Lipoxygenase;
a game-changing enzyme

Ruud Heshof

Thesis committee

Promotors
Prof. Dr W.M. de Vos
Professor of Microbiology
Wageningen University

Prof. Dr V.A.P. Martins dos Santos
Professor of Systems and Synthetic Biology
Wageningen University

Co-promotor
Dr L.H. de Graaff
Associate professor, Systems and Synthetic Biology
Wageningen University

Other members
Prof. Dr W.J.H. van Berkel, Wageningen University
Prof. Dr F.P.M. Govers, Wageningen University
Prof. Dr D. de Ridder, Wageningen University
Dr R. de Jong, DSM, Delft

This research was conducted under the auspices of the Graduate School VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences).

Lipoxygenase;
a game-changing enzyme

Ruud Heshof

Thesis
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Academic Board,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Monday 1 June 2015
at 11 a.m. in the Aula.

"Our greatest weakness lies in giving up. The most certain way to succeed is always to try just one more time."

Thomas A. Edison

Table of contents

Chapter 1

Introduction

We now have reached an era in which a transition takes place from a petroleum-based economy towards a biobased economy. The foundation of a biobased economy is the conversion of biological waste into valuable chemicals and energy. Biological waste streams, like plant based agricultural waste, contain many sugars that can be used to grow microorganisms. These microorganisms can be developed to cell-factories for the production of all sorts of chemicals and proteins. Plant waste residues also contain polyunsaturated fatty acids (PUFAs), which are very valuable raw materials that can be used by industry for production of biopolymers such as plastics. Since the biobased economy concept is relatively young, new processes have to be developed to make this type of economy viable for industry.

I aim to contribute to the development of novel concepts for a biobased economy by exploring the potential of lipoxygenase (LOX) for the production of valuable chemicals. LOX catalyzes the dioxygenation of PUFAs, thereby rendering them suitable for further degradation reactions into oleochemicals. These oleochemicals can be used as building blocks for the chemical industry. Different oleochemicals can be formed depending on the position of the introduced peroxide group to the PUFA. Large quantities of LOX, however, are needed for this process to become industrially relevant. Therefore we aimed to use *Aspergillus niger* as an expression host. *A. niger* is a fungus used by industry to generate organic compound like citric acid, and can be used for production of enzymes. *A. niger* is also capable to consume the sugar xylose, which is abundant in plant waste material. Therefore the degradation of plant waste material and the LOX enzyme production can in principle be performed in one step, which is an important contribution to the concept of a biobased process.

This thesis is a result of an approach to contribute to a sustainable environment using LOX. To achieve this goal it is important to ascertain the biological role of LOX. This identification assesses the potential that this enzyme can have in industrial processes. These different potentials are described in **Chapter 2**. Also the current state of heterologous production of LOX using microorganisms is discussed.

Since LOX is abundant in the kingdom of life, a good selection of LOXs is needed for heterologous expression. For this selection of LOXs a bioinformatics script was written as discussed in **Chapter 3.** We identified a novel class of fungal LOXs that we wanted to exploit for its potential in a biobased economy. Four potential candidates were selected: *Pleurotus sapidus* LOX, two *Aspergillus fumigatus* LOXs, and *Gaeumannomyces graminis* LOX. These LOXs were chosen for their potential in food industry, to investigate the potential pathogenic role in lung tissue, and the potential in plastic production respectively.

To select the desired transformants of *A. niger* expressing different *lox* genes high-throughput assays are needed. In **Chapter 4** several plate assays are discussed, which could serve as valuable assays in selecting wanted transformants. Using this screening method, hundreds of clones can be screened simultaneously for LOX expression and can be applied to different expression hosts. Since the results of the described potassium iodide assay were negative for *A. niger*, we decided to switch to *Aspergillus nidulans* as an expression host. In this thesis we show these plate assays are effective using expression hosts such as *Escherichia coli*, *A. nidulans*, *P. pastoris*, and *Trichoderma reesei*.

Chapter 5 describes the results using *A. nidulans* as an expression host for heterologous production of LOX. Unfortunately, *A. nidulans* did not give the results we hoped for. Therefore we investigated the cause for the ineffective use of *A. nidulans* in producing LOX at various levels. We analyzed the LOX production on mRNA level, we used Western blotting techniques to identify small quantities of LOX, and eventually used a proteomics approach to verify the presence of LOX. We concluded *A. nidulans* produces proteases able to degrade the LOX. Thus making *A. nidulans* unsuitable for the production of LOX in high quantities.

Finally, in **Chapter 6** we reflect on the steps taken during this thesis and analyze what would be a better approach to produce LOX in high quantities. We discuss the use of LOX in a biobased economy in a broader perspective, *e.g.* the origin of the needed PUFA for LOX. Also it is discussed how enzyme engineering can help in making LOX more specific or even broaden its use in the production of oleochemicals.

This thesis research is combined with an ERA-IB (European Research Area in Industrial Biotechnology) collaboration of six partners in Europe. The project was called "Novel enzyme tools for production of functional oleochemicals from unsaturated lipids" (ERA-NOEL). The project partners worked in two areas: one on the biochemical aspects and one on the oleochemical and downstream processing aspects. AB Enzymes in Germany, VTT in Finland, and Wageningen University in the Netherlands were the partners responsible for heterologous expression of LOX by using different fungal expression hosts. Where AB Enzymes worked with *T. reesei* and VTT worked with *P. pastoris*, Wageningen University was responsible to investigate the potential of *A. niger*. in heterologous expression of LOX. Aarhus University in Denmark and the University of Aveiro in Portugal were responsible for the chemical procedures in making oleochemicals suited for industry with use of LOX. Finally, Fraunhofer Institute in Germany was responsible for the chemical upscaling and downstream processing. After three years of collaboration we were able to manufacture chunks of plastic that

can be used to produce plastic products. This illustrates the basic concept of the project: that using fatty acids from plant waste material plastics can be produced by use of LOX as is shown in Fig. 1.

a   b
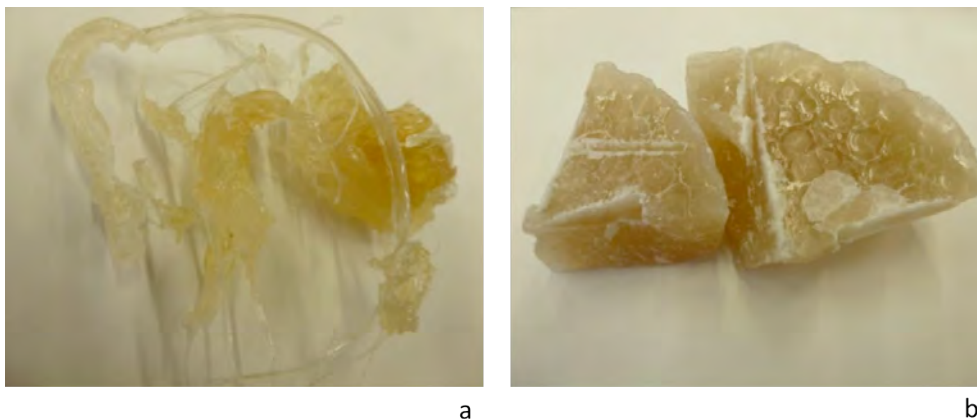
Figure 1: Plastics produced by from the reaction products of linoleic acid and LOX. The chunks had a size varying from 5 cm to 10 cm and can be used as start material to be moulded into plastic products. a) Product of dodecanedioic acid with a diamine and b) dodecanedioic acid with PEG 1000. The pictures are from the final report of the ERA-NOEL project. Pictures are courtesy of Prof. J. Buchert, final report ERA-NOEL project 053.80.702

13

Chapter 2

# Industrial potential of lipoxygenases

Abstract

Lipoxygenases (LOXs) are iron- or manganese-containing oxidative enzymes found in plants, animals, bacteria, and fungi. Lipoxygenases catalyze the oxidation of polyunsaturated fatty acids to the corresponding highly reactive hydroperoxides. Production of hydroperoxides by LOX can be exploited in different applications such as in bleaching of coloured components, in modification of lipids originating from different raw materials, in production of lipid derived chemicals, and in production of aroma compounds. Most application research has been carried out using soybean LOX, but currently also the use of microbial LOXs has been reported. Development of LOX compositions with high activities by *e.g.* heterologous expression in suitable production hosts would enable full exploitation of the potential of LOX derived reactions in different applications. Here we review the biological role of LOXs, their heterologous production, as well as potential uses in different applications. Lipoxygenases may fulfil an important role in the design of processes that are far more environmental friendly than currently used chemical reactions. Difficulties in screening for the optimal enzymes and in producing LOX enzymes in sufficient amounts prevent large-scale application so far. With this review we summarize the current knowledge of LOX enzymes and the way in which they can be produced and applied.

17

1. Lipoxygenase and its biological role

Lipoxygenases (LOX) are found in a wide variety of organisms throughout the kingdoms of *Planta*, *Animalia*, *Bacteria,* and *Fungi* (Nemchenko et al. 2006, Gilbert et al. 2011, Oliw 2002, Hansen et al. 2013). LOXs are iron- or manganese-containing dioxygenases that catalyze the conversion of unsaturated fatty acids (UFA) into the corresponding hydroperoxides called oxylipins (Mita et al. 2007). These oxylipins have important signalling functions in many organisms, though the language of oxylipins is not fully understood. Fungal LOXs are involved in interaction between plants and fungi by using the LOX signalling pathway to infect plant tissue (Oliw 2002, Tsitsigiannis et al. 2005). Oxylipins are also reported to have a role in antimicrobial or defence mechanisms in plants and animals, in cell division in animals, and to play a role in seed germination in plants (Prost et al. 2005, Peters-Golden 1998, Feussner et al. 2001, Pidgeon et al. 2007). The phylogenetic relation of LOXs has been investigated using bioinformatics as shown in Fig. 1 (Heshof et al. 2014a). Based on this analysis it was found LOXs can be divided into two groups: plant LOXs vs. other LOXs. LOXs can be subdivided into classes based on the C-atom they oxygenate in the UFA. For example, a 9-LOX generates the hydroperoxide group to C-9 position of

the substrate UFA. The main difference between LOXs of plant and animal origin is their specificity. Plant LOXs consist of 9- and 13-LOXs and mainly use linoleic acid as a substrate, while animal LOXs consist of 5-, 8-, 12-, and 15-LOXs and use arachidonic acid as a substrate (Brash 1999, Tsitsigiannis et al. 2007). Compared to plant and animal LOXs, LOXs from microbial origin have gained less attention. However, some LOXs from microbial origin are identified as 10- and 11-LOXs using the substrates oleic and linoleic acid respectively (Vidal-Mas et al. 2005, Su & Oliw 1998). LOXs can produce enantiomeric specific oxylipins and the substrate can enter the binding-pocket in head-first or tail-first position (Coffa et al. 2005). Therefore thirty-two different oxylipins can be generated (Coffa et al. 2004). A schematic overview of the structures of the oxylipins produced by LOXs is shown in Fig. 2.
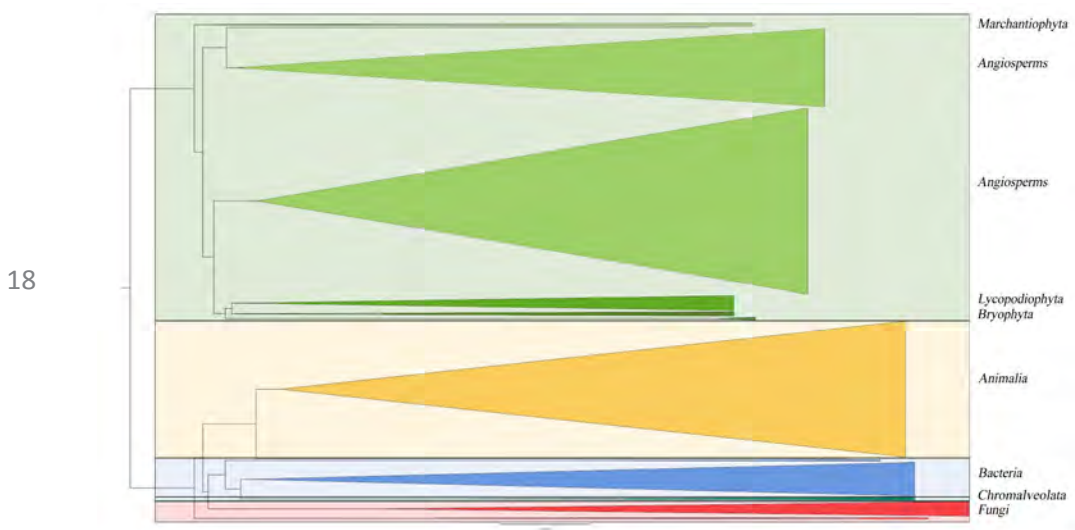
Figure 1: A schematic presentation of the phylogenetic relationship of LOXs throughout the kingdom of life. *Planta* LOXs are coloured in light green and divided into subclasses: *Marchantiophyta*, *Angiosperms*, *Lycodiophyta*, and *Bryophyta*. In yellow *Animalia* LOXs, in blue *Bacteria* LOXs, in dark green a *Chromalveolata* LOX, and in red *Fungi* LOXs. This figure is reproduced with permission of the publisher from the original paper (Heshof et al. 2014a).
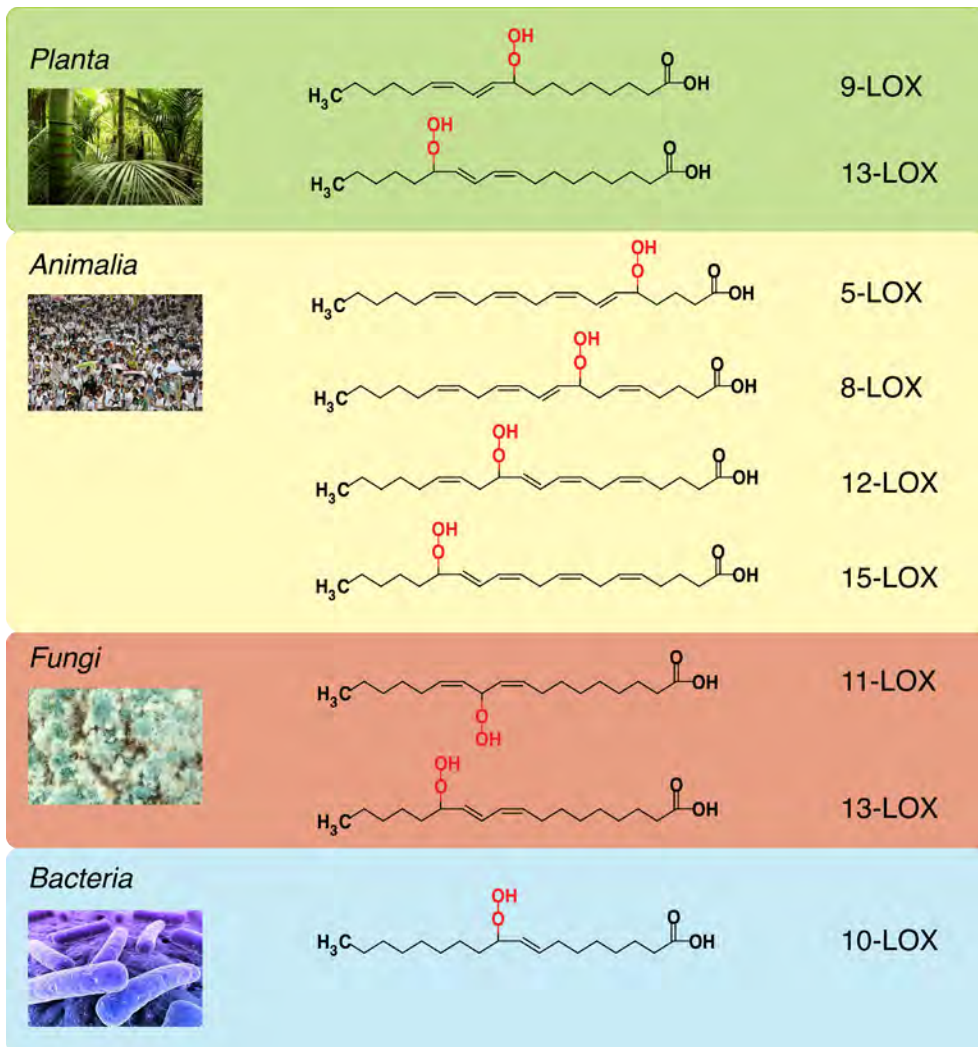
Figure 2: Overview of different oxylipins that are produced by LOXs from various origins. *Planta* use linoleic acid for 9-, and 13-LOXs, *Animalia* use arachidonic acid for 5-, 8-, 12-, and 15-LOXs. In addition to these LOXs the *Fungi* kingdom revealed a so far unique 11/13-LOX, and in the *Bacteria* kingdom a 10-LOX was identified using oleic acid as a substrate.

