% glycerol, 2% SDS) and applied to electrophoresis. Proteins were stained with ready-to-use Instant Blue solution (0.1%, Expedeon, Cambridge, UK). Laccase activity staining was performed directly on the gel using ABTS (5 mM in 100 mM sodium phosphate buffer pH 4.5).

2.4.5 Analysis of Amino Acid Sequence

The identities of the purified laccases, as well as the sequence of LccAbi were deduced from the amino acid sequence of tryptic peptides of cut out protein bands from SDS gel electrophoresis. De-staining and tryptic digestions of the respective protein bands were carried out as described elsewhere (Plagemann *et al.* 2014). Tryptic peptides were analyzed by means of nano-LC EASY-nLC II (Bruker Daltronik, Bremen, Germany) equipped with a 20-mm pre-column (C18-A1 3PCS; ThermoFisher Scientific, Dreieich, Germany) and a capillary column (0.1 mm × 150 mm) packed with Magic C18 AQ (3-mm particle size, 200-Å pore size; Michrom Bioresources, Inc., Auburn, CA, USA) eluted by a linear gradient (300 nL min^{-1}) of water and acetonitrile, each with 0:1 % formic acid v/v from 95 % water to 95 % acetonitrile within 25 min and held for 15 min. The amino acid sequences elucidated were subjected to protein database (NCBI, Mascot search algorithm). Sequences were aligned using the ClustalW2 multiple sequence alignment database.

2.4.6 Enzyme Assays

2.4.6.1 Laccase Activity

The activity of each laccase was determined with ABTS as the substrate. The change in the absorbance was recorded at 420 nm using a Biotek Eon 2 Microplate reader (Biotek, Winooski, VT, USA) at 30 °C. In brief, 15 µL of enzyme solution were mixed with 0.5 mM substrate in 50 mM phosphate buffer at pH 3.0 in a total volume of 300 µL. The change in the absorbance was monitored over ten minutes. One unit of enzyme activity was defined as 1 µmol of substrate (ε = 36,000 $L \cdot mol^{-1} \cdot cm^{-1}$) oxidized per minute under the experimental conditions (Linke *et al.* 2013).

2.4.6.2 Esterase Activity

Esterase activity was assayed using *p*-nitrophenyl butanoate as the substrate and monitoring the change in absorbance at 410 nm (15,000 $L \cdot mol^{-1} \cdot cm^{-1}$, pH 8.0) over ten minutes with a Biotek Eon 2 Microplate reader (Biotek, Winooski, VT, USA) at 37 °C. Twenty microliters of sample were mixed with 175 µL of 100 mM sodium phosphate buffer (pH 6.0) and 5 µL of 50 mM *p*-nitrophenyl butanoate in ethanol. The increase of absorbance was monitored at 37 °C at 410 nm for 20 min. One unit of enzyme activity was defined as the release of 1 µmol nitrophenol per minute under the specified conditions (Haase-Aschoff *et al.* 2013).

2.4.6.3 Lipase Activity

This assay was performed by measuring the increase in absorbance at 410 nm produced by *p*-nitrophenol released from 0.4 mM *p*-nitrophenyl butanoate in sodium phosphate buffer (50 mM, pH 7.0) at 37 °C. To start the reaction, the lipase solution or suspension (20 µL) was added to the substrate solution (175 µL buffer, 5 µL *p*-nitrophenyl butanoate). One international unit of activity was defined as the amount of enzyme that hydrolyzed one µmol of *p*-nitrophenol butanoate per minute under the conditions (Escorcia *et al.* 2013). All enzyme assays were performed in duplicate, and the standard deviation was found below 5 %.

2.4.7 Curcumin Transformation

2.4.7.1 Chemical Acetylation of Curcumin

Acetylated curcumins were synthesized chemically as reference compounds using acetic anhydride. One mmol of curcumin was dissolved in 150 mL ethyl acetate and mixed for 20 min. After dissolving was completed, four mmol acetic anhydride were carefully added. After six hours, the reaction was stopped by adding one drop H_2O_2. Reaction yield (consumption of curcumin) and product identification were carried out by LC-MS.

2.4.7.2 Lipase-Catalyzed Acetylation of Curcumin

Before each experiment, ethyl acetate and toluene as the solvent and the acyl donor (vinyl or geranyl acetate) were stored over Na_2SO_4. The reaction was carried out in 2 mL ethyl acetate and toluene (10:90) in sealed 30-mL glass vials at 40 °C with continuous stirring using a glass magnetic stir bar (150 rpm). The powdered CAL was added to a final concentration of five $mg·mL^{-1}$. Sodium acetate buffer 50 mM, pH 6, was added at 4 % to the reaction solution. Over the incubation, time samples were taken, filtered using 0.45 µm filter (Chromafil PET 45/25, Macherey-Nagel) and then analyzed directly by LC-MS.

2.4.7.3 Monoacetyl Curcumin Degradation

The two-phase reaction system was made up of 2.0 mL of 0.5 mM monoacetyl curcumin (concentration calculated according to a curcumin standard) together with a minor impurity of diacetyl curcumin (concentration not affected by the laccase present) in 2.0 mL diethyl ether and 2.0 mL respective enzyme solution (1.19 $U·mL^{-1}$, ABTS-assay, 30 °C, pH 3) in 50 mM sodium phosphate buffer, pH 5.5, under continuous vortexing at 1300 rpm (Heidolph, Germany) for 20 h at room temperature. A control sample with the buffer, but without enzyme, was treated under the same conditions. After separation of the diethyl ether phase, the aqueous buffer was re-extracted three times with 2.0 mL diethyl ether, and the combined organic phases were dried over night with Na2SO4. The degradation rate of acetyl curcumin was determined by LC-MS, and volatile degradation products, such as vanillin acetate, were analyzed by GC-MS.

2.4.7.4 Esterase-Catalyzed Deacetylation of Vanillin Acetate

Three esterases were used for the hydrolysis of vanillin acetate. The hydrolysis was carried out in a glass vial containing 2 mL of 1 mM vanillin acetate dissolved in diethyl ether/hexane and 2 mL

sodium phosphate buffer 50 mM, pH 5 and 6.5. The reactions were initiated by adding enzyme solution with an activity of one $U \cdot mL^{-1}$ to the reaction mixture and placed in a vortex shaker at 37 °C for five hours. The samples were extracted three times with diethyl ether, and the combined fractions were dried over sodium sulfate and analyzed by GC-MS using the external standard, 3,4-dimethoxybenzaldehyde (final concentration 125 mg L^{-1}). The reproducibility of three repeated transformations showed a relative standard deviation of typically 3 %.

2.4.8 Gas Chromatography

2.4.8.1 Gas Chromatography/Flame Ionization Detection

For each sample, 1 µL was injected on-column in an Agilent 7890A gas chromatograph (Agilent, Waldbronn, Germany) equipped with a cool on-column injection port and a 30 m × 0.32 mm i. d. × 0.25 µm CP-Wax 52 CB column (Varian, Darmstadt, Germany). The oven temperature program was 40 ° C for 3 min, raised at 3 °C per minute to 230 °C and held for 10 min. Hydrogen was used as the carrier gas at a flow rate of 2 mL per minute. Quantification was carried out according to the external standard (3,4-dimethoxybenzaldehyde).