1.1 Lipoxygenase in *Planta*

In *Planta* two types of LOXs are found. These LOXs are called *type 1* and *type 2* LOXs. *Type 1* LOXs consist of cytosolic 9- and 13-LOXs and these LOXs share high similarity with each other. *Type 2* LOXs only consist of 13-LOXs and have a putative chloroplast targeting sequence (Nemchenko et al. 2006). Plant LOXs have a function in seed maturation by converting storage lipids to acetyl–CoA to be used as carbon feedstock by the seed. LOXs also have a role in defence mechanisms due to the production of jasmonic acid, which in turn functions as a signal molecule in wound healing and programmed cell death (Howe et al. 2008, Mosblech et al. 2009, Reinbothe et al. 2009). Initial steps in jasmonic acid production are caused by LOX as the hydroperoxide fatty acids produced by 13-LOX are further converted to 12-oxo phytodienoic acid (OPDA) and finally reduced *via* β-oxidation to jasmonic acid (Mosblech et al. 2009). An antimicrobial defence mechanism is induced *via* hydroperoxides produced by cytosolic 9-LOXs (Blee 2002, Hughes et al. 2009, Vellosillo et al. 2013). For example, in *Nicotiana tabacum* down-regulation of 9-LOX causes infection by *Phytophthora parasiticus* while over expressing this 9-LOX results in higher resistance against the pathogen (Rance et al. 1998, Mene-Saffrane et al. 2003). The susceptibility of microorganisms towards different oxylipins was investigated by Prost *et al.* (Prost et al. 2005).

1.2 Lipoxygenase in *Animalia*

LOXs are present as 5-, 8-, 12-, and 15-LOXs in the majority of *Animalia* such as *Homo sapiens*, *Sus scrofa*, *Oryctolagus cuniculus*, and in the coral *Plexaura homomalla*. (Gilbert et al. 2011, Xu et al. 2012, Choi et al. 2008, Oldham et al. 2005). *Animalia* LOXs and their oxylipins play a role in the biosynthesis of leukotrienes and in the cell cycle (Lewis et al. 1990, Tang et al. 2002, Pidgeon et al. 2007). Inhibition or overexpression of *Animalia* LOXs can lead to carcinogenesis, atherosclerosis, neurotransmission, allergic rhinitis, astma or osteoporosis (Toledo et al. 2011, Jiang et al. 2003, Kandouz et al. 2003, Duroudier et al. 2009, Yoshimoto et al. 2002, McAlexander et al. 1998, Plewako et al. 2006, Dahlen et al. 1998, Serhan et al. 2004). Thus the role of LOXs and their oxylipins in *Animalia* is being studied intensively in order to prevent these diseases (Riccioni et al. 2009, Hammond et al. 2012, Shureiqi et al. 2001, Tang et al. 2002).

1.3 Lipoxygenase in *Bacteria*

LOXs are found in *Bacteria* although they are not very abundant. So far LOXs have been found in Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Cyanothece* sp., *Anabaena* sp., and *Myxococcus xanthus* (Vidal-Mas et al. 2005, Andreou et al. 2010, Hansen et al. 2013, Zheng et al. 2008, Goldman et al. 2006). The only Gram-positive bacteria known so far to have LOXs are *Streptomyces canosus* and *Streptomyces massasporeus* (Hansen et al. 2013). A secreted LOX from *P. aeruginosa* has been successfully expressed in *Escherichia coli* and its structure has been solved (Vidal-Mas et al. 2005, Garetta et al. 2013). This LOX is known to function as a 10-LOX with oleic acid as a substrate, but also as a 13-LOX with linoleic acid, and a 15-LOX with arachidonic acid as a substrate (Vance et al. 2004, Vidal-Mas et al. 2005, Garetta et al. 2013).

1.4 Lipoxygenase in *Fungi*

In *Fungi* genes encoding extracellular LOXs have been found in *Gaeumannomyces graminis, Aspergillus fumigatus*, *Magnaporthe salvinii*, *Magnaporthe grisea*, and *Botryotinia fuckeliana* (Hörnsten et al. 2002, Nierman et al. 2005, Wennman et al. 2013, Dean et al. 2005, Amselem et al. 2011). Although their function is still speculative, it is hypothesized that fungal LOXs can interfere with plant signalling cascades (Rance et al. 1998, Mene-Saffrane et al. 2003, Gao et al. 2008). For example, the pathogenic fungus *G. graminis* secretes a manganese-containing 11/13-LOX, which is hypothesized to interfere with the host-cell signalling cascade upon infection (Su et al. 1998, Oliw 2002). Also, oxylipins produced in *Aspergillus flavus* are suggested to act as quorum sensing signals (Tsitsigiannis et al. 2007, Horowitz Brown et al. 2008).

21

2. The structure of lipoxygenases

The 3D structures of LOXs originating from *Glycine max*, *O. cuniculus*, *P. homomalla*, *H. sapiens*, *S. scrofa, Gersemia fructicosa*, and *P. aeruginosa* have been determined (Minor et al. 1996, Skrzypczak-Jankun et al. 1997, Gillmor et al. 1997, Oldham et al. 2005, Gilbert et al. 2011, Xu et al. 2012, Eek et al. 2012, Garetta et al. 2013). These LOXs generally consist of two domains: an antiparallel N-terminal β-barrel domain and a helical catalytic domain, shown in Fig. 3 (Kuhn et al. 2005, Ivanov et al. 2010). The N-terminal β-barrel of LOX shows homology with pancreatic lipase bound to thylakoid membranes (Ivanov et al. 2010, Chahinian et al. 2000, Emek et al. 2013). The role of the β-barrel in membrane binding and its role in catalytic properties of the

enzyme has been elucidated by its deletion from LOXs originating from *H. sapiens*, *Mus musculus*, *Macaca mulatta*, and *Pongo pygmaeus*. Although the N-terminal β-barrel was not necessary for membrane binding, binding capacity to membranes of mutants was lower. The mutant LOXs showed decreased activity as compared to wild type LOXs, whereas product specificity was not changed (Walther et al. 2011). LOXs devoid of N-terminal β-barrel have also been identified in *e.g. P. aeruginosa* and *Anabaena* sp. (Garetta et al. 2013, Zheng et al. 2008). These LOXs are called mini-LOXs due to their relatively low molecular weight (Zheng et al. 2008). Instead of an N-terminal β-barrel the *P. aeruginosa* LOX contains an extension of about 100 amino acid residues that forms a flexible lid covering the entrance to the binding pocket (Garetta et al. 2013). Another example is the *Anabaena* sp. LOX that is fused to a catalase-like hemoprotein (Zheng et al. 2008). Deletion of this catalase-like hemoprotein did not affect the LOX activity when expressed in *E. coli*.
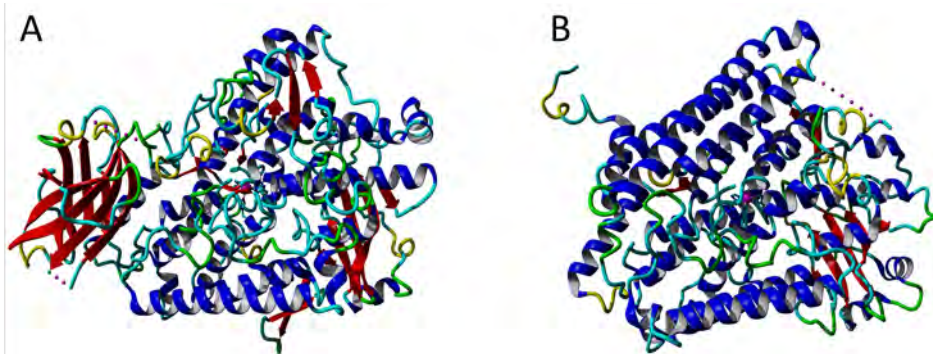
Figure 3: 3D-models of the (A) *Glycine max* (PDB 3PWZ) and (B) *Pseudomonas aeruginosa* (PDB 4G33) LOX. The *G. max* LOX has a membrane binding β-barrel, whereas the *P. aeruginosa* LOX has a removable lid covering the entrance over the binding pocket.

The quaternary structure of the catalytic domain of LOXs consists mainly of α-helices in which the catalytic centre and the substrate-binding pocket are located (Ivanov et al. 2010). The proposed reaction mechanism of LOX is schematically drawn in Fig. 4 (Zoia et al. 2011). The cofactor is activated from Fe^{2+} to Fe^{3+} using its product or atmospheric O_2 (Gardner et al. 1991). The Fe^{3+} cofactor binds to the PUFA (1), abstracts a hydrogen, and changes to Fe^{2+}, which results in a radical formation on the PUFA (2). The radical rearranges with a double bond (3), and oxygen can be inserted (4). *Via* peroxy radical reduction (5) the hydroperoxy fatty acid product is formed. This results in active Fe^{3+}, which creates a chain reaction effect. The cofactor can be inactivated to its Fe^{2+} state by free radical decomposition that occurs when oxygen becomes the rate-limiting step, which was investigated by Zoia *et al.* (Zoia et al. 2011). They

determined the amount of radical products by performing the LOX reaction with and without oxygen. Their findings show that a lower oxygen concentration leads to an increase of free radicals formed in the reaction solution.
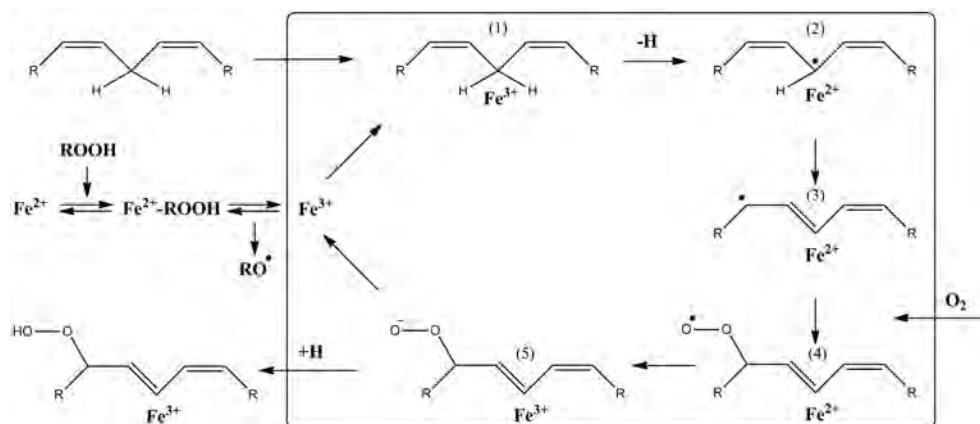
Figure 4: Schematic overview of the proposed reaction mechanism of LOX (Zoia et al. 2011). The reaction consists of active Fe^{3+} that binds to the PUFA (1), hydrogen abstraction (2) radical rearrangement (3), insertion of oxygen (4), and peroxy radical reduction (5). The reaction is stopped when oxygen or substrate becomes limiting. The cofactor will then turn to its resting state Fe^{2+} *via* free radical decomposition.

The cofactor in the catalytic domain is held in place by a water molecule and five amino acids; three histidines, a fourth serine, histidine or asparagine, and a fifth C-terminal amino acid as shown in Fig. 5 (Brash 1999, Skrzypczak-Jankun et al. 1997). The cofactor is usually iron, but the secreted LOXs from the fungi *G. graminis*, *M. salvinii*, and *A. fumigatus* have manganese as a cofactor (Cristea et al. 2005, Wenmnan et al. 2012, Heshof et al. 2014a). The substrate-binding pocket is located near the cofactor-binding site (Minor et al. 1996). Site-directed mutagenesis of the binding pocket revealed that conserved amino acids alanine or glycine in the so-called Coffa-Brash site are involved in product specificity. An alanine in Coffa-Brash site will result in an *S*-enantiomer oxylipin while a glycinecine will result in an *R*-enantiomer oxylipin (Coffa et al. 2005). Exceptions of this rule, however, are known in *e.g.* the *Danio rerio* LOX (Jansen et al. 2011). Sloane *et al.* identified amino acids that are involved in the product specificity of human 15-LOX. A M418V mutation resulted in a conversion of 15-LOX to a 12/15-LOX with an equal ratio of oxylipin-products (Sloane et al. 1991). By mutating the neighbouring amino acids Q416K and I417A the 15-LOX was converted to a 12-LOX with a ratio of 15:1. Borngräber *et al.* discovered that a phenylalanine at position 353 in the rabbit 15-LOX forces the substrate to bend in the substrate-binding pocket. By introduction of a F353L mutation the substrate is able to get deeper into the binding-pocket, resulting in a 12-LOX (Börngraber et al. 1996).

23
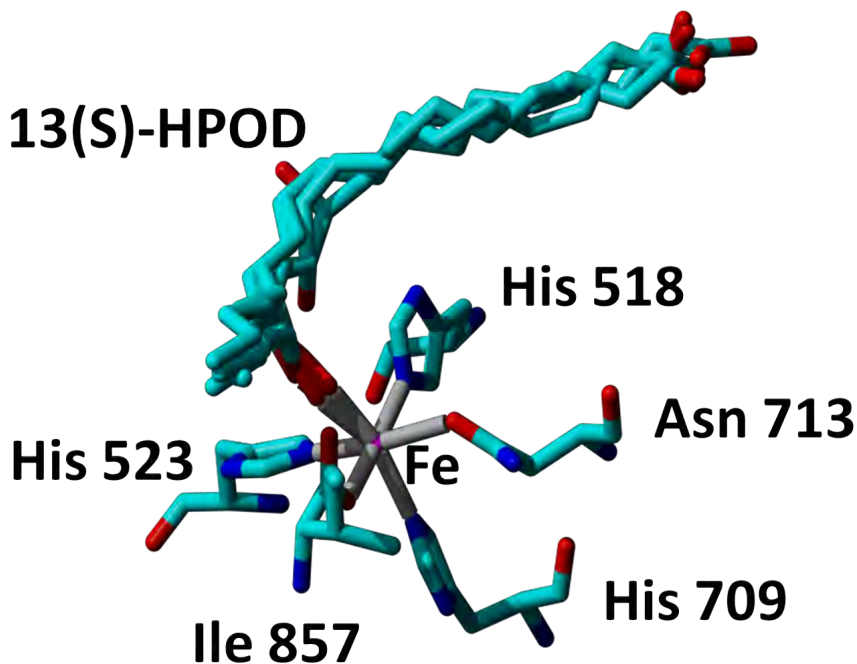
Figure 5: 3D-model of the catalytic site of the *Glycine max* LOX (PDB 1IK3) in complex with 13(S)-hydroperoxy-9(Z),11(E)-octadecadienoic acid (13(S)-HPOD). The iron cofactor is held in place by three histidines, an asparagine, an isoleucine, and a water molecule. The substrate linoleic acid substitutes the water molecule and binds to the iron cofactor. When this complex has been formed oxygen will attach to the substrate generating 13(S)-HPOD.

3. Expression and production of LOX in microbial hosts

So far there are only a few reports of the successful heterologous production of LOXs in microbial hosts. Several attempts have been made to express LOXs from a eukaryotic origin in prokaryotic hosts such as *E. coli*. As extracellular fungal LOXs are glycosylated, the prokaryotic expression system is not the preferred one as only few bacteria can glycosylate proteins (Nothaft et al. 2010). However, plant LOX-2 and LOX-3 from *Pisum sativum*, and human reticulocyte-type 15-LOX have been successfully expressed in *E. coli* (Hughes et al. 1998, Walther et al. 2002). The extensively glycosylated *G. graminis* LOX could not be expressed in active form in *E. coli*, whereas its expression was successful using *Pichia pastoris* as an expression host with yields of 30 mg/l (Cristea et al. 2005). Knust and Wettstein tried the extracellular production of pea-seed LOXs in *Saccharomyces cerevisiae* by using the yeast invertase secretion signal. Despite the addition of a secretion signal, most of the enzyme remained inside the cell in an inactive form (Knust & Wettstein 1992). Several bacterial LOXs

have been successfully expressed in prokaryotes. As claimed by Lu *et al.* the highest level of expressed LOX so far is 3.89 U/ml and has been achieved by expressing *P. aeruginosa* LOX in *E. coli* (Lu et al. 2013). Most heterologous production of LOXs in *E. coli* is performed at temperatures ranging from 8°C – 20°C. This low temperature can cause additional production costs in up-scaling the production process due to the cooling energy that is required for fermenters during growth of the expression host. An overview of successful heterologous expression of LOXs is given in Table 1. The limited number of examples present in scientific and patent literature is indicative that the expression of LOX of any origin in microbial hosts is not a trivial matter. The success of expression of LOX can probably be increased by using a host that is genetically closely related to the organism that produces the enzyme. Also the biological role of the LOX produced oxylipins as precursors for signal molecules, *e.g.* inducing cell death or cell division, can be anticipated to interfere with the expression system used (Heshof et al. 2014b). Extracellular production of LOX would thus be beneficial and this can be achieved by selecting a naturally extracellular LOX or, alternatively incorporating a secretion signal to the LOX protein. Recent developments in synthetic biology will further contribute to improved expression levels. The use of BioBricks allows rapid screening of codon-optimized constructs that carry promoter or secretion signal variants (Shetty et al. 2008). Mutagenesis of the LOX of interest can be a useful strategy in production of specific oxylipins. This can be achieved by changing amino acids affecting the substrate specificity (Sloane et al. 1991). Alternatively the catalytic activity of the molecule can be altered by removing the N-terminal β-barrel, either by tryptic digestion or genetic modification. Resulting mini-LOXs are catalytically complete, but seem to be more flexible and less stable (Di Venere et al. 2003, Zheng et al. 2008). Although the N-terminal β-barrel is considered to be non-essential for enzymatic activity, it may have other functions, *e.g.* in membrane binding or calcium binding (Walther et al. 2002). In all cases, successful production of industrial quantities of this enzyme class will require more fundamental research and also needs improvements in bioprocess engineering.

25

Table 1: Overview of heterologous expressed LOXs in different expression systems. The LOXs can be classified as 5-, 8-, 9-, 10-, 11-, 12-, 13-, or 15-LOXs. The number indicates the attachment of the peroxide group to the fatty acid. The ValLOX represents a LOX that uses valencene as a substrate.

| LOX-type	LOX host organism	Expression organism	Yield	Reference
5-LOX	*Homo sapiens*	*Saccharomyces cerevisiae*	50 µg/l	Nakamura et al. 1990
9-LOX	*Oryza sativa*	*Escherichia coli*	3% of total soluble protein	Schirano et al. 1990
13-LOX	*Glycine max*	*Escherichia coli*	22-30 mg/l	Steczko et al. 1991
9/13-LOX	*Pisum sativum*	*Saccharomyces cerevisiae*	100-200 U/µl	Knust et al. 1992
13-LOX	*Lens culinaris*	*Escherichia coli*	N/A	Hilbers et al. 1996
9/13-LOX	*Pisum sativum LOX-2*	*Escherichia coli*	N/A	Hughes et al. 1998
9/13-LOX	*Pisum sativum LOX-3*	*Escherichia coli*	N/A	Hughes et al. 1998
9-LOX	*Magnaporthe salvinii*	*Aspergillus oryzae*	N/A	Sugio et al. 2002
15-LOX	*Oryctolagus cuniculus*	*Escherichia coli*	0.33 mg/l	Walther et al. 2002
10/13-LOX	*Pseudomonas aeruginosa*	*Escherichia coli*	N/A	Vidal-Mas et al. 2005
11/13-LOX	*Gaeumannomyces graminis*	*Pichia pastoris*	30 mg/l	Cristea et al. 2005
13-LOX	*Nostoc punctiforme*	*Escherichia coli*	N/A	Koeduka et al. 2007
9-LOX	*Anabaena sp.*	*Escherichia coli*	85 mg/l	Zheng et al. 2008
11/13-LOX	*Gaeumannomyces graminis*	*Aspergillus oryzae*	N/A	Christensen et al. 2008
9/13-LOX	*Nicotiana benthamiana*	*Saccharomyces cerevisiae*	0.9 U/ml	Huang & Schwab 2011
11/13-LOX	*Gaeumannomyces graminis*	*Trichoderma reesei*	N/A	Nyyssölä et al. 2012
9-LOX	*Anabaena PCC7120*	*Bacillus subtilis*	171.9 µg/ml	Zhang et al. 2012
ValLOX	*Pleurotus sapidus*	*Escherichia coli*	60 mg/l	Zelena et al. 2012
10/13-LOX	*Pseudomonas aeruginosa*	*Escherichia coli*	3.89 U/ml	Lu et al. 2013
9-LOX	*Magnaporthe salvinii*	*Pichia pastoris*	N/A	Wennman et al. 2013
13-LOX	*Fusarium oxysporum*	*Escherichia coli*	30 mg/l	Brodhun et al. 2013
13-LOX	*Aspergillus fumigatus*	*Pichia pastoris*	1 mg/l	Heshof et al. 2014a
ValLOX	*Pleurotus sapidus*	*Pichia pastoris*	100-150 mg/l	Kelle et al. 2014

4. Potential applications of LOXs

The primary reaction of LOX, *e.g.* production of hydroperoxides, can be exploited in different industrial applications ranging from food processing to oleochemical production as summarized in Table 2. Fatty acid derived hydroperoxides produced by LOXs can be converted to a wide variety of oleochemicals and material precursors by different chemical reactions. On the other hand, co-oxidation reactions caused by the hydroperoxide can be exploited in different bleaching applications in food, pulping, and textile applications. Formation of hydroperoxides by intrinsic LOXs present in food raw materials, *e.g.* in cereals or legumes, and its subsequent reactions can, however, be harmful to the final product quality due to off-flavour formation as reviewed by Robinson *et al.* (Robinson et al. 1995). Although the potential of LOXs has been widely discussed in different scientific papers, the only commercial applications so far is the use of LOX enriched soybean flour in baking for bleaching of wheat flour (de Roos et al. 2006). Up to now most of the application research has also been carried out using soybean LOX, although recently the use of microbial LOXs in different applications have been reported. Development of LOX compositions with high activities, by *e.g.* heterologous expression in suitable production hosts, would enable full exploitation of the potential of LOX derived reactions in different applications.

27

Table 2: Potential applications of LOX catalysed reactions.

Application	LOX source	Key reaction	Reference
Flavour production	*Glycine max*	Cleavage of LOX produced hydroperoxide to e.g. hexanal by hydroperoxide lyase	Muller et al. 1998 Furusawa et al. 2005 Gigot et al. 2010 Noordermeer et al. 2002 Márczy et al. 2002
Flour bleaching in bread or noodle manufacture	*Glycine max*	Co-oxidation of carotenoids by LOX produced hydroperoxide	Faubion & Hoseney 1981 Cato & Halmos 2006 Frazier et al. 1977
Annatto decolourization in waste whey	*Glycine max*	Co-oxidation of annatto colour by LOX produced hydroperoxide	de Roos et al. 2006 Kang et al. 2010
Dough structure improvement by protein crosslinking	*Glycine max*	Oxidation of SH-groups by LOX produced hydroperoxide	Faubion & Hoseney 1981 Frazier et al. 1977 Hoseney et al. 1980
Production of oleochemicals	*Gaeumannomyces graminis, Glycine max, Pseudomonas aeruginosa*	Hydroperoxide production and its further chemical reactions	Villaverde et al, 2013a Villaverde et al. 2013b Hayes 2004 Maurer & Schmid 2005
Removal of wood extractives in mechanical pulping	*Gaeumannomyces graminis, Glycine max*	Oxidative degradation of extractives	Zhang et al. 2007 Marques et al. 2011
Textile bleaching during manufacture or washing	*Magnaporthe salvinii*	Co-oxidation of coloured components	Salmon et al. 2004 Adriaanse et al. 2000 Sugio & Takagi 2002

4.1. LOX as tool in oleochemistry

Biomass derived lipids are potential renewable raw materials for the chemical industry (Metzger et al. 2006). Lipid derived hydroperoxides produced by LOX can be converted to a wide variety of functional oleochemicals precursors by three main conversion routes: acid catalyzed decomposition, free radical decomposition, and base catalyzed decomposition as schematically illustrated in Fig. 5 (Gardner 1984, Gardner 1989, Frankel 1989, Blanksby et al. 2002, Frankel et al. 2005). LOX-catalyzed production of hydroperoxides can be regarded more sustainable than chemical technologies using *e.g.* ozone oxidation (Nishikawa et al. 1995, Reynolds et al. 2006). In contrast to enzymatic oxidation, chemical oxidation is non-specific. Therefore specific regio-isomers can be obtained in a low yield. In addition, the chemical reaction is difficult to control. Most reactions yield degradation products like the radicals formed in a Fenton's reaction (Villaverde et al. 2011, Villaverde et al. 2012). *G. graminis* LOX has been shown to have a high specificity for generation of 13-hydroperoxy-(9Z,11E)-octodecadienoic acid when linoleic was used as substrate (Su & Oliw 1998). This result was recently confirmed by Villaverde *et al.* (Villaverde et al. 2013a). They obtained high yields of hydroperoxides (88.0%) at low linoleic acid concentrations and a selectivity for 13-(Z,E)-isomer of around 74%. Furthermore, cloned *Pseudomonas aeruginosa* LOXs also demonstrated, at laboratory scale, to efficiently convert linoleic acid into hydroperoxides with yields higher than 75% and selectivity for the 13-(Z,E)-isomer up to around 70% (Villaverde et al. 2013b).