2.4.8.2 Gas Chromatography/Mass Spectrometry

Gas chromatography-mass spectrometry (GC-MS) was conducted using a GC 8000 coupled to an MD 800 mass-selective detector (Fisons, Mainz-Kastel, Germany) equipped with a cool-on-column injection port and a 30 m × 0.32 mm i.d. × 0.25 µm CP-Wax 52 CB column (Varian). The samples were injected using the same oven program as for GC/FID, but helium at a flow rate of 1.2 mL per minute was the carrier gas. Mass spectra were acquired using electron impact ionization at 70 eV and a 200 °C source temperature. Reaction products were identified by comparing their RIs (Resonance-ionization) and mass spectra with those of authentic standards.

2.4.9 Liquid Chromatography/Mass Spectrometry

For the identification of vanillin, curcumin and acetylated curcumins, as well as for the determination of the molar mass of the expected oxidation/polymerization products thereof, high performance liquid chromatography coupled to a triple quadrupole mass analyzer was used (Varian 212 LC pump, Pro Star 325 UV-Vis detector, 320 TQ-MS mass spectrometer). The MS

was conducted simultaneously in the ESI positive and negative mode with a scan range of *m/z* 110–500 or *m/z* 300–1200, respectively. The MS parameters for ESI(+)/ESI(−) were: capillary voltage +30 V/−40 V, needle voltage 5000 V/−4500 V, nebulizer gas (N2) 379 kPa, drying gas 207 kPa at 350 °C. For HPLC, water and acetonitrile (MS-grade), both containing 0.1 % formic acid, were used as the mobile phase, and the following linear gradient was used: 10 % acetonitrile for three minutes, up to 90% acetonitrile within 20 min, hold for five minutes and back to start conditions. The separation was performed on an RP-18 HD column (Eurosphere 100-C18-5-HD, 250 × 4 mm, 5 µm, Macherey-Nagel) at a flow rate of 0.3 mL per minute. Additionally, UV absorption was monitored at 280 and 425 nm.

2.5 Conclusions

During the coming years, the flavour market is expected to increase, and biotechnology will contribute to guaranteeing the supply (Berger 2015; Wells and Meyer 2014). This work showed that laccases are suitable for the oxidative cleavage of acetyl curcumin in a cofactor- and mediator-independent reaction. A three-enzyme system provided protection/deprotection chemistry, with the selective acetylation of one of the two phenolic hydroxyl groups of curcumin as the key step. As a result, one phenol moiety of the symmetric molecule was protected against attack of the laccase, while the other vanillin moiety was inevitably lost to oligomerization. Laccase-catalyzed reactions are governed by the structure of the phenolic substrate, the redox potential of the enzyme (high, middle or low), the presence and choice of a mediator and the usual parameters, such as reaction pH, temperature and solvent composition (Kunamneni *et al.* 2008a). A refined system, including a food-grade mediator, might convert both acetyl vanillin moieties of an enzymatically-synthesized diacetyl curcumin into ‘natural’ vanillin. To this end, the first enzymatic step must be made more efficient by using a lipase able to accept both curcumin and monoacetyl curcumin as substrates. Some representatives out of the large set of lignolytic enzymes of higher fungi might be even more suitable for the acetylation, cleavage and hydrolysis of phenolic substrate molecules to yield ‘natural’ flavor compounds.

2.6 Acknowledgments

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Author Contributions

Vida Esparan conducted most of the laboratory work, evaluated the data and wrote major sections of the paper. Ulrich Krings operated the mass spectrometers and interpreted the data. Marlene Struch contributed a purified and characterized laccase from *Funalia trogii*. Ralf G. Berger supervised the work and edited the manuscript. All authors have read and approved the manuscript.

The taste enhancer divanillin: sources and enzymatic generation

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3.1 Abstract

Dehydrodivanillin, the symmetrical dimer of vanillin, is a taste enhancer which imparts pleasant impressions of creaminess to food. Found in vanilla pods in traces only, a co-substrate independent dimerization of vanillin, conducted in a co-solvent system to improve the solubility of vanillin, was developed using iso-active fungal laccases from *Meripilus giganteus*, *Agaricus bisporus* and *Funalia trogii*. The yields were compared with a peroxidase from *Marasmius scorodonius* (MsP2) and horseradish peroxidase (HRP), both supplied with hydrogen peroxide. Using laccase catalysis, the kinetically preferred reaction product, 5,5'-dehydrodivanillin, rapidly reached saturation and precipitated *in situ*, thus shifting the reaction equilibrium to the product. Yields of > 95 % were obtained with the high-redox-potential laccase of *Funalia trogii*, while HRP gave 18 %.

Keywords: dehydrodivanillin, vanillin, laccase, peroxidase, co-solvent, reaction equilibrium

3.1 Introduction

The discovery of divanillin (6,6′-dihydroxy-5,5′-dimethoxy-(1,1′-biphenyl)-3,3′-dicarboxaldehyde) dates back to *Tiemann* and the year 1885 (Tiemann 1885). A first comprehensive report on the chemical synthesis and properties of divanillin appeared as early as in 1916 (Elbs and Lerch 1916). In contrast to the original method of *Tiemann*, the use of $Na_2S_2O_8$ as an oxidant instead of $FeCl_3$ resulted in a nearly quantitative conversion of vanillin to its symmetric dehydrodimer.

Divanillin occurs in nature as an occasional sub-unit of lignin, and it is detectable upon chemical or enzymatic degradation of wood (Freudenberg and Renner 1965; Lahtinen *et al.* 2009b; Martinez *et al.* 2005). A Japanese patent claimed properties as an antioxidant, skin-lightening and fixative in perfume oils, but described the substance as being odorless and tasteless (Ikemoto *et al.* 1995). In-depth sensorial evaluations showed divanillin to possess a pleasant, adherent and rich taste impression of creaminess, milk fattiness and sweetness, improving the quality of low-fat and reduced fat, semi-finished food products and ready-to-eat foodstuffs (Reiss *et al.* 2006). A subsequent patent claimed divanillin as a bitterness masking substance which extended the scope of applications for the food industry (Ley *et al.* 2008). With 5 µg per kg skim milk, the orosensory perception threshold of divanillin is remarkably low (Schwarz and Hofmann 2009).

3.2 Sources of divanillin

Divanillin is primarily formed during the curing of the vanilla pods, a process of repeated wetting, warming (‘sweating’), drying and conditioning aiming at the liberation of vanillin (1.5 to 2.5 % w/w) from its glucoside precursor. The traces of natural divanillin present (5 to 50 mg per kg) are much too small to allow for an economic isolation, but suggest a slow autoxidative formation once free vanillin is accumulating in the pods. In a situation where consumers worldwide increasingly call for ‘natural’ food ingredients and natural sources are economically unavailable, biotechnology can provide solutions without using toxic chemicals and harsh reaction conditions (Berger 2015). According to effective European law (EG 1334/2008) a ‘natural flavouring substance’ shall mean a compound ‘obtained by appropriate physical, enzymatic or microbiological processes....’. The Code of Federal Regulation (CFR - Title 21) of the Food and Drug Administration of the United States contains the terms ‘enzymolysis’ and ‘fermentation’. Transferred onto the divanillin case, a bioprocess could be envisaged mimicking

the above pathway in the vanilla tissue, if a rich source of 'natural' vanillin as a precursor existed. This is the case (Berger 2015; Walton *et al*. 2000].