29
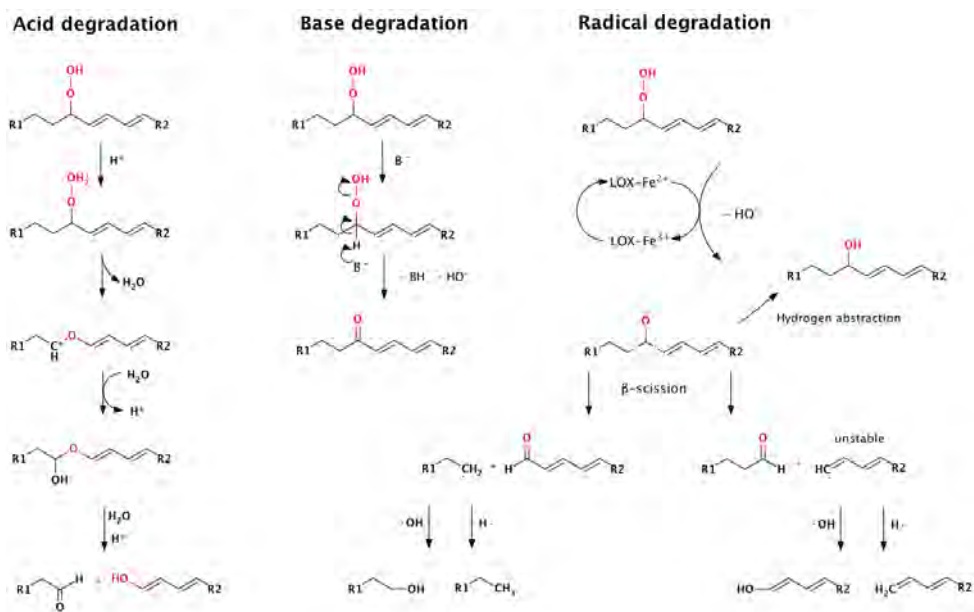
Figure 6: Schematic overview of the chemical conversion of unsaturated fatty acids by acid and base and enzymatic conversion by radical degradation. The chain length of the degradation products depends on the position of the peroxide group on the fatty acid. By using specific LOXs and/or fatty acids various sizes of polymers can be generated.

Production of hydroperoxides by LOX has been investigated on laboratory scale using different process conditions. The process efficiency is hampered by low solubility of the lipid and oxygen substrates in water. The use of detergents or organic solvents that enable a higher accessibility of the fatty acid substrate to the enzyme, using pressurized oxygen to improve solubility of O_2, or using immobilized enzymes, have been studied as reviewed by Hayes, and Maurer and Schmid (Hayes 2004, Maurer & Schmid 2005). Studies on the production of hydroperoxides by *G. graminis* LOX using industrially feasible linoleic acid concentrations (100-300 g/l) concluded that normal aeration can be used as oxygen source thus lowering the costs of the process (Villaverde et al. 2013a). Fatty acid hydroperoxides can be used as intermediates for the preparation different oleochemicals, *e.g.* mid-chain hydroxy fatty acids, epoxides, alcohols, carbonylic compounds, and diacids all being monomers for polymer production (Kharasch et al. 1950, Hamberg & Gotthammar 1973, Gardner 1989, Frankel 2005). Fatty acid hydroperoxides are also involved in the mechanism of oils polymerization in painting formulations (van Gorkum & Bouwman 2005).

4.2. Use of LOX in food applications

The use LOXs in food applications has been reviewed by Casey and Hughes (Casey & Hughes 2004). Depending on the targeted food product, LOX catalysed lipid oxidation can be regarded as harmful or beneficial. The intrinsic LOX present in *e.g.* cereals may cause formation of off-flavours in the final product such as in beer (Robinson et al. 1995, Hirota et al. 2006). Thus, significant amount of research has been carried out to develop methods for LOX inhibition during food processing (Baysal & Demirdöven 2007). Another application is the production of nootkatone from valencene using soy bean LOX (Muller et al. 2998). Nootkatone is a grapefruit flavour that can be used in industry for food applications and cosmetics (Furusawa et al. 2005). To date no known commercial application using LOX in the production of nootkatone is known. The main commercial LOX application so far is co-oxidation of carotenoid pigments in cereal flour by LOX enriched soya flour. As a result a bleaching effect is obtained. LOX can also co-oxidize SH-groups in proteins resulting in crosslinking or aggregate formation, which in turn affects the structure of the product (Permyakova & Trufanov 2011). The role of LOXs in bread and pasta quality has been addressed in several studies (Frazier et al. 1977, Hoseney et al. 1980, Faubion & Hoseney 1981, Cato et al. 2006, Junguera et al. 2007). The bleaching effect caused by hydroperoxides can also be exploited in dairy products, *e.g.* cheese, milk, butter oil, cream, and whey products (de Roos et al. 2006). The use of LOX to bleach annatto, a carotenoid colourant used in the manufacture of certain cheeses, has also been reported as whey decolourisation treatment instead of chemical bleaching agents (Kang et al. 2010). LOX catalysed bleaching in food processing is expected to be more positively perceived by the consumers as compared to chemical bleaching.

31

LOX and hydroperoxide lyase catalysed fatty acid conversion *in vivo* is responsible of the aroma of *e.g.* freshly cut grass, cucumbers, or green apples (Gardner et al. 1991, Gigot et al. 2010). Traditionally these natural green notes have been produced by extraction from plants, but currently more efficient means for their production are investigated. Production of hexanal and hexenal from vegetable oils by LOX in combination with hydroperoxide lyase reaction has been reported by many papers (Cass et al. 2000, Noordermeer et al. 2002, Santano et al. 2002, Omar et al. 2003, Gigot et al. 2010). A two-step process with soybean LOX and spinach leaf hydroperoxide lyase was reported resulting in hexanal production with a yield of 54% (et al. 2002). The LOX catalysed production of the aroma compound β-ionone from β-carotene

has also been reported (Waché et al. 2006). Limitation of the processes using plant homogenates as source of LOX and hydroperoxide lyase is low enzyme activities and low specificity of these crude enzyme preparations (Gigot et al. 2010, Buchhaupt et al. 2012).

4.3. Other LOX applications

Lipophilic extractives from lignocellulosic materials, *e.g.* alkanes, fatty acids, steroids, and triglycerides, cause pitch deposits in papermaking (Dubé et al. 2008). This can result in product defects or problems in paper runnability (Gutiérrez et al. 2009). Global paper and board production volumes are significant, accounting for 400 million tonnes annually (FAO 2013). Therefore different chemical and biotechnical approaches to minimize the amount of lipids, especially during mechanical pulping, are being pursued. LOXs can be used for pitch control of mechanical pulps (Zhang et al. 2007). The role of *G. graminis* LOX to remove lipophilic extractives from chemical eucalyptus and flax pulps in presence and absence of linoleic acid followed by peroxide bleaching was studied by Marques *et al.* (Marques et al. 2011). The main lipophilic extractives in eucalyptus pulp were reduced by 40% and 7% in presence and absence of linoleic acid respectively, with simultaneous pulp delignification (Marques et al. 2011). Degradation of xenobiotics, *e.g.* chlorpromazine, by immobilized LOX has also been reported by Santano *et al.* (Santano et al. 2002). LOX derived bleaching effect used in food and pulp processing can also be exploited in textile bleaching or in detergents, *e.g.* triglycerides (Adriaanse et al. 2002, Sugio & Shinobu 2002, Salmon et al. 2004).

5. Conclusions

LOX catalysed hydroperoxide formation and the reactivity of hydroperoxides produced can be exploited in many different applications ranging from production of oleochemical compounds or flavour compounds to bleaching of different materials. Despite the potential, the development of applications is hampered by lack of commercial LOXs with high activity. So far only LOX enriched soya flour is commercially available for application development. LOXs may fulfil an important role in the design of novel environmentally friendly processes especially in oleochemistry and in this way partially replacing chemical reactions. The major problem thus far, however, is to produce LOX enzymes in sufficient amounts for large-scale application. Although there are reports on successful heterologous expression of LOXs in microorganisms, development of efficient production systems for LOXs with different specificities are needed in order to harness its potential as industrial biocatalyst.

6. Declaration of interest

Chapter 3

A novel class of
fungal lipoxygenases

Abstract

Lipoxygenases (LOXs) are enzymes that have been well-studied enzymes in plants and mammals but less studied in fungi. In this study we compared fungal LOX protein sequences to all known characterized LOXs. To do this, a script was written using Shell commands to extract sequences from the NCBI database. The extracted sequences were then aligned using MUltiple Sequence Comparison by Log-Expectation (MUSCLE). We constructed a phylogenetic tree with the use of Quicktree to visualize the relationship of fungal LOXs with other LOXs. Sequences were analyzed with respect to the signal sequence, C-terminal amino acid, the stereochemistry of the formed oxylipin, and the metal ion cofactor usage. This study shows fungal LOXs are divided into two groups: the Ile-group and the Val-group. The Ile-group has a conserved WRYAK sequence that appears to be characteristic for fungal LOXs and has as a C-terminal amino acid isoleucine. The Val-group has a highly conserved WL-L/F-AK sequence that is also found in LOXs of plant and animal origin. We found that fungal LOXs with this conserved sequence have a valine at the C-terminus in contrast to other LOXs of fungal origin. We also found that these LOXs have signal sequences, implying they will be expressed extracellular. Our results show that in this group, in addition to the *Gaeumannomyces graminis* and the *Magnaporthe salvinii* LOXs, the *Aspergillus fumigatus* LOX uses manganese as a cofactor.

Introduction

Lipoxygenase (LOX) is a non-heme iron-containing or manganese-containing dioxygenase that catalyzes the conversion of polyunsaturated fatty acids (PUFAs) to fatty acid hydroperoxides. Substrates such as linoleic acid (C18:2ω6), linolenic acid (C18:3ω3) and arachidonic acid (C20:4ω6) are converted to hydroperoxy octadecadienoic acid (HPOD), hydroperoxy octadecatrienoic acid (HPOT) and hydroperoxy eicosatetraenoic acid (HPETE), respectively (Andreou et al. 2009). In many LOX enzymes the catalytic center consists of the sequence $H-X_4-H$; $H-X_3-N$ and the enzyme has an isoleucine as a C-terminal amino acid. The composition of the catalytic center, however, can vary as is found for *Gaeumannomyces graminis* ($H-X_3-H$; $H-X_3-N$), *Homo sapiens* ($H-X_4-H$; $H-X_3-H$) and *Mus musculus* ($H-X_4-H$; $H-X_3-S$) LOXs (Brash 1999; Hornsten et al. 2002; Jisaka et al. 1997; Wennman and Oliw 2012). LOX reaction products are called oxylipins and have different functions in various organisms. In vertebrates, 5-LOXs regulate the biosynthesis of leukotrienes and are involved as signal molecules in the inflammatory response in asthma and allergic rhinitis. In platelets, 12-LOX products contribute to chemotactic and mitogenic responses in the smooth muscle cells of the circulatory system (Schwarz et al. 2001; Lagarde et al. 2010; Duroudier et al. 2009; Izumi et al. 1990). In plants, oxylipins function as signal molecules in the development of seeds but are also involved in the initiation of a defensive system against pathogenic fungi. The hydroperoxides formed are further converted to jasmonic acid, which activates proteinase inhibitor encoding genes in wounded and non-wounded leafs (Jensen et al. 1997; Hamberg et al. 2003; Baysal and Demirdoven 2007).

So far, two fungal LOXs are characterized that use manganese as a cofactor instead of iron (Su and Oliw 1998; Wennman and Oliw 2012). These Mn-LOXs are found in the "take-all" fungus *G. graminis* and the fungus *Magnaporthe salvinii* and the biological function of Mn-LOXs is unknown. It has been suggested that these Mn-LOXs play a role in programing cell death and damaging the root cells of wheat as part of the invasion process (Wennman et al. 2012; Andreou et al. 2009).

Several classification systems for LOXs have been developed and are based on information regarding substrate, phylogenetic relatedness, and function. However, no unifying nomenclature of LOXs has been developed (Ivanov et al. 2010). Much is known of *Planta* and *Animalia* LOXs but LOXs have only been partially described for other organisms such as bacteria, algae, and fungi (Hansen et al. 2013; Kuo et al. 1996; Oliw 2002). To gain more insight in the characteristics of LOXs throughout the kingdom of life, we analyzed these enzymes based on their amino acid sequence and ordered them in a phylogenetic tree.

Materials and methods

Databases and sequences used for the analysis

NCBI's protein database was searched for LOXs containing the tag "lipoxygenase OR lox". The identified LOXs were filtered according to five criteria: a) the sequences should start with methionine to be a complete sequence b) contain the highly conserved sequence $W-X_2-AK$ where X is any amino acid (Brash 1999; Hornsten et al. 2002), c) sequence titles do not contain the tags putative, hypothetical, patent, predicted, unnamed, unknown, and related in the title, d) the C-terminal amino acid ends with valine or isoleucine, and e) the sequences have the catalytic center $H-X_{3/4}-H$; $H-X_3-N/H/S$ (Tomchick et al. 2001). All these criteria were written in a script using Shell commands (see Supplementary material 1). We chose the X_2 combination because not all LOXs contain the WLLAK sequence but have variants. The tags in the titles were eliminated from selection, however, these settings were set as a filter and could be turned on or off. The C-terminal amino acid is an important part of the catalytic center as it is involved in coordinating the catalytic metal. In most cases this is isoleucine but also sometimes valine is found.

MUltiple Sequence Comparison by Log-Expectation (MUSCLE) was used to align the isolated sequences and this alignment was then analyzed with ClustalX to manually delete improperly aligned sequences (Edgar 2004; Larkin et al. 2007). This concerns sequences the script selected relating to $H-X_{3/4}-H$; $H-X_3-N/H/S$ fragments existing in another position in the sequence. Sequences were then re-aligned with the use of MUSCLE. A phylogenetic tree was constructed with the use of Quicktree and this was then visualized using the program FigTree (v1.2.3) (Howe et al. 2002; Page 1996).