First bioprocesses towards natural vanillin used plant cell cultures, or *ß*- glucosidases for an improved release; first concerted microbial transformations attempted the conversion of eugenol, isoeugenol, aromatic amino acids, vanillic acid, stilbenes or ferulic acid (Walton *et al.* 2000). As ferulic acid is the most abundant hydroxycinnamic acid in plants, its microbial side chain degradation by Gram-negative bacteria has been recognized as the prime rose path (Muheim and Lerch 1999). Various mechanisms of chain shortening by prokaryotics were suggested, and a dioxygenase of vanilla was assumed to present the key enzyme of the pod (Negishi *et al.* 2009). Catabolism of the target compound, a severe problem of the earlier systems, was combatted by pathway engineering of producer strains of *Amycolatopsis* (genus *Actinomycetes*), and has resulted in yields of 20 grams of 'biotech-vanillin' per liter of culture medium, presenting a rich source of the divanillin precursor (Fleige 2013).

3.4 Enzyme catalyzed routes to divanillin

With an abundant supply of 'natural' vanillin at hand, oxidoreductases specializing on phenolic substrates lend themselves not only for hydroxylation or epoxidation reactions, but also for the aspired dimerization (Martinez *et al.* 2014). Commercial horseradish peroxidase (HRP) biomimetically oxidized resveratrol, a trihydroxystilben, and the reaction was tuned from decomposition to dimerization by incorporation of various metal ions (Li *et al.* 2014). Immobilized HRP formed radicals on phenols which reacted to linear polymers via C-O bridges (Nanayakkara *et al.* 2014). In the presence of a slight molar excess of hydrogen peroxide HRP was also the enzyme of choice for the patented production of natural divanillin (Reiss *et al.* 2006). In spite of the requirement of the reactive co-substrate, the procedure was recommended as a 'green chemistry' prototype (Nishimura *et al.* 2010). A previous report on the laccase catalyzed dimerization of resveratrol and other stilbenes indicated that this class of enzymes was able to replace HRP or other peroxidases (Ponzoni *et al.* 2007). Three dimers were obtained from resveratrol, the main product being an oxygen-bridged dihydrofuran structure.

Laccases are widely distributed in nature. They couple the one electron oxidation of phenols with the reduction of oxygen to water, and were thoroughly investigated down to the gene level (Diaz-Godinez *et al.* 2013). Compared to peroxidases, the peroxide free action of laccases represents a

significant advantage. However, the random character of radical reactions typically resulted in complex mixtures of oligomers and polymers (Ponzoni *et al.* 2007; Shumakovich *et al.* 2014). Another expected disadvantage was the possible requirement of a mediator for achieving reasonable yields (Calcaterra *et al.* 2008; Ihssen *et al.* 2014). To explore the pros and cons, three fungal laccases were compared under the same experimental conditions with a fungal peroxidase and HRP for their ability to react vanillin to the desired C-C bridged dimer; both peroxidases were supplied with H_2O_2 (Table 3.1).

Table 3.1 Enzymes used for the oxidative dimerization of vanillin

Enzyme	Origin	Enzyme description
HRP	*Horseradish* Peroxidase	lyophilized powder (Sigma-Aldrich, 150 U mg^{-1} specified)
MsP2	*Marasmiusscorodonius* eroxidase 2	Solution, recombinant from *E. coli* (2 U mL^{-1})
Abi	*Agaricus bisporus*	lyophilized powder (Sigma-Aldrich, 4 U mg^{-1} specified)
Mgi	*Meripilus giganteus*	solution, purified by IEX, SEC (12 U mL^{-1})
Ftr	*Funalia trogii*	solution, purified by IEX (52 U mL^{-1})

3.5 Generation of divanillin

In a first screening, the fungal oxidases and HRP were reacted with vanillin under the same conditions: One U mL^{-1} of enzyme, sodium phosphate buffer pH 4.5, one mg vanillin mL^{-1}, and equimolar concentration of H_2O_2 for the peroxidases. All enzymes were applied in iso-active form as measured using the common synthetic substrate ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)). Fastest reaction rates were obtained with the laccase Ftr from *Funalia trogii* (Ftr, related to the *Trametes* genus, a high-redox-potential enzyme from a white-rot fungus). LC-ESI(-)-MS and MS/MS collision experiments showed a broad range of reaction products. At least five divanillin structures were tentatively identified through their molecular ions. A number of trimers and higher oligomerization products were also tentatively

identified indicating that the oligomers reacted further with the primary phenolic radicals delivered by the laccase. The samples containing the laccases from Ftr and Mgi (*Meripilus giganteus*, giant polypore) showed cloudiness and numerous reaction products after 30 minutes of reaction, whereas the laccase from Abi (*Agaricus bisporus,* button mushroom) did not.

During the incubation with the laccase from Ftr, precipitation of some crystals was observed. The precipitate was filtered off, re-dissolved and analyzed as chemically almost pure divanillin. Compared to vanillin, divanillin is less soluble in water (3.7 g L^{-1} vs. 0.26 g L^{-1} at pH 5). Therefore, it was attempted to favor the formation of the desired reaction product divanillin and to reduce the possibly inevitable formation of oligomers by changing the ratio of enzyme activity to substrate concentration. The final transformation conditions are summarized in Table 3.2. As expected, vanillin became the predominant substrate, and the divanillin formed was removed from the dynamic equilibria, otherwise leading to a mixture of oligomers, by rapid *in situ* precipitation as soon as its solubility product was exceeded.

Table 3.2 Comparison of biotransformation systems for the formation of divanillin

Parameter	**Patent** (Reiss *et al.* 2006)	**This work**
Aqueous buffer	Tris Acetate pH 5.0	50 mM Sodium Phosphate pH 4.5
Vanillin concentration	10 g L^{-1} [66 mM]	10 g L^{-1} [66 mM]
Solubilization	Heated to 40 − 50 °C	Addition of 5 % (v/v) ethanol
Enzyme activity	11 U mL^{-1}	1 U mL^{-1}
[H_2O_2]	> 2.25 g L^{-1} [66 mM]	2.25 g L^{-1} [66 mM]; HRP and MsP2 only
Incubation time	18 h	5 h

For both types of oxidases, laccases and peroxidases, the initial reaction step is the formation of a phenolic radical which is stabilized over the aromatic structure. Hence, oxygen and carbon centered radicals may result in the formation of C-O-C and C-C-bridged products. Recombination of two radicals occurs preferably at position five of the aromatic ring of vanillin.

To clarify whether a C-O-C or a C-C-bridged dimer was generated, the precipitate of the reaction catalyzed by the laccase from Ftr was re-dissolved and silylated after appropriate dilution in dry dichloromethane using MSTFA (N-methyl-N-(trimethylsilyl) trifluoroacetamide). In a C-C-bridged dimer, both phenolic hydroxyl groups of vanillin are maintained. Accordingly, upon silylation of this divanillin isomer, its mass must be increased by 144 Da, whereas the C-O-C-bridged dimer should show an increase of 72 Da. GC-EI-MS analysis showed a dominating peak (> 99 % of total area) which was attributed to the C-C-bridged form of divanillin. The C-O-C-bridged dimer, like other side products, was detected in traces only.

The product yields for all enzymes tested are compiled in Figure 3.1. High yields were obtained with the laccases, exceeding 95 % (w/w) when using the laccase of Ftr, followed by the laccase of Mgi with > 60 %. No product was obtained from the reaction catalyzed by the laccase of Abi. Under the same conditions, the divanillin yield for HRP reached 18 %, whereas MsP2 (from *Marasmius scorodonius,* garlic parachute, peroxidase 2) gave around 50 % (w/w). This difference may be explained by the different structure, but also by the different origins of both peroxidases. Commercial HRP is heterologously expressed in *Pichia* and produced in a hyperglycosilated form (Capone *et al.* 2014), whereas MsP2 was expressed in *E. coli* BL21(DE3) using the cold shock vector pCOLD I (Zelena *et al.* 2012).

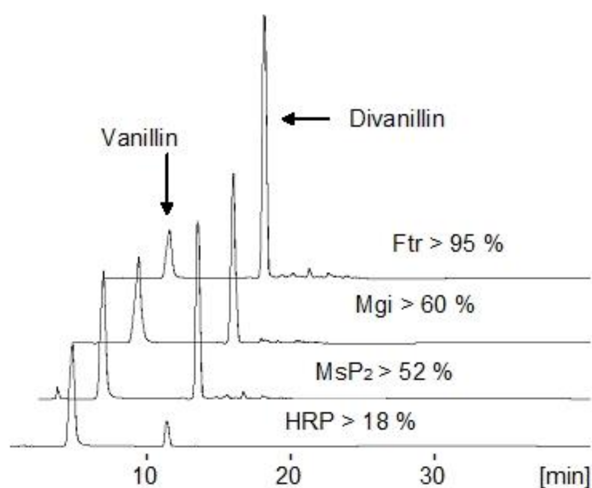

Figure 3.1 HPLC- DAD chromatograms showing the formation of divanillin. Transformation conditions were as shown in Table 3.2

Activity measurements were carried out after the incubation. More than 40 % of the initial activity of the laccase of Ftr was recovered compared to 16 % and 10 % of the activity of MsP2 and HRP, respectively. Laccases of both bacterial and fungal origin frequently showed excellent thermo-stability (Wang *et al.* 2006). Likewise favorable for technical applications are the good operational stability, particularly of fungal laccases, in the presence of organic solvents (Zumarraga *et al.* 2007) and surfactants (Schmidt *et al.* 2012).

The different product yields of laccases from different sources may be attributed to their respective redox potentials (Macellaro *et al.* 2014). The T1 copper site of a laccase and its substrate binding properties allowed to classify them into low, medium and high redox potential types. Sequence alignments suggested that laccase harboring an axial phenylalanine residue at the T1 center, such as the laccase of Ftr, should have a high redox potential, while those with a methionine, such as the laccase of Abi, have a lower redox potential. Accordingly, the laccase of Abi with a middle redox potential was unable to oxidize vanillin, whereas the high-redox-potential types, Ftr and Mgi, were active. However, the laccase of Mgi oxidized vanillin at a lower rate than the enzyme from Ftr, although the redox potentials of both were estimated as high. Apparently, other factors, such as the degree of glycosylation of the enzyme, the molecular shape of the substrate, the solvent system and perhaps other reaction conditions affect the observed reaction rate.

3.6 Conclusion

Laccases of the high-redox-type were found superior to H_2O_2 dependent peroxidases for the production of divanillin. The driving forces for high yields were a high concentration of the substrate vanillin, achieved with the aid of the co-solvent ethanol, an apparent lack of substrate inhibition, and the operational stability of the laccase in this chemical environment. High concentrations of divanillin above its solubility were immediately formed resulting in its *in situ* precipitation, thereby protecting the product, avoiding product inhibition, and shifting the equilibrium continuously towards the product side. An almost quantitative formation (> 95 % conversion) of natural divanillin was obtained in the absence of a mediator. This showed that the random formation of phenolic radicals may be channeled into a concerted dimerization. Different reaction conditions may favor polymerization (Zhang *et al.* 2014) or substitution (Hahn *et al.* 2014) and cleavage reactions (Moreira *et al.* 2014) depending on the type of laccase and

mediator. No H_2O_2 or other co-substratesm (except oxygen) are required for the action of laccases, while peroxidases, as used previously, (Gatfield *et al.* 2006) depend on H_2O_2 by definition. Because of this distinct advantage, laccases (and laccase/mediator systems (Fillat *et al.* 2012)) may find wider synthetic and degradative applications to produce 'natural flavours' according to EG 1334/2008.

Acknowledgments

We thank Katja Zelena, Gunnar Schmidt and Marlene Struch for kindly providing enzyme isolates and helpful information. The laccase Mgi originated from the research cluster Biokatalyse 2021 (FKZ 0315172F) of the German Ministry of Education and Research, and the laccase Ftr from a project supported by the Forschungskreis der Ernährungsindustrie FEI e. V., Bonn (17475N) through AIF and the German Ministry of Economics.

4. Laccase catalyzed generation of lignans in a biphasic system

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4.1 Abstract

Laccase (EC 1.10.3.2) catalyzed transformations of monomer lignin precursors were investigated. Coniferyl alcohol, coniferyl aldehyde, sinapic acid, ferulic acid, caffeic acid, gallic acid and *p*-coumaric acid were oxidized by commercial laccases from *Agaricus bisporus*, *Trametes versicolor* and the new high redox potential laccase from *Funalia trogii*. Rapid and random oligo-/polymerization instead of the desired dimerization occurred in aequeous standard buffers. In 80 % aequeous ethanol and in the biphasic system 80 % ethyl acetate plus 20 % buffer, dimerization was more favored. The solvent-tolerant laccase of *Funalia trogii* gave the best conversion retaining 25 % and 35 % of its activity, respectively, after 30 minutes of incubation in these solvents, and dimers of coniferyl alcohol, sinapic acid and ferulic acid were generated with more than 95 % conversion rate. Structural assignments of products by mass spectrometry of silylated products showed the formation of the β–β dimer of sinapic acid, and of both the β–β and the β-5-dimer of ferulic acid, while several dimers of coniferyl alcohol and coniferyl aldehyde were formed concurrently.

KEYWORDS: Lignin precursors, laccase, biotransformation, lignans, dimerization, taste modifiers, Bioactives

4.2 Introduction

Lignans are widely distributed in roots, leaves, seeds and fruit of plants and are derived from the oxidative dimerization of phenylpropanoid precursors (Slanina and Glatz 2004). The two phenyl propane moieties occur connected through the formation of C-C or C-O bounds, and are precursors for higher molecular mass oligomers and finally the lignins. Dimerization, oxygen incorporation, and skeleton functionalization reactions result in a great variety of structures (Suzuki and Umezawa 2007). A wide range of biological and pharmaceutical activities has been claimed for lignans, such as anti-tumor (Zhu *et al.* 2013), especially anti-breast and colon cancer (Lee and Xiao 2004), cardiovasculoprotective and neuroprotective (Sok *et al.* 2009), anti-Alzheimer, anti-HIV (Jiuan *et al.* 1993), anti-viral and anti-inflammatory properties (Saleem *et al.* 2005; Pan *et al.* 2014).

Foods naturally often contain specific bitter compounds which negatively affect the sensorial quality of a product. In foods, such as tea, coffee, *Citrus* products and dark chocolate bitter tastants are inevitable and regarded as character impact compounds. More recently they were supposed to have a positive effect on human health (Drewnowski and Gomez-Carneros 2000), but if the bioactivity of the bitter compounds should be preserved, it is required for the bitter-sensitive consumers to mask bitter and astringent tastes. Lignans appear to be a possible solution to the problem (Backes *et al.* 2014). The use of lignans as potent natural antioxidants and nutritional supplements are other possible applications in the food industry (Kumar and Singh 2015). Extraction of lignans from plants using enzymatic and chemical methods is complicated, because the covalent lignin structures cannot be easily opened without simultaneously changing the lignan structures (Guerra *et al.* 2006). Thus, isolation of pure lignans from natural sources suffers from a scarcity of suitable natural sources and the risk of structural alterations during extraction (Willför *et al.* 2006).