Production and purification of LOXs

Purification of Glycine max *LOX*

G. max 15-LOX was obtained from Cayman Chemical (Estland, no. 60700). We purified the protein by loading 250 μl onto a Superdex 200 10/300 GL (Pharmacia Biotech) using 0.1 M Tris-HCl 0.15 M NaCl pH 7.0 as a running buffer with a flow rate of 0.75 ml/min. Fractions of 1 ml were collected and stored. LOX activity containing fractions were separated again on the column with the same settings to further purify the enzyme.

Production and purification of G. graminis *LOX*

G. graminis LOX has been produced by AB enzymes, Darmstadt, Germany as described earlier (Nyyssölä et al. 2012). The *G. graminis* lipoxygenase-encoding gene AAK81882.1 was codon-optimized for *Trichoderma reesei* [GI:500229533] and synthesized by GeneArt (Regensburg, Germany). To facilitate cloning in *Trichoderma*, *Sac*II and *Bam*HI restriction sites were added at the 5' and 3' ends of the fragment, respectively. The DNA fragment of the synthesized gene was ligated into the *Sac*II and *Bam*HI sites of the expression vector pAB100 containing the strong *cbhI-*promoter of *T. reesei*. The native signal sequence of the recombinant LOX was used for secretion. *T. reesei* strain RH32578 was transformed as described and transformants were screened for growth on plates with acetamide as sole nitrogen source (Penttilä et al. 1987). Resulting transformants were analyzed by PCR for integration of the LOX gene by isolating genomic DNA (Cenis 1992). A PCR reaction was performed using the forward 5'-ATGCAGGCCTTCGTCGACTC-3' and reverse 5'-GGATCCTTAGACGCTCAGGAAGAAGG-3' primers resulting in a band of 630 bp.
Crude *G. graminis* LOX extract was filtered using 0.45 µm filters. The 50 ml was loaded manually onto an equilibrated G75 column (GE Healthcare, Brøndby, Denmark) (phosphate buffer, 678 cm bed). Fractions of 14 ml were eluted with a flow rate of 0.5 ml/min. Dialysis was performed of the pooled fraction in 8 L of 20 mM $NaH_2PO_4 H_2O$ pH 6.6 (Buffer A) at 4°C overnight. The dialyzed pool was diluted 1:1 in Buffer A and the 28 ml sample was loaded onto the Resource S column (GE Healthcare, Brøndby, Denmark) with a flow rate of 2 ml/min. Samples of 1 ml were eluted using a linear gradient (Buffer A + 1 M NaCl; 10 column volumes).

Production and purification of A. fumigatus LOX

A. fumigatus LOX was codon-optimized for *Pichia pastoris* [GI:500229553] and has been produced by VTT, Espoo, Finland as previously described (Nyyssölä et al. 2012). A *P. pastoris* transformant expressing LOX of *A. fumigatus* was selected for growth in a 1 L Sartorius Stedim Biotech Biostat Qplus fermenter containing 0.4 L basal salt medium (Sartorius, Goettingen, Germany) (Stratton et al. 1998). One colony was pre-grown in 50 ml YNB medium containing 2% glycerol at 30 °C for two days (Madzak et al. 2005). This culture was added to the fermenter to an OD_{600} of 2.5. The transformant was grown at 30 °C until the glycerol in the medium was completely consumed. A glycerol fed-batch was initiated for 8 h to increase biomass in the fermenter before methanol induction at 25 °C was started. The medium was controlled with 35% dissolved oxygen at pH 5.0 by addition of NH_3 and H_3PO_4. After 4 days the culture reached an OD_{600} of ~800 and the fermentation process was ceased.

The supernatant was isolated by centrifugation and the pH was adjusted to pH 8.0. Precipitation was removed by centrifugation and the supernatant was filtered using a 0.22 μm filter and loaded onto a HisPrep FF 16/10 column (GE Healthcare, Diegem, Belgium) using 300 mM NaCl, 50 mM Tris-HCl pH 8.0 as a running buffer at a flow rate of 2 ml/min. Active fractions of 1 ml were isolated using a gradient at 1 ml/min for 60 min. using 300 mM NaCl, 50 mM Tris-HCl, 500 mM imidazole pH 8.0 as elution buffer. Finally, the protein was stored at 4 °C.

Lipid hydroperoxide diene isomers

Three independent samples of 10 μl of reaction mixture were injected at room temperature onto a reverse phase Kinetex column (2.6 μm C18 100 Å column 150 x 2.1 mm, Phenomenex, Værløse, Denmark) that was equilibrated with an aqueous 0.1% formic acid 10% acetonitrile solution with a flow of 1.0 ml/min. Elution was achieved with 0.1% formic acid in water (eluent A) and 0.1% formic acid in 95% acetonitrile (eluent B). Elution started with 10% B for 2 min. and was increased to 55% B in 9 min., to 70% B in 15 min. and to 95% B in 5 min where it was then held for 10 min. Analyzes were performed using an Agilent Technologies (Waldbronn, Germany) HPLC series 1100 comprising a model G1312A binary pump, a model G1379A microvacuum degasser, a model G1327A thermostated auto sampler, a model G1316A thermostated column compartment, a model G1315B diode-array detector, and a model G2707DA LC/MSD SL detector fitted with a model G1948A electrospray source. The station was controlled and the results were analyzed with Agilent's ChemStation software (Rev. A.10.02). UV spectra were recorded from 190 to 400 nm using a reference of 700 nm and the secondary derivative was used to differentiate between cis and trans forms of the different hydroperoxides isomers (Banni et al. 1996). The MS-scan spectrum was recorded in negative mode using a fragmenter voltage of 200 V and a capillary voltage of 3500 V. The ESI ion-source was operating at 350°C with nebulizer pressure of 55 psig and drying gas flow of 12 l/min. The identification of the hydroperoxide isomers with respect to the position of the hydroperoxide was performed according to the fragmentation pattern (Haefliger et al. 2007).

ICP-AES

To determine the metal ion cofactor in LOXs, Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) was used for three different LOXs: Purified *G. max* 15-LOX and *G. graminis* LOX were used as references to determine the iron and

manganese cofactors, respectively and purified *A. fumigatus* LOX was used to confirm the hypothesis. Measurements were carried out using a radial ICP-AES of Varian Vista Pro (Varian, Mulgrave, Australia). RvA certified (Dutch Accreditation Council) solutions of 0, 10, and 20 mg/l iron in dH_2O were used as standards. For manganese measurements, RVA certified solutions of 0, 1, and 2 mg/l manganese in dH_2O were used. Samples containing purified 0.96 mg *G. max* LOX, 2.53 mg *G. graminis* LOX, and 1.00 mg *A. fumigatus* LOX were used to determine the cofactor.

Results

Bioinformatics scripts

The script-identified LOX protein sequences from the NCBI database all contained the highly conserved W-X_2-AK sequence, the catalytic H-X_4-H; H-X_3-N amino acid sequence, and the C-terminal isoleucine or valine. Variants of the catalytic site were described and different script settings were used to select these variants (Table 1). These settings selected for four different catalytic variants (A, B, C and D) divided in four subsections (filter on/off, C-terminal Ile/Val) as presented in Table 1.

Script A1 identified 314 LOX amino acid sequences containing the H-X_4-H; H-X_3-N catalytic variant. Most of these sequences are found in plant sequences but are also present in mammalian, bacterial, algae, and fungal LOXs. Script B1I identified 26 sequences that are all 12/15-LOXs *Animalia* LOXs and script B1V identified an arachidonic 5-LOX originating from the bacterium *Nitrosomonas europaea* [GI:30180541].

Another variant of the LOX catalytic site is H-X_4-H; H-X_3-S with a C-terminal isoleucine. The number of sequences extracted by script C1I was 17 that code for 15-LOXs with exception of a *M. musculus* 8-LOX [GI:56800274]. The only non-*Animalia* LOX in this group is a 9-LOX originating from *Brassica napus* [GI:27372773] (Terp et al. 2006). Script C2I (filter off) identified 45 sequences. The addition of these sequences resulted in the identification of another LOX sequence that does not code for a 15-LOX: a predicted 12*S*-LOX originating from *Monodelphis domestica* [GI:126309204], which makes a total of three non-15-LOX serine catalyzed LOXs. The variant with a C-terminal valine identified by script C1V finds its origin in two different organisms: *Zea mays* LOX6 [GI:162463394] and *Sorghum bicolor* LOX6 [GI:258618873]. Script C2V identified seven different LOXs originating from *Hordeum vulgare* [326494396], *Z. mays*, and *S. bicolor*, which are all cereal-like organisms.

Two different LOXs were found using script D1: *G. graminis* Mn-LOX [GI:17861374] and *Arabidopsis thaliana* LOX2 [GI:6911852]. The *G. graminis* LOX has a C-terminal valine whereas the *A. thaliana* LOX has a C-terminal isoleucine.

43

Table 1: Criteria used in the scripts for selection of LOXs and the number of identified sequences.

| Catalytic sequence	Filter used	C-terminal amino acid	Script name	Number of sequences
$H-X_4-H$; $H-X_3-N$	Yes	Ile	A1I	314
		Val	A1V	7
	No	Ile	A2I	575
		Val	A2V	24
$H-X_4-H$; $H-X_3-H$	Yes	Ile	B1I	26
		Val	B1V	1
	No	Ile	B2I	45
		Val	B2V	1
$H-X_4-H$; $H-X_3-S$	Yes	Ile	C1I	17
		Val	C1V	2
	No	Ile	C2I	45
		Val	C2V	7
$H-X_3-H$; $H-X_3-N$	Yes	Ile	D1I	1
		Val	D1V	1
	No	Ile	D2I	2
		Val	D2V	2

Phylogenetic tree

A total of 369 LOX amino acid sequences were identified from the NCBI database using the scripts A1, B1, C1 and D1. These sequences were used to generate a phylogenetic tree (Fig. 1). The different colours in the figure identify the various groups of the kingdom of life where all LOXs from plant origin are coloured green. The plant group is a large group and is further categorized into four sub-classes: *Machantiophyta*, *Angiosperms*, *Lycopodiophyta*, and *Bryophyta*. The plant group in the phylogenetic tree differs from the other LOXs. This is probably a reflection of the substrate difference, since plants commonly use linoleic acid, while mammalians use arachidonic acid (Tsitsigiannis and Keller 2007).

The group of LOXs clustered in yellow is found in *Animalia*, which is an isolated group. The blue group represents *Bacteria*, which has three branches. The smallest branch splits up *via* the route of *Animalia* LOXs according to Fig. 1 and is a single LOX with a C-terminal valine belonging to the Gram-negative bacteria *Myxococcus xanthus* [GI:108463641]. The group in dark green represents *Chromalveolata* LOXs. This group currently exists of *Ectocarpus siliculosus* LOXs. The *Fungi* group is coloured red and is sub-divided into two groups: the Iso-group LOXs and the Val-group LOXs.

The sequences used to prepare Fig. 1 are listed from top to bottom in Supplementary material 2.
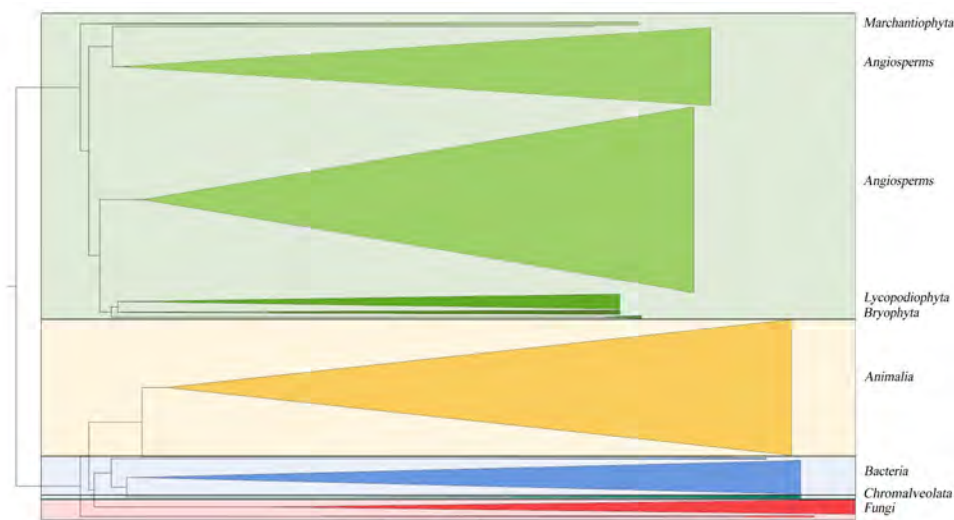
Figure 1: Alignment of 369 LOX amino acid sequences extracted from the NCBI database. The upper branches (with green background) are all classified Plant LOXs and the lower branches (no background) are classified as Other LOXs. The colours represent different organism groups. Plants (light green) are divided into four subclasses: *Machantiophyta*, *Angiosperms*, *Lycopodiophyta* and *Bryophyta*. The other colored groups represent *Animalia* (yellow), *Bacteria* (blue), *Chromoveolata* (dark green) and *Fungi* (red). The number of sequences that were identified determines branch width and the evolutionary length of the LOX determines branch length.