Enzymatic dimerization of phenolic substrates could be a practical option for the production of bioactive lignans. One-electron oxidation reaction by peroxidase/H_2O_2 works well for the dimerization of phenols (Agha *et al.* 2008). Laccases (EC 1.10.3.2) with their preference for phenolic substrates appear to be particularly suitable for the aspired dimerization possessing the additional advantages of co-factor independency and operational stability. Three-dimensional structures have been resolved to discover the mechanism of oxidation of the substrate (Kudanga *et al.* 2011). A detailed comparison between different laccases showed that the T1 copper site

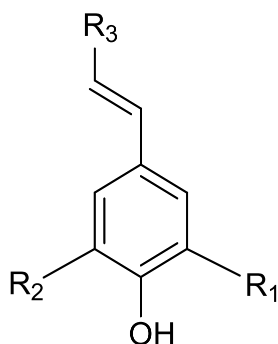
determined the redox potential of the enzyme (Xu 1996a; 2005). Although the redox potential is an important factor related to radical formation, the reaction is also affected by the structure of the substrate, the redox potential difference between a phenolic substrate and the T1 copper of a laccase, the pH optimum of the enzyme, substrate and product solubility, incubation time, and the presence of organic solvents (Hahn *et al.* 2014; Wan *et al.* 2010).

In this study seven phenols, coniferyl alcohol, coniferyl aldehyde, sinapic acid, ferulic acid, *p*-coumaric acid and gallic acid served as substrates for three laccases with middle or high redox potential in various solvent systems with the aim of a preferred production of dimers with lignan structure.

4.3 Material and Method

4.3.1 Chemicals

All phenolic substrates, coniferyl alcohol, coniferyl aldehyde, sinapic acid, *p*-coumaric acid, caffeic acid, ferulic acid and gallic acid (Figure 4-1) were provided from Sigma-Aldrich (Taufkirchen, Germany). 2,2`-Azino-bis (3–ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) was obtained from ICN Biochemicals (Muenchen, Germany). Ethyl acetate, acetonitrile, ethanol, and methanol were from Karl Roth (Karlsruhe, Germany), and solvents (all MS grade) used for GC-MS and HPLC-MS were from Carlo Erba Reactifs (Peypin, France).



	R_1	**R_2**	**R_3**
Coniferyl alcohol	OCH_3	H	OH
Coniferyl aldehyde	OCH_3	H	COH
Sinapic acid	OCH_3	OCH_3	COOH
Ferulic acid	OCH_3	H	COOH
p-Coumaric acid	H-	H	COOH
Caffeic acid	H	OH	COOH
Gallic acid	OH	OH	COOH

Figure 4.1 Phenolic substrates.

4.3.2 Enzymes

Laccase Abi from *Agaricus bisporus* (6.8 U mg^{-1}) and laccase C from *Trametes versicolor* (10 U mg^{-1}) were provided from Sigma-Aldrich (Taufkirchen, Germany) and laccase Ftr from *Funalia trogii* was isolated from fungal culture supernatants as described elsewhere (Esparan *et al.* 2015). The strain was purchased from the German Collection of Microorganisms and Cell Cultures (*Funalia trogii*, DSMZ).

4.3.3 Enzyme activity

The activity of each laccase was determined with ABTS as the substrate. The change in the absorbance was recorded at 420 nm using a Biotek Eon 2 Microplate reader (Biotek, Winooski, United States) at 30 °C. In brief, 15 µL of enzyme solution was mixed with 0.5mM substrate in 50 mM phosphate buffer at pH 3.0 in a total volume of 300 µL. The change in the absorbance was monitored over ten min. One unit of enzyme activity was defined as one µmol of substrate ($\varepsilon = 36{,}000$ L mol^{-1} cm^{-1}) oxidized per minute under the experimental conditions (Linke *et al.* 2013).

4.3.4 Oxidation of phenolic substrates by laccases

The oxidation reactions were carried out in a two solvent mixture (water plus an organic solvent) in a monophasic (methanol, EtOH, acetonitrile) or a biphasic system (ethyl acetate) with concentration of the organic solvent varying from 10 %, 50 % and 80 % (v/v). For both reaction systems, the mixture contained 10 mM of the respective phenolic substrate which was dissolved in the respective volume of each solvent (to obtain final volumetric ratio of 10, 50, and 80 % solvent) and mixed with aequeous (sodium phosphate buffer 100 mM, pH 5.0) enzyme solution containing 1.5 U mL^{-1} activity (ABTS assay). The reaction batch was stirred with glass magnet stirrer with 500 rpm at 35 °C under the same conditions for both mono and biphasic systems. Samples were taken every ten min. After continued shaking for 30 min, the reaction was stopped by adding 20 µL of HCl (5 mM); the reaction mixture was extracted three times with 1 mL EtAc which contained 25 ng μL^{-1} n-tetracosane as an external standard. Then, 100 µL of the organic layer were derivatized after evaporation of the solvent under nitrogen stream. For the silylation procedure 100 µL of a mixture of TMCS and BSTFA (1:99, v/v) were added and vortexed in screw cap glass tubes at 60 °C for one hour. To remove remaining silylating agent, methanol was added to the solution.

4.3.5 Gas chromatography flame ionization detection (GC-FID)

For each silylated sample, one µL was injected on-column in an Agilent 7890A gas chromatograph (Agilent, Waldbronn, Germany) equipped with a cool on-column injection port and a 30 m × 0.32 mm i. d. ×0.25 µm Optima 5 column (Macherey-Nagel , Dueren, Germany). The temperature program was from 40 °C to 250 °C with 2 °C/min, hold for 10 min; from 250 °C to 320 °C with 4 °C/min, hold for 15 min; from 325 °C to 350 °C with 3.5 °C/min and then hold for 5 min. A post run time of 10 min at 40 °C was found sufficient for re-conditioning of the column for the next injection. The flow rate of carrier gas hydrogen was maintained at 2 mL min^{-1}. Quantification was carried out according to the external standard n-tetracosane.

4.3.6 Gas Chromatography/Mass Spectrometry (GC-MS)

Gas chromatography-mass spectrometry (GC-MS) was conducted using a GC 8000 coupled to an MD 800 mass-selective detector (Fisons, Mainz-Kastel, Germany) equipped with a cool-on-

column injection port and a 30 m × 0.32 mm i.d. × 0.25 µm CP-Wax 52 CB column (Varian). The samples were injected using the same oven program as for GC-FID, but helium at a flow rate of 1.2 mL min $^{-1}$ was the carrier gas. Mass spectra were acquired using electron impact ionization at 70 eV, 200 °C source temperature and mass range from 70 to 817 (m/z) was used to detect all products. Reaction products were identified by comparing their linear retention indices (RI) and mass spectral data with those of authentic compounds or with spectra from spectral databases (Wiley NIST08, 2008).

4.3.7 Liquid Chromatography/Mass Spectrometry

For the detection of non-volatile oligomerization products of phenolic precursors, high performance liquid chromatography coupled to a triple quadrupole mass analyzer was used (Varian 212 LC pump, Pro Star 325 UV-Vis detector, 320 TQ-MS mass spectrometer). The MS was conducted simultaneously in the ESI positive and negative mode with a scan range of *m/z* 110–500 or *m/z* 300–1200, respectively. The MS parameters for ESI(+)/ESI(−) were: capillary voltage +30 V/−40 V, needle voltage 5000 V/−4500 V, nebulizer gas (N_2) 379 kPa, drying gas 207 kPa at 350 °C. For HPLC, water and acetonitrile (MS-grade), both containing 0.1 % formic acid, were used as the mobile phase. The following linear gradient was used: 10% acetonitrile for three min, up to 90 % acetonitrile within 20 min, hold for five min and back to start conditions. The separation was performed on an RP-18 HD column (Eurosphere 100-C18-5-HD, 250×4 mm, 5 µm, Macherey-Nagel) at a flow rate of 0.3 mL min^{-1}. Additionally, UV absorption was monitored at 280 and 425 nm.

4.4 Result and Discussion

Oxidation of phenolic substrates by laccases leads to radical intermediates (Figure 4.2). Depending on the type of substrate, the radicals react to quinones or cleavage products of the aromatic ring. With phenolic substrates, radical pairing creates a new C-C or C-O-C bond and connects two monomers (Hofer and Schlosser 1999). To overcome the problem of further ongoing crosslinking and polymerization, organic solvents were used to control the laccase activity in the reaction mixture (Mattinen *et al.* 2011; Krings *et al.* 2015). Solvents may affect an enzyme reaction by changing the actual concentration of educts or products, or by changing the tertiary structure of the enzyme; in the case of laccases the initial activity was lost upon small

changes of the three-dimensional structure (Rodakiewicz-Nowak *et al.* 2000). The data below show that suitable solvents direct the laccase-catalyzed reaction towards the concerted formation of dimers while the activity of the enzyme is maintained long enough to convert the substrate almost completely.

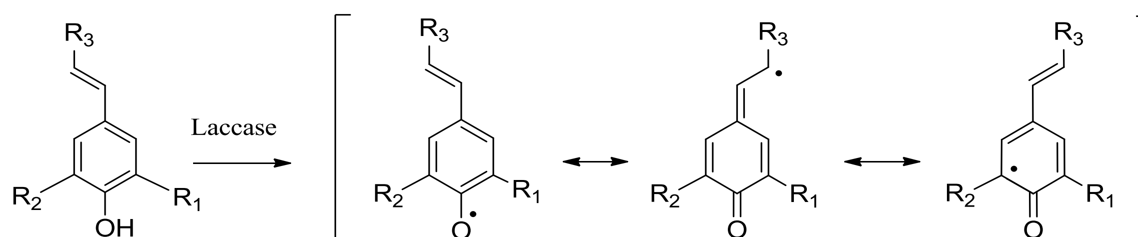


Figure 4.2 Oxygen and carbon centered radicals formed after incubation of phenolic substrates with laccases. Positions relevant for dimer formation are shown, only; R_{1-3} refers to Figure 4.1.

4.4.1 Oxidation of phenolic substrates in organic solvents

Phenolic substrates were dissolved in mono and biphasic systems to find reaction conditions favoring the production of lignin-type dimers instead of polymerization. First, the concentration of substrates and the enzyme activity were kept constant, and the nature of the organic solvent and the volumetric ratio (10, 50 and 80 % (v/v)) to water were modified. All organic solvents adversely influenced the initial enzyme activity regardless of the redox potential of the respective laccase. Water miscible organic solvents, such as methanol and acetonitrile, inactivated each of the three laccases over time. The fastest inactivation was found for laccase Abi which was already completely inactivated after 10 min in a 1:1 mixture of methanol with water. Based on this result, further experiments were carried out in EtOH-water (one phase system) or EtAc-water (biphasic system) mixtures, respectively. Table 4.1 shows the relative decrease of enzyme activity in 80 % EtOH and 80 % EtAc. The two high redox potential laccases (C, Ftr) retained some activity even at higher solvent concentration, and this activity was higher in the biphasic EtAc-water system than in EtOH-water. It should be noted that around 8 % w/v of ethyl acetate are soluble in water at 20 °C.

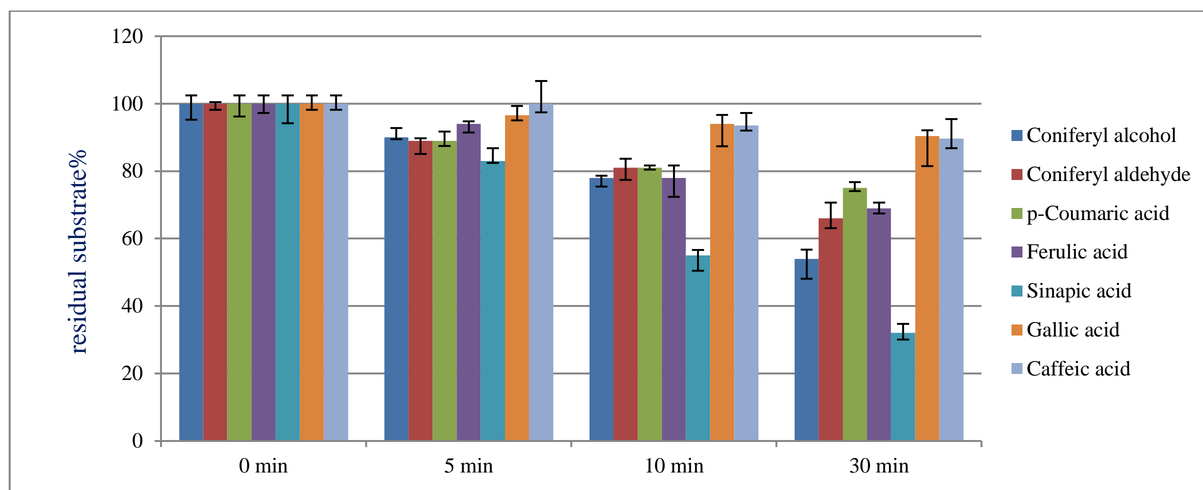
Table 4.1 Residual laccase (Lcc Abi, Ftr, C) activity according to the ABTS assay in 80 % EtOH / 20 % H_2O, and 80 % EtAc / 20 % H_2O (30 °C, after 0, 10, and 30 min). The numbers indicate the rate of oxidation (%) compared to the activity in buffer.

	Ethanol/water		
Enzyme	**0 min**	**10 min**	**30 min**
Abi	100%	1.9%	0
C	100%	12%	0.9%
Ftr	100%	25%	1.7%

	Ethyl acetate/water		
Enzyme	**0 min**	**10 min**	**30 min**
Abi	100%	9%	0.7%
C	100%	18%	7.2%
Ftr	100%	35%	12.4%

The impact of EtOH and EtAc in both mono and biphasic system on the laccase catalyzed oxidation of phenolic substrates is shown in Figures 4.3 a and b. The bars show the relative decrease of the respective phenolic substrate as quantified by GC-FID, compared to the concentration at t_0. The data are shown for laccase Ftr, only, because of its good stability in the organic solvents. The residual concentrations of substrates in both solvent systems showed that the consumption of substrates was faster in the biphasic system. For some phenolic substrates, such as ferulic acid, sinapic acid and coniferyl alcohol the oxidation was almost complete. Using the same laccase activity in the monophasic system, sinapic acid (> 30 %), coniferyl alcohol (> 50 %) and ferulic acid (around 70 %) remained in the reaction mixture. Analysis of the samples using HPLC-MS and GC-MS showed that in the monophasic system most of the phenolic substrates were converted to polymers (data not shown), whereas the reaction was directed to preferred production of targeted dimers in the biphasic system.

a)



b)