Fungal LOXs

Phylogenetic tree

The phylogenetic tree displayed in Fig. 1 consists of 14 different fungal LOXs. Switching off the filter, implicating also hypothetical sequences are selected, results in 40 different fungal sequences. In this study we use these sequences since they have the conserved W-X_2-AK sequence, the H-X_4-H; H-X_3-N or the H-X_3-H; H-X_3-N catalytic center, and an isoleucine or valine as a C-terminal amino acid. The sequences were aligned using MUSCLE and the phylogenetic tree is shown in Fig. 2. This phylogenetic tree splits into two different groups. In this study we refer to these groups as the "Ile-group" and the "Val-group". The Ile-group contains sequences with a C-terminal

isoleucine and the conserved WRYAK sequence. This conserved sequence is only found in fungal LOXs. The Val-group consists of the WL-L/F-AK sequence that has a C-terminal valine with exception of the *M. salvinii* LOX.
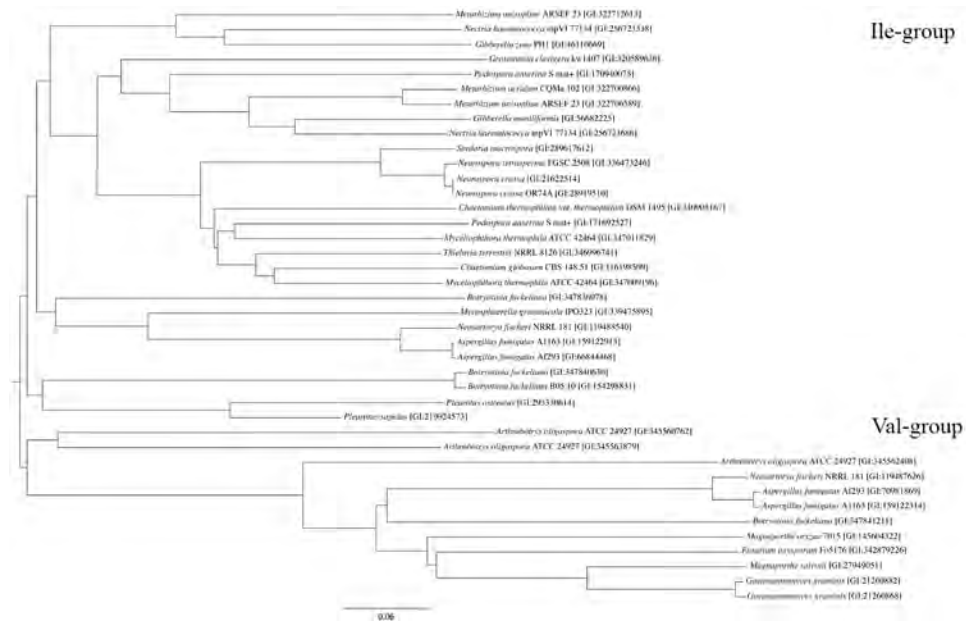

Figure 2: Phylogenetic tree derived from 40 LOX amino acid sequences of fungal origin. Two groups can be distinguished: the Ile-group and the Val-group. The Ile-group has a conserved WRYAK sequence and a C-terminal isoleucine. With exception of the *Magnaporthe salvinii* LOX, the Val-group has a conserved WL-L/F-AK sequence and a C-terminal valine residue.

Stereochemistry

An alanine or a glycine at a specific location in the sequence, called the Coffa-Brash site, is important with respect to the stereochemistry of the formed oxylipin called the A/G stereo specificity rule (Coffa and Brash 2004; Cristea and Oliw 2006). We analyzed the application of this rule for the fungal LOX sequences. Box 3 in Fig. 3 highlights the part of the alignment where the A/G stereo specificity rule applied to LOXs. All variations of the A/G stereo specificity rule regarding the sequences shown in Fig. 1 are listed in Table 2.
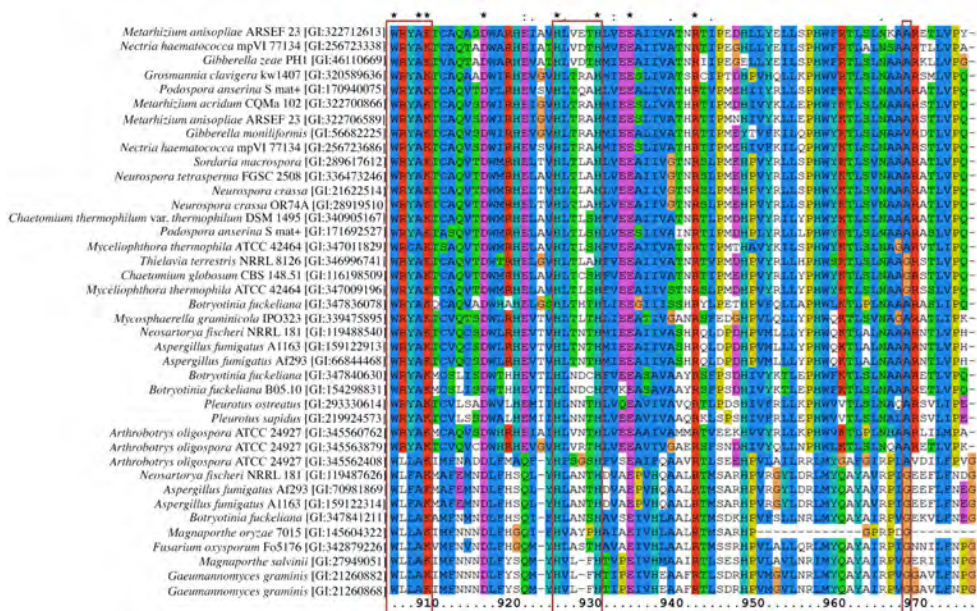
Figure 3: Alignment of fungal LOXs with the conserved W-X$_2$-AK (box 1; 905-909), catalytic H-X$_4$-H (box 2; 925-930) and the Coffa-Brash site (box 3; 968). Box 3 indicates most of the LOXs in the Ile-group 1 will form *S*-hydroperoxides due to the alanine at the specific location, while the Val-group will form *R*-hydroperoxides due to the glycine.

47

Table 2: The variance of amino acids seen in the 369 LOX sequences regarding the Coffa-Brash site.

| GI number	Accession number	Coffa-Brash site amino acid	LOX-type	Organism
56682225	AAW21637	V	N/A	*Gibberella moniliformis*
302796934	XP_002980228	D	N/A	*Selaginella moellendorffii*
56800273	CAI35250	-	Ara 8-lipoxygenase	*Mus musculus*
85067503	NP_001034220	-	Ara 15-lipoxygenase B isoform b	*Homo sapiens*
85067499	NP_001034219	-	Ara 15-lipoxygenase B isoform a	*Homo sapiens*
18643347	AAL76275	-	Ara 15-lipoxygenase 2 splice variant a	*Homo sapiens*
119610507	EAW90101	-	Ara 15-lipoxygenase, type B, isoform CRA_d	*Homo sapiens*
119610508	EAW90102	-	Ara 15-lipoxygenase, type B, isoform CRA_e	*Homo sapiens*
344237821	EGV93924	S	Ara 12-lipoxygenase, 12S-type	*Cricetulus griseus*
157786628	NP_001099268	S	Ara 12-lipoxygenase, 12S-type	*Rattus norvegicus*
56800471	CAI35235	S	Ara 12-lipoxygenase	*Mus musculus*
148680604	EDL12551	S	Ara 12-lipoxygenase isoform CRA_b	*Mus musculus*
148680605	EDL12552	S	Ara 12-lipoxygenase isoform CRA_c	*Mus musculus*
467221	AAA20659	T	Ara 12-lipoxygenase	*Mus musculus*
1246125	AAB36013	T	Ara 12-lipoxygenase	*Mus sp.*
296476804	DAA18919	-	Ara 12-lipoxygenase isoform 2	*Bos taurus*

Cofactor

With the exception of *Neosartorya fischeri* [GI:119487626] and *Arthrobotrys oligospora* [GI:345562408] LOXs (Emanuelsson et al. 2007), LOXs that belong to the Val-group originate from plant pathogenic fungi and have a secretion signal according to SignalP 4.0 server. The *N. fischeri* LOX is identical to the *A. fumigatus* [GI:70981869] LOX but it lacks the secretion signal and the *A. oligospora* LOX has a SignalP prediction of 0.449 where the cutoff is 0.450 in value.

Given that *G. graminis* is classified into the Val-group, we investigated whether more LOXs in this group have a manganese cofactor. Based on the amino acid sequence of the *G. graminis* LOX and the similarities found in this group, we hypothesized that the Val-group LOXs use manganese as a cofactor. The 14 fungal sequences displayed in Fig. 1 all split into either the Ile-group or Val-group, where the Val-group consists of the *G. graminis* and *A. fumigatus* LOX sequence. Therefore, we elected to analyze the *A. fumigatus* LOX for cofactor usage.

ICP-AES analysis showed purified *A. fumigatus* LOX uses manganese and not iron as a cofactor. No iron could be detected in the purified *A. fumigatus* LOX sample, whereas 0.058 mg/l manganese was measured. This resulted in a manganese usage of > 0.50 manganese molecules per enzyme molecule and indicates the *A. fumigatus* LOX uses manganese instead of iron as a cofactor.

49
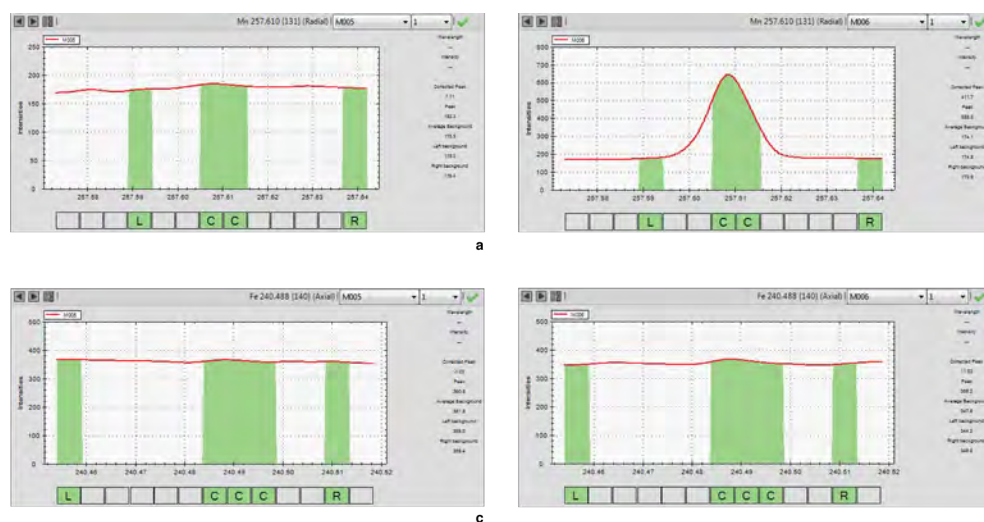
Figure 4: ICP-AES results of the *A. fumigatus* LOX. a) The blank measurement indicates no manganese. b) The purified *A. fumigatus* LOX contains manganese in a concentration of 0.058 mg/l. c) The blank measurement contains no iron and d) the purifed *A. fumigatus* LOX also shows no iron content.

Fungal LOXs lipid hydroperoxide diene isomers

The specificity of the *A. fumigatus* LOX was measured by identifying the presence of hydroperoxide isomers that are generated after reaction with linoleic acid. Reverse phase separation coupled with absorbance detection at 234 nm and MS was used to detect hydroperoxide conjugated isomer dienes. As illustrated in Fig. 5, four isomeric forms of conjugated dienes were identified in each chromatogram according to the UV-spectra and the MS-ions fragmentation patterns (Banni et al. 1996; Haefliger et al. 2007). The interpretation of the UV-absorbance spectra was performed using two derivatives showing local minimum according to the cis/trans conformation. The position of hydroperoxide was identified by EIS-MS; the ions 293, 249, 223, 195, and 113 m/z being specific for 13-HPOD and 293, 249, 185, 183, and 169 m/z being specific for 9-HPOD, respectively. The identification was performed by LC-UV/MS and the LOX was highly specific towards the 13-Z,E-HPOD yielding 89% of the total HPOD. The other HPOD isomers 9-Z,E-HPOD, 13E,E-HPOD, and 9-E,E-HPOD were 1, 4, and 5% respectively.
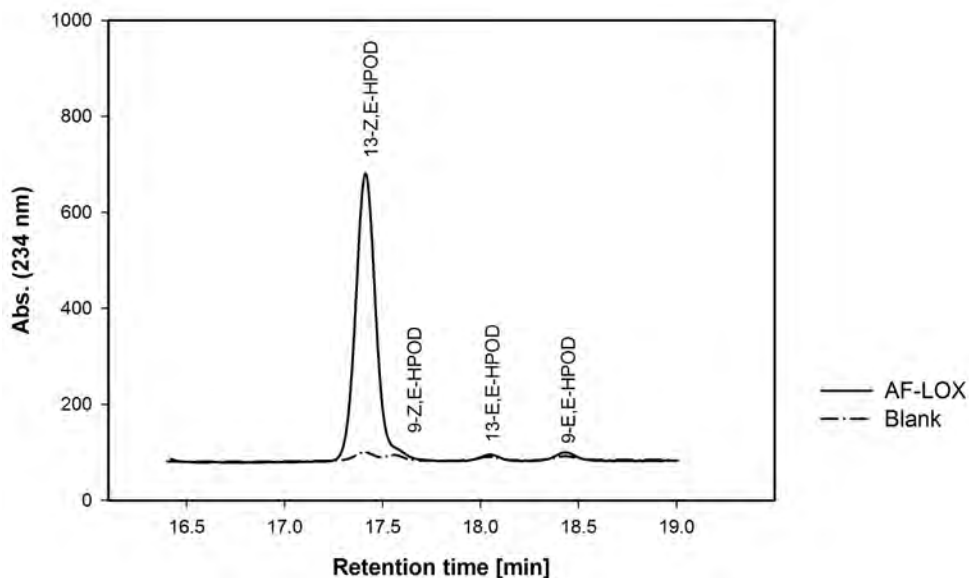
Figure 5: Conjugated dienes produced by the *Aspergillus fumigatus* LOX yield in 89% specificity for 13-Z,E-HPOD. The other HPOD isomers 9-Z,E-HPOD, 13-E,E-HPOD, and 9-E,E-HPOD were 1, 4, 5% respectively. Percentages are relative to the absorption (Abs.).

Discussion

Bioinformatic scripts

Several bioinformatics scripts were used to identify and cluster LOXs from the kingdom of life. Script C2V identified LOXs with a C-terminal valine and a serine in the catalytic site and these LOXs belong to cereal-like organisms originating from *H. vulgare*, *Z. mays*, and *S. bicolor*. This result raises the question whether plant pathogenic fungal LOXs with a C-terminal valine such as *G. graminis* have relatedness towards these specific plant LOXs regarding infection and intracellular communication.