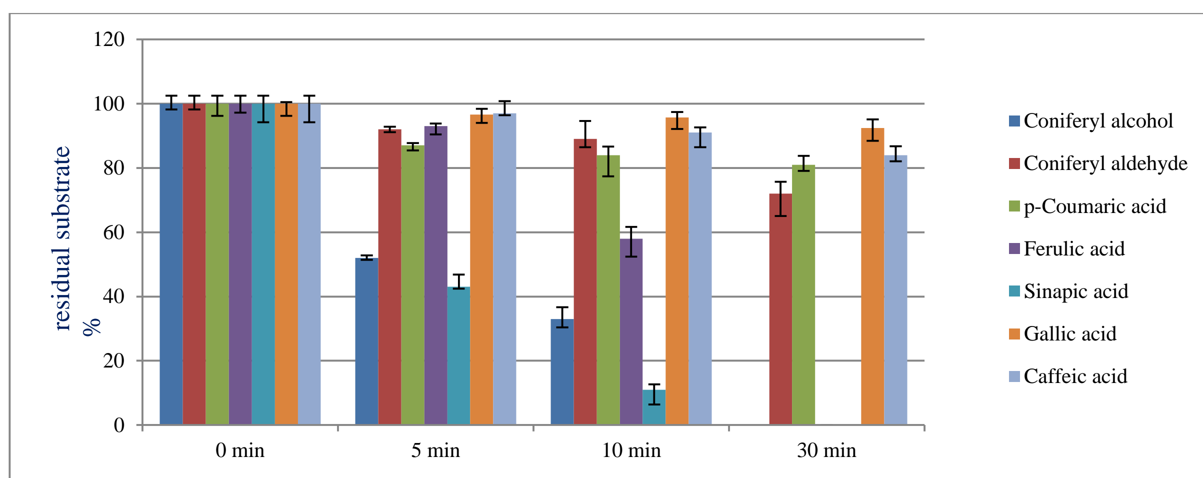


Figure 4.3 Reaction rate of the laccase catalyzed oxidation of phenolic substrates (laccase Ftr, 1.5 U mL^{-1}, at 30 °C) in a) monophasic two-solvent system (80 % EtOH in water (v/v)) and b) biphasic system (80 % EtAc in water (v/v))

Laccases possessing different redox potentials were compared for the oxidation of the phenolic substrates. The laccases from *Funalia trogii* and *Trametes versicolor* (C) were classified as high, and the commercial laccase from *Agaricus bisporus* (Abi) as laccase with middle range redox potential (Table 4.2) (Strong and Claus 2011). As predictable from the residual activity of laccases in the presence of 80 % EtAc or EtOH, respectively, the fastest oxidation reaction was found for laccases Ftr and C in the biphasic system. The initial rate of substrate reduction indicated that both high redox potential laccases oxidized the substrates at different oxidation

rates. The oxidation rate was faster using laccase Ftr than using laccase C, especially for coniferyl alcohol, ferulic acid and sinapic acid (Figure 4.4). The oxidation of *p*-coumaric acid, coniferyl aldehyde and caffeic acid proceeded more slowly, even with laccase Ftr: After 30 min 72 % of coniferyl aldehyde, 80 % of *p*-coumaric acid and 80 % of caffeic acid remained in the reaction solution. The results confirm that reaction kinetics and selectivity of laccases in terms of dimerization of phenolic substrates were different, although they possessed the same (high) redox potential. These differences are probably related to the nature of the enzyme and the ligand binding site of type 1 copper in these two laccases (Gunne *et al.* 2014). The solvent inactivation kinetics of the laccase Abi were too fast to give significant product yields after 30 minutes (Figure 4.4).

Table 4.2 Redox potential of laccases and phenolic substrates (Strong *et al.* Claus 2011)

Laccase[a]	Redox potential E° (V)
Lcc Abi	Middle (0.47-0.71 V)
Lcc Ftr	High (0.73-0.78 V)
Lcc C	High (0.73-0.78 V)

[a] according to ABTS enzyme activity (Gonza´lez *et al.* 2009)

Phenolic substrates pH 4.0	Redox potential E°(V)
p-Coumaric acid	0.70
Ferulic acid	0.66
Coniferyl aldehyde	0.63
Coniferyl alcohol	0.61
Sinapic acid	0.53
Gallic acid	0.37
Caffeic acid	0.37

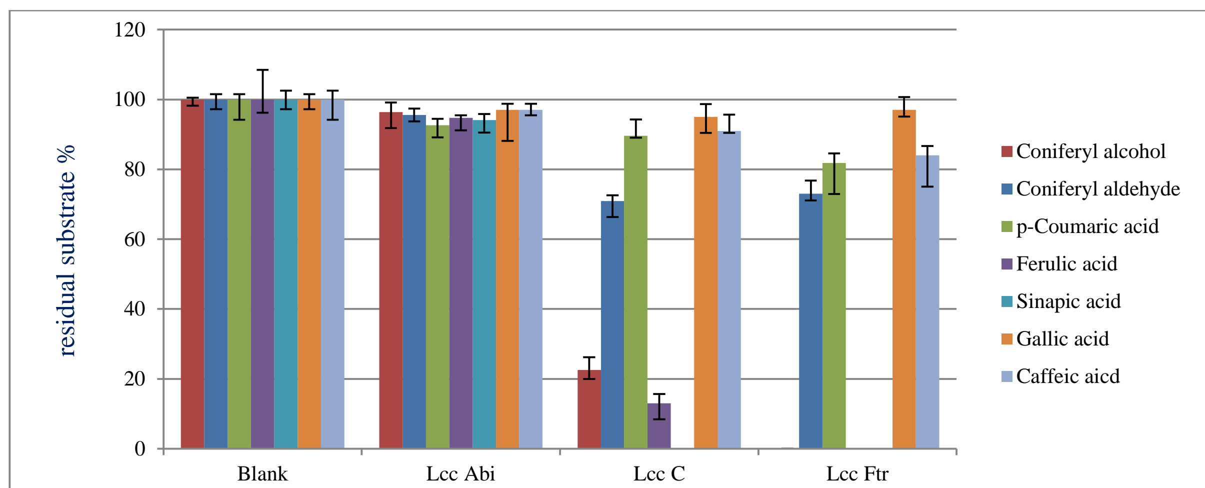


Figure 4.4 Oxidation of phenolic substrate in biphasic system (80 % EtAc in water (v/v)) by three laccases (Ftr, C, Abi), activity 1.5 U mL^{-1}, 30 min, 30 °C

4.4.3 Identification of products

Structural analysis of oxidized products was done by HPLC-MS and GC-MS (Table 4.3). Coniferyl alcohol was completely converted to dimers, while coniferyl aldehyde was converted more slowly. Analysis of products by mass spectrometry showed at least three dimers as major products from coniferyl alcohol. The main product was ascribed to the β-5 dimer with m/z 574, and the second biggest was pinoresinol with m/z 502. Pinoresinol is known as a phytoestrogen and taste modifier; moreover it is precursor to other lignans, such as matairesinol (Ito *et al.* 2002; Kuo *et al.* 2014). Compared to a previous study using a peroxidase for the dimerization of coniferyl alcohol or chemical oxidative reaction, the dimer with the β-O-4 structure (m/z 664) was not detected which is explained by the different mechanism of laccase Ftr (Figure 4.5 a, b) (Houtman 1999).

Oxidation of ferulic acid in the biphasic system (80 % EtAc) resulted in the formation of two dimers, and the substrate was completely converted when the incubation time was extended to 30 minutes. The largest peak in the GC-MS chromatogram showed a m/z ratio of 530 after silylation which was related to the β-β dimer or dehydrodiferulic acid. Dehydrodiferulic acid which is the

main product of this reaction can be used as precursor of matairesinol-type lignans and has application in medicine, food and beverage industry (Backes *et al.* 2014; Takei *et al.*1972). The second peak represented the β-5 dimer of ferulic acid (Figure 4.5 c) (Carunchio *et al.* 2001). This dimer is known for its high antioxidant activity, with possible application in the health and cosmetic industries (Sanchez-Moreno *et al.* 1998). In contrast, oxidized products with higher molecular masses were detected in the mono phasic systems, even in 80 % EtOH, by LC-MS.