Script D1 identified two LOXs originating from *G. graminis* and *A. thaliana*. The sequence of the *A. thaliana* LOX [GI:6911852] is almost identical to the *A. thaliana* LOX2 protein [GI:17065070]. It only differs in an insertion of the amino acid sequence RTHACTEPYIIAANRQLSAMHPIYRL at position 557-582, which is the region of the alignment where the catalytic site is found. Therefore, it is unlikely that sequence [GI:6911852] is a splice variant from *A. thaliana* LOX2 [GI:17065070]. Based on our alignment sequence, [GI:17065070] belongs to the (A) H-X$_4$-H; H-X$_3$-N family whereas sequence [GI:6911852] belongs to the (D) H-X$_3$-H; H-X$_3$-N family. Sequence [GI:17065070] fits the alignment better because more LOXs have the RTHACTEPYIIAANRQLSAMHPIYRL-like sequence. It could be that sequence [GI:6911852] is incorrectly sequenced.

51

Phylogenetic tree

The phylogenetic tree given in Fig. 1 contains a bacterial LOX sequence originating from *M. xanthus*. According to MUSCLE, this LOX is clustered in the *Animalia* group. *M. xanthus* is a spore-forming predatory proteobacterium that interacts with conspecifics as a social group and is typically found in marine topsoil (Konovalova et al. 2010). *M. xanthus* is known for its large genome in which gene families have likely been inserted *via* horizontal gene transfer (Goldman et al. 2006). This might explain the presence of a bacterial LOX in a group that has more characteristics of LOXs of marine origin. Analysis of the *M. xanthus* genome shows two different LOX encoding genes incorporated in the genome: one encoding a C-terminal valine LOX [GI:108463641] and the other encoding a hypothetical C-terminal isoleucine LOX [GI:108461295]. Both LOXs meet the criteria set in the methods section to be classified as a LOX. One may speculate these LOXs have functions in the processes of spore formation and predatory behavior.

Cofactor

Fig. 2 shows the Ile-group and the Val-group of fungal LOXs. We hypothesized the fungal Val-group LOXs use manganese as cofactor. This opens the way to a discussion on why this group would use manganese as a cofactor. *G. graminis* can infect the root cells of wheat and other grasses and releases Mn-LOX to oxidize fatty acids to generate hydroperoxides and reactive oxygen species (Cristea and Oliw 2007). As stated earlier, fungal LOXs of the Val-group are plant pathogenic and their LOXs show a secretion signal and a high similarity in primary protein structure. Mn-LOX is heavily glycosylated, has a broad pH spectrum, and is heat stable. Mn^{2+} is chemically more stable than Fe^{2+} suggesting these features are useful in an extracellular expressed enzyme (Oliw 2002).

Another interesting feature of the *G. graminis* Mn-LOX is it produces 11*S*-oxylipins and it is able to isomerize this product to 13*R*-oxylipins because Mn-LOX has the capacity for suprafacial hydrogen extraction from its substrate rather than antarafacial extraction such as occurs with Fe-LOX (Cristea and Oliw 2007). These Mn-LOX features suggest that manganese is a better choice of cofactor than iron because this LOX has the capacity to be a more stable enzyme and have a broader use in product formation.

Stereochemistry

An alanine or a glycine at the Coffa-Brash site is important with respect to the stereochemistry of the formed oxylipin (Coffa and Brash 2004; Cristea and Oliw 2006). glycine is a small amino acid and can penetrate the substrate into the LOX binding pocket and results in *R*-enantiomer oxylipins, where the methyl group of alanine diminishes the space and resulting in the production of *S*-enantiomer oxylipins. In contrast, this rule is not applicable for *Danio rerio* LOX1, because this LOX produces *S*-enantiomer oxylipins although a glycine is present at the specific position. Another variation of the A/G stereo specificity rule is found in the *G. graminis* LOX, because it has a glycine at the specific location. This LOX can produce both *S*- and *R*-enantiomer oxylipins (Hamberg et al. 1998). Also the secretable 9*S*-LOX from *M. salvinii* has a glycine at the Coffa-Brash site, which contradicts the A/G stereo specificity rule (Wennman et al. 2012). These results suggest the Coffa-Brash site does not determine the stereochemistry of the produced oxylipin for fungal LOXs.

Comparing the two fungal groups (Ile and Val groups) indicates Ile-group LOXs tend to have alanine at the specific location. There are three exceptions to this: LOXs originating from *Chaetomium globosum* [GI:88178689], *Thielavia terrestris* [GI:346996741] and *Myceliophthora thermophila* [GI:347009196]. With exception of

A. oligospora, which has an alanine at the specific location, the Val-group tends to use glycine.

Fungal LOXs

On the basis of primary structures we have shown that fungal LOXs can be divided into two groups: the Ile-group and the Val-group. Unlike other known LOXs, the Ile-group has a unique conserved WRYAK sequence and all these sequences have a C-terminal isoleucine. With the exception of the *M. salvinii* LOX, the Val-group has the conserved WL-L/F-AK sequence seen mostly in plants and end with a C-terminal valine. The Ile-group is composed of plant pathogenic fungal LOXs, which are expressed extracellular. Therefore, it is likely they use manganese as a cofactor instead of iron because Mn^{2+} is more stable than Fe^{2+}.

The *G. graminis* and *M. salvinii* LOXs use manganese as a cofactor and we have found that the *A. fumigatus* LOX in the Val-group also contains manganese. Our results show that with use of a bioinformatics script, amino acid sequences can be compared and patterns can be revealed. Pattern identification then leads to new hypotheses and new research questions. In this way we have predicted and acknowledged the use of a cofactor in a novel class of fungal lipoxygenases.

53

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

Supplementary materials

Supplementary material 1 – Bioinformatics script used in isolating 369 LOXs

```
cat all_lox.fasta|sed '/>/s/$/@/' |tr -d "\n" |tr ">" "\n" |grep @M |grep W..AK |grep
H....H |grep H...N |rev |grep -f endlist |rev |grep -f filter -i -v |awk -F\@ '{print $2}'
|sort -u >lox1

cat -n lox1 |awk '{print ">@"$1"@""\n"$2}' >lox2

cat all_lox.fasta |grep -f filter -i -v >database_blast/.../bit_prog/blast-2.2.22/bin/
formatdb -i database_blast/.../bit_prog/blast-2.2.22/bin/blastall -pblastp -i lox2 -d
database_blast -FF -m9 -b10 -v10 |tee lox3

cat lox3 |grep gi |awk '$3 == 100.00 && $7==$9 && $8 ==$10 {print
"s/"$1"/"$2"/"}' >sedin

cat lox2 |sed -f sedin >./../lox_final.fasta/.../bit_prog/muscle -in lox_final.fasta -out
lox_final_align.fasta
```

Supplementary material 2 – GI-numbers belonging to Fig. 1

gi|315360477|dbj|BAJ46516.1|_lipoxygenase_[Marchantia_polymorpha]
gi|315360475|dbj|BAJ46515.1|_lipoxygenase_[Marchantia_polymorpha]
gi|315360473|dbj|BAJ46514.1|_lipoxygenase_[Marchantia_polymorpha]
gi|99083505|gb|ABF66653.1|_lipoxygenase7_[Physcomitrella_patens]
gi|170280121|gb|ACB12039.1|_lipoxygenase_[Oryza_sativa_Indica_Group]
gi|297613351|ref|NP_001067015.2|_Os12g0560200_[Oryza_sativa_Japonica_Group]
gi|170280123|gb|ACB12040.1|_lipoxygenase_[Oryza_sativa_Japonica_Group]
gi|113649518|dbj|BAF30030.1|_Os12g0559200_[Oryza_sativa_Japonica_Group]
gi|34922539|sp|Q8GSM3.1|LOX22_HORVU_RecName__Full=Lipoxygenase_2.2__
chloroplastic__AltName__Full=LOX2_Hv_2__Flags__Precursor
gi|34922469|sp|P93184.1|LOX21_HORVU_RecName__Full=Lipoxygenase_2.1__
chloroplastic__AltName__Full=LOX100__AltName__Full=LOX2_Hv_1__Flags__
Precursor
gi|115444801|ref|NP_001046180.1|_Os02g0194700_[Oryza_sativa_Japonica_Group]
gi|34922538|sp|Q8GSM2.1|LOX23_HORVU_RecName__Full=Lipoxygenase_2.3__
chloroplastic__AltName__Full=LOX2_Hv_3__Flags__Precursor
gi|93211180|gb|ABF01001.1|_lipoxygenase_[Zea_mays]
gi|162464003|ref|NP_001105980.1|_lipoxygenase10_[Zea_mays]
gi|162464186|ref|NP_001105981.1|_lipoxygenase11_[Zea_mays]
gi|93211182|gb|ABF01002.1|_lipoxygenase_[Zea_mays]
gi|113624169|dbj|BAF24114.1|_Os08g0509100_[Oryza_sativa_Japonica_Group]
gi|156187364|gb|ABU55902.1|_lipoxygenase2_[Oryza_sativa_Japonica_Group]
gi|563985|dbj|BAA03102.1|_lipoxygenase_[Oryza_sativa_Japonica_Group]
gi|626032|pir||A53054_lipoxygenase__EC_1.13.11.12__L2__rice
gi|113624168|dbj|BAF24113.1|_Os08g0508800_[Oryza_sativa_Japonica_Group]
gi|258618875|gb|ACV84255.1|_LOX7_[Sorghum_bicolor]
gi|27372775|gb|AAO03559.1|_lipoxygenase_2_[Brassica_napus]
gi|130845790|gb|ABO32545.1|_LOX_[Brassica_oleracea_var._gemmifera]
gi|585421|sp|P38418.1|LOX2_ARATH_RecName__Full=Lipoxygenase_2__
chloroplastic__Short=AtLOX2__Flags__Precursor
gi|6911852|emb|CAB72152.1|_lipoxygenase_AtLOX2_[Arabidopsis_thaliana]
gi|17065070|gb|AAL32689.1|_lipoxygenase_AtLOX2_[Arabidopsis_thaliana]
gi|71999169|gb|AAZ57444.1|_lipoxygenase_LOX1_[Populus_deltoides]
gi|71999171|gb|AAZ57445.1|_lipoxygenase_LOX2_[Populus_deltoides]

gi|229554825|gb|ACQ76787.1|_lipoxygenase_[Camellia_sinensis]
gi|308943877|gb|ADO51752.1|_lipoxygenase_[Camellia_sinensis]
gi|213876486|gb|ACJ54281.1|_lipoxygenase_[Camellia_sinensis]
gi|312837045|dbj|BAJ34928.1|_lipoxygenase_[Vitis_hybrid_cultivar]
gi|268636245|gb|ACZ17391.1|_lipoxygenase_[Vitis_vinifera]
gi|1495802|emb|CAA65268.1|_13lipoxygenase_[Solanum_tuberosum]
gi|1654138|gb|AAB65766.1|_lipoxygenase_[Solanum_lycopersicum]
gi|223016547|gb|ACM77790.1|_lipoxygenase_[Solanum_lycopersicum]
gi|187960379|gb|ACD43485.1|_lipoxygenase_2_[Olea_europaea]
gi|1853970|dbj|BAA13542.1|_CPRD46_protein_[Vigna_unguiculata]
gi|124014020|gb|ABM88259.1|_lipoxygenase_[Phaseolus_vulgaris]
gi|18461098|dbj|BAB84352.1|_lipoxygenase_[Citrus_jambhiri]
gi|84626293|gb|ABC59691.1|_lipoxygenase_[Zea_mays]
gi|220715236|gb|ACL81190.1|_tasselseed_1_[Zea_mays]
gi|258618871|gb|ACV84253.1|_LOX5_[Sorghum_bicolor]
gi|84626291|gb|ABC59690.1|_lipoxygenase_[Zea_mays]
gi|220715238|gb|ACL81191.1|_tasselseed_1b_[Zea_mays]
gi|116310177|emb|CAH67189.1|_OSIGBa0152K17.1_[Oryza_sativa_Indica_Group]
gi|62867565|emb|CAI84707.1|_lipoxygenaselike_protein_[Hordeum_vulgare_subsp._vulgare]
gi|258618865|gb|ACV84250.1|_LOX2_[Sorghum_bicolor]
gi|162463530|ref|NP_001105977.1|_lipoxygenase9_[Zea_mays]
gi|113547629|dbj|BAF11072.1|_Os03g0179900_[Oryza_sativa_Japonica_Group]
gi|1654140|gb|AAB65767.1|_lipoxygenase_[Solanum_lycopersicum]
gi|1495804|emb|CAA65269.1|_13lipoxygenase_[Solanum_tuberosum]
gi|32454714|gb|AAP83138.1|_lipoxygenase_[Nicotiana_attenuata]
gi|187960377|gb|ACD43484.1|_lipoxygenase_2_[Olea_europaea]
gi|40362875|gb|AAR84664.1|_lipoxygenase_[Carica_papaya]
gi|268636249|gb|ACZ17393.1|_lipoxygenase_[Vitis_vinifera]
gi|18394479|ref|NP_564021.1|_lipoxygenase_3_[Arabidopsis_thaliana]
gi|6002055|emb|CAB56692.1|_lipoxygenase_[Arabidopsis_thaliana]
gi|9665131|gb|AAF97315.1|AC007843_18_lipoxygenase_[Arabidopsis_thaliana]
gi|75309247|sp|Q9FNX8.1|LOX4_ARATH_RecName__Full=Lipoxygenase_4__
chloroplastic__Short=AtLOX4__AltName__Full=LOX3like_protein__Flags__Precursor
gi|48958183|emb|CAG38328.1|_13lipoxygenase_[Arabidopsis_thaliana]
gi|297332971|gb|EFH63389.1|_lipoxygenase_family_protein_[Arabidopsis_lyrata_subsp._lyrata]
gi|99083495|gb|ABF66648.1|_lipoxygenase2_[Physcomitrella_patens]