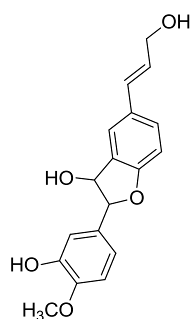
Sinapic acid was completely converted by laccase Ftr in the biphasic system. After 10 min only 10 % of residual substrate was detected, and after 30 min, it was completely oxidized in the biphasic system. In the oxidation reaction at pH 5, one product with a molecular ion 590 (m/z) was detected which was tentatively attributed to dehydrodisinapic acid dilactone (Figure 4.5 d) (Wang *et al.* 2004). Comparing the decrease of sinapic acid with dimerization products, the substrate was completely converted to the dimer. Sinapic acid oxidation by a peroxidase from *Momordica charantia* was shown to produce the lactone type dimer at pH 5, but more than one product was detected in case of the peroxidase (Liu *et al.* 2007). Sinapic acid bears two methoxy groups *ortho* to the phenolic hydroxyl group, which prevents docking of a radical partner.

The oxidation of *p*-coumaric acid was very slow even with the high redox potential laccases (Koschorreck *et al.* 2008). Gallic acid with its three hydroxyl groups at the aromatic ring was not even oxidized by high potential laccases in the biphasic system. The residual activity of laccase in solution after the reaction showed that gallic acid, adding to the inactivating effect of the organic solvent, inhibited all three laccases completely after 30 minutes. Caffeic acid solution showed the lowest laccase activity after 30 min of incubation confirming that other factors, such as the immediate inhibitory characters of substrates will also affect the activity of laccases (data not shown).

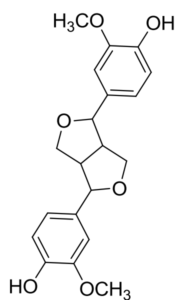
Table 4.3 Dimers identified after incubation of phenolic substrates with laccase Ftr (fragmentation of silyl-derivatives of dimers (1.5 U mL^{-1}, 30 min, 30 °C)

Substrate	molecular ion (m/z)	dimer type
Ferulic acid	530,530	(β- β), (β-5)
Coniferyl alcohol	574, 502, 634	(β-5), (β-β),(β-O-4)
Coniferyl aldehyde	498, 498, 426	(β-5), (β-β),(β-O-4)
Sinapic acid	586	(β-β)
Gallic acid	no dimers	-
p-Coumaric acid	no dimers	-

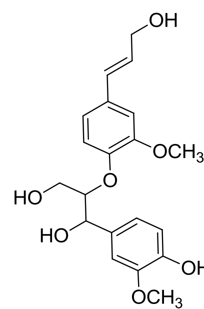
a)



ß-5 dimer

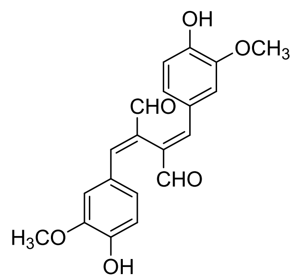


ß-ß dimer

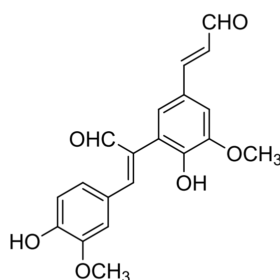


ß-O-4 dimer

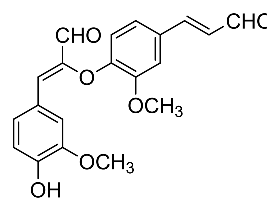
b)



ß-ß dimer



ß-5 dimer



ß-O-4 dimer

c)

d)

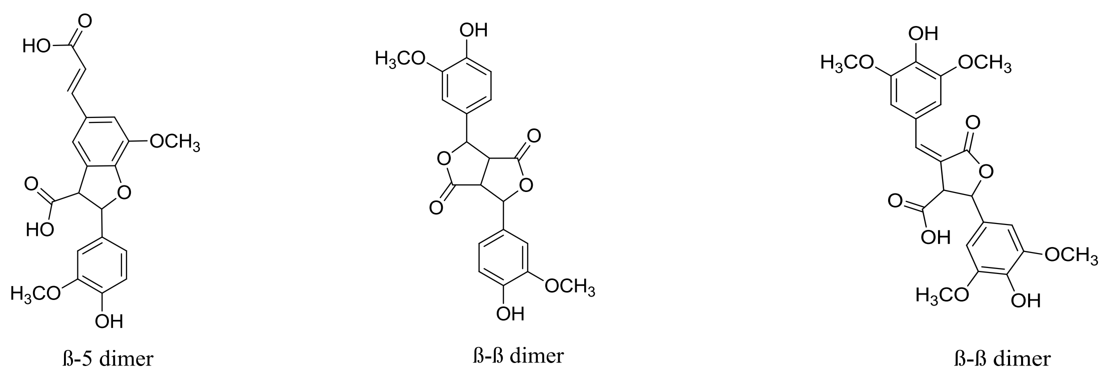


Figure 4.5 Dimers identified after incubation with laccase Ftr in a biphasic system (80 % EtAc / 20 % H_2O (v/v). a) coniferyl alcohol, b) coniferyl aldehyde, c) ferulic acid, d) sinapic acid

4.5 Conclusion

Lignans may gain importance for foods and beverages, because they can act as taste modifiers to cover bitter and astringent notes, as anti-oxidants, anti-cancer, anti-viral and anti-inflammatory compounds (Ríos *et al.* 2002). As isolation of pure lignans from plant sources is difficult, the laccase catalyzed dimerization of easily available phenolic precursors offers an alternative access. Two high and a middle redox potential laccase were investigated in mono and biphasic systems to increase the solubility of the substrates and to prevent polymerization. By selecting an appropriate solvent system and enzyme, the reaction was found to yield more than 95 % of desired products. As in the case of divanillin formation (Krings *et al.* 2015), more refined reaction conditions and subsequent chemical or enzymatic modifications may open access to sought-after lignans, such as pinoresinol or matairesinol.

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5. Outlook

Beside many well-known bacterial and yeast strains, fungi, especially basidiomycetes are able to synthesise potentially valuable flavour or fragrance compounds by whole-cell processes or isolated enzyme. Because of the economic challenges for the development of whole-cell process, research interest is shifting more and more to the enzyme catalysts reaction.

In this thesis, new enzymatic route for the production of vanillin, divanillin and lignans was investigated. Although, the products were promising but further investigation for the production of these products in the industrial scale is needed.

Additionally to this work which focused on the biotransformation of cheap substrates to valuable products such as vanillin, divanillin and lignans, high redox potential laccases from basidiomycetes, were found as promising candidate for biotransformation reaction especially where, organic solvents because of the high concentration of substrate are required.

A better understanding of the fungal biochemistry, screening of new isolated enzymes, optimizing reaction condition and supplementing with cheap and natural precursors substrates for enzymatic reactions, could open the way to high-yielding processes for the future work.

Meanwhile, for the future scientific interest, choosing appropriate substrate which has similar phenolic residues and double bounds in their structure such as carotenoids and capsaicin, with using same biotransformation system and isolated enzyme, can be an alternative for the production of valuable natural flavour and taste modifier.

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7. Curriculum vitae

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