gi|258618877|gb|ACV84256.1|_LOX8_[Sorghum_bicolor]
gi|84626301|gb|ABC59695.1|_lipoxygenase_[Zea_mays]
gi|115463087|ref|NP_001055143.1|_Os05g0304600_[Oryza_sativa_Japonica_Group]
gi|258618873|gb|ACV84254.1|_LOX6_[Sorghum_bicolor]
gi|162463394|ref|NP_001105976.1|_lipoxygenase6_[Zea_mays]
gi|113645357|dbj|BAF28498.1|_Os11g0575600_[Oryza_sativa_Japonica_Group]
gi|157061188|gb|ABV03556.1|_lipoxygenase3_[Oryza_sativa_Japonica_Group]
gi|313661589|gb|ADR71856.1|_lipoxygenase1_[Triticum_turgidum_subsp._durum]
gi|313661593|gb|ADR71858.1|_lipoxygenase1_[Triticum_turgidum_subsp._durum]
gi|239923159|gb|ACS34909.1|_lipoxygenase_1_[Triticum_aestivum]
gi|328942163|gb|AEB70991.1|_lipoxygenase_4_[Triticum_aestivum]
gi|328942161|gb|AEB70990.1|_lipoxygenase_3_[Triticum_aestivum]
gi|313661591|gb|ADR71857.1|_lipoxygenase1_[Triticum_turgidum_subsp._durum]
gi|313661595|gb|ADR71859.1|_lipoxygenase1_[Triticum_turgidum_subsp._durum]
gi|2506825|sp|P29114.2|LOX1_HORVU_RecName__
Full=Linoleate_9Slipoxygenase_1__AltName__Full=Lipoxygenase_1
gi|341926404|gb|AEL03787.1|_lipoxygenase_[Avena_sativa]
gi|162459823|ref|NP_001105515.1|_lipoxygenase_[Zea_mays]
gi|12620877|gb|AAG61118.1|_lipoxygenase_[Zea_mays]
gi|258618863|gb|ACV84249.1|_LOX1_[Sorghum_bicolor]
gi|113549464|dbj|BAF12907.1|_Os03g0700400_[Oryza_sativa_Japonica_Group]
gi|20267|emb|CAA45738.1|_lipoxygenase_[Oryza_sativa_Japonica_Group]
gi|113549683|dbj|BAF13126.1|_Os03g0738600_[Oryza_sativa_Japonica_Group]
gi|258618869|gb|ACV84252.1|_LOX4_[Sorghum_bicolor]
gi|84626283|gb|ABC59686.1|_lipoxygenase_[Zea_mays]
gi|195648024|gb|ACG43480.1|_lipoxygenase_2_[Zea_mays]
gi|84626281|gb|ABC59685.1|_lipoxygenase_[Zea_mays]
gi|258618867|gb|ACV84251.1|_LOX3_[Sorghum_bicolor]
gi|2429087|gb|AAB70865.1|_lipoxygenase_2_[Hordeum_vulgare_subsp._vulgare]
gi|239923157|gb|ACS34908.1|_lipoxygenase_2_[Triticum_aestivum]
gi|190606635|gb|ACE79245.1|_lipoxygenase1_[Oryza_sativa_Indica_Group]
gi|154293784|gb|ABS72448.1|_lipoxygenase1_[Oryza_sativa_Japonica_Group]
gi|113549461|dbj|BAF12904.1|_Os03g0699700_[Oryza_sativa_Japonica_Group]
gi|38636549|dbj|BAD02945.1|_9lipoxigenase_[Oryza_sativa_Japonica_Group]
gi|161137615|gb|ABX57825.1|_lipoxygenase1_[Oryza_sativa_Indica_Group]
gi|84626287|gb|ABC59688.1|_lipoxygenase_[Zea_mays]
gi|84626285|gb|ABC59687.1|_lipoxygenase_[Zea_mays]
gi|73920878|sp|Q53RB0.1|LOX4_ORYSJ_RecName__Full=Probable_

linoleate_9Slipoxygenase_4__AltName__Full=Lipoxygenase_4
gi|88657483|gb|ABD47523.1|_dual_positional_specificity_lipoxygenase_[Oryza_
sativa_Japonica_Group]
gi|255674812|dbj|BAF12909.2|_Os03g0700700_[Oryza_sativa_Japonica_Group]
gi|325560590|gb|ADZ31265.1|_lipoxygenase_3_[Triticum_aestivum]
gi|2182267|gb|AAB60715.1|_lipoxygenase_[Hordeum_vulgare_subsp._vulgare]
gi|345648556|gb|AEO13837.1|_lipoxygenase_[Gladiolus_hybrid_cultivar]
gi|3668063|gb|AAC61785.1|_lipoxygenase_1_[Cucumis_sativus]
gi|1296512|emb|CAA63483.1|_lipoxygenase_[Cucumis_sativus]
gi|218092006|emb|CAP59449.1|_lipoxygenase_[Momordica_charantia]
gi|82547876|gb|ABB82552.1|_13Slipoxygenase_[Cucumis_melo_var._inodorus]
gi|256489082|gb|ACU81176.1|_13Slipoxygenase_[Cucumis_melo_var._inodorus]
gi|1017772|gb|AAA79186.1|_lipoxygenase_[Cucumis_sativus]
gi|14719443|pdb|1F8N|A_Chain_A__Lipoxygenase1__Soybean__At_100k__New_
Refinement
gi|16975077|pdb|1FGM|A_Chain_A__Lipoxygenase1__Soybean__At_100k__N694h_
Mutant
gi|14719447|pdb|1FGT|A_Chain_A__Lipoxygenase1__Soybean__At_100k__Q697n_
Mutant
gi|14719446|pdb|1FGR|A_Chain_A__Lipoxygenase1__Soybean__At_100k__Q697e_
Mutant
gi|14719445|pdb|1FGQ|A_Chain_A__Lipoxygenase1__Soybean__At_100k__Q495e_
Mutant
gi|14719444|pdb|1FGO|A_Chain_A__Lipoxygenase1__Soybean__At_100k__Q495a_
Mutant
gi|319443825|pdb|3PZW|A_Chain_A__Soybean_Lipoxygenase1__ReRefinement
gi|295388399|gb|ADG03093.1|_lipoxygenase_1_[Glycine_max]
gi|171849011|pdb|3BND|A_Chain_A__Lipoxygenase1__Soybean___I553v_Mutant
gi|171849010|pdb|3BNC|A_Chain_A__Lipoxygenase1__Soybean__I553g_Mutant
gi|171849012|pdb|3BNE|A_Chain_A__Lipoxygenase1__Soybean__I553a_Mutant
gi|171849009|pdb|3BNB|A_Chain_A__Lipoxygenase1__Soybean__I553l_Mutant
gi|295388395|gb|ADG03091.1|_lipoxygenase_2_[Glycine_max]
gi|126404|sp|P09439.1|LOX2_SOYBN_RecName__Full=Seed_linoleate_9Slipoxy
genase2__AltName__Full=Lipoxygenase2__Short=L2
gi|126402|sp|P14856.1|LOX2_PEA_RecName__Full=Seed_
linoleate_9Slipoxygenase2__AltName__Full=Lipoxygenase2
gi|493730|emb|CAA55318.1|_lipoxygenase_[Pisum_sativum]
gi|68161356|gb|AAY87056.1|_13lipoxygenase_[Arachis_hypogaea]

gi|68161358|gb|AAY87057.1|_13lipoxygenase_[Arachis_hypogaea]
gi|9909849|emb|CAC04380.1|_lipoxygenase_[Pisum_sativum]
gi|2459611|gb|AAB71759.1|_lipoxygenase_[Pisum_sativum]
gi|436169|gb|AAA03728.1|_lipoxygenase_[Glycine_max]
gi|118138512|pdb|2IUK|A_Chain_A__Crystal_Structure_Of_Soybean_LipoxygenaseD
gi|126411|sp|P24095.1|LOXX_SOYBN_RecName__Full=Seed_
linoleate_9Slipoxygenase__AltName__Full=Lipoxygenase
gi|1262440|gb|AAA96817.1|_lipoxygenase_[Glycine_max]
gi|152926332|gb|ABS32275.1|_lipoxygenase9_[Glycine_max]
gi|4464185|gb|AAB18970.2|_lipoxygenase_[Phaseolus_vulgaris]
gi|126409|sp|P27480.1|LOXA_PHAVU_RecName__Full=Linoleate_9Slipoxygenase_1__
AltName__Full=Lipoxygenase_1
gi|161318157|gb|ABX60408.1|_lipoxygenase_L3_[Glycine_max]
gi|31615698|pdb|1NO3|A_Chain_A__Refined_Structure_Of_Soybean_Lipoxygenase3_
With_4_Nitrocatechol_At_2.15_Angstrom_Resolution
gi|47168807|pdb|1ROV|A_Chain_A__Lipoxygenase3_Treated_With_Cumene_
Hydroperoxide
gi|295388403|gb|ADG03095.1|_lipoxygenase_3_[Glycine_max]
gi|126406|sp|P09186.1|LOX3_SOYBN_RecName__Full=Seed_
linoleate_9Slipoxygenase3__AltName__Full=Lipoxygenase3__Short=L3
gi|226237|prf||1502333A_lipoxygenase_3gi|126405|sp|P09918.1|LOX3_PEA_
RecName__Full=Seed_linol
eate_9Slipoxygenase3__AltName__Full=Lipoxygenase3
gi|2143422|emb|CAA97845.1|_lipoxygenase_[Vicia_faba]
gi|7024230|gb|AAF15296.2|AF204210_1_lipoxygenase_[Phaseolus_vulgaris]
gi|11991840|gb|AAG42354.1|_lipoxygenase_[Phaseolus_vulgaris]
gi|161318159|gb|ABX60409.1|_lipoxygease_L4_[Glycine_max]
gi|2160320|dbj|BAA03101.1|_lipxygenase_L4_[Glycine_max]
gi|439857|gb|AAA03726.1|_lipoxygenase_[Glycine_max]
gi|118138511|pdb|2IUJ|A_Chain_A__Crystal_Structure_Of_Soybean_LipoxygenaseB
gi|1588566|prf||2208476A_lipoxygenase
gi|161318163|gb|ABX60411.1|_lipoxygeaselike_protein_[Glycine_max]
gi|219935421|emb|CAG44503.1|_lipoxygenase_LOXN3_[Pisum_sativum]
gi|219935419|emb|CAG44501.1|_lipoxygenase_LOXN3_[Pisum_sativum]
gi|585416|sp|P38414.1|LOX1_LENCU_RecName__Full=Linoleate_9Slipoxygenase__
AltName__Full=Lipoxygenase
gi|541746|emb|CAA53730.1|_lipoxygenase_[Pisum_sativum]
gi|152926334|gb|ABS32276.1|_lipoxygenase10_[Glycine_max]

gi|110319961|emb|CAG44504.1|_lipoxygenase_loxN2_[Pisum_sativum]
gi|110319959|emb|CAG44502.1|_lipoxygenase_loxN2_[Pisum_sativum]
gi|337732519|gb|AEI71780.1|_bacterialinduced_lipoxygenase_[Gossypium_hirsutum]
gi|27372773|gb|AAO03558.1|_lipoxygenase_1_[Brassica_napus]
gi|34098837|gb|AAQ56801.1|_At1g55020_[Arabidopsis_thaliana]
gi|124365601|gb|AAK50778.4|AF361893_1_bacterialinduced_lipoxygenase_
[Gossypium_hirsutum]
gi|199584368|gb|ACH90245.1|_lipoxygenase_LOX2_[Prunus_persica]
gi|33235471|emb|CAE17327.1|_lipoxygenase_[Fragaria_x_ananassa]
gi|296476804|gb|DAA18919.1|_arachidonate_12lipoxygenase_isoform_2_[Bos_
taurus]
gi|1246125|gb|AAB36013.1|_12lipoxygenase_[Mus_sp.]
gi|467221|gb|AAA20659.1|_platelettype_12lipoxygenase_[Mus_musculus]
gi|148680605|gb|EDL12552.1|_arachidonate_12lipoxygenase__isoform_CRA_c_[Mus_
musculus]
gi|148680604|gb|EDL12551.1|_arachidonate_12lipoxygenase__isoform_CRA_b_[Mus_
musculus]
gi|56800471|emb|CAI35235.1|_arachidonate_12lipoxygenase_[Mus_musculus]
gi|157786628|ref|NP_001099268.1|_arachidonate_12lipoxygenase__12Stype_[Rattus_
norvegicus]
gi|344237821|gb|EGV93924.1|_Arachidonate_12lipoxygenase__12Stype_[Cricetulus_
griseus]
gi|32451918|gb|AAH54621.1|_Arachidonate_12lipoxygenase_[Danio_rerio]
gi|220678977|emb|CAX14764.1|_novel_protein_similar_to_vertebrate_
arachidonate_5lipoxygenase__ALOX5__[Danio_rerio]
gi|209149946|gb|ACI33000.1|_Arachidonate_5lipoxygenase_[Salmo_salar]
gi|344237698|gb|EGV93801.1|_Arachidonate_5lipoxygenase_[Cricetulus_griseus]
gi|1151265|gb|AAA85257.1|_5lipoxygenase_[Mesocricetus_auratus]
gi|729945|sp|P12527.3|LOX5_RAT_RecName__Full=Arachidonate_5lipoxygenase__
Short=5LO__Short=5lipoxygenase
gi|6978493|ref|NP_036954.1|_arachidonate_5lipoxygenase_[Rattus_norvegicus]
gi|149049669|gb|EDM02123.1|_arachidonate_5lipoxygenase_[Rattus_norvegicus]
gi|148667143|gb|EDK99559.1|_arachidonate_5lipoxygenase__isoform_CRA_a_[Mus_
musculus]
gi|886333|gb|AAC37673.1|_arachidonate_5lipoxygenase_[Mus_musculus]
gi|187954199|gb|AAI39103.1|_Arachidonate_5lipoxygenase_[Mus_musculus]
gi|318055294|gb|ADV36116.1|_arachidonate_5lipoxygenase_[Canis_lupus_familiaris]
gi|300796056|ref|NP_001179721.1|_arachidonate_5lipoxygenase_[Bos_taurus]

gi|4502057|ref|NP_000689.1|_arachidonate_5lipoxygenase_[Homo_sapiens]
gi|312985301|gb|ADR30801.1|_arachidonic_5lipoxygenase_deltap10_isoform_
[Homo_sapiens]
gi|219520700|gb|AAI43986.1|_ALOX5_protein_[Homo_sapiens]
gi|119607048|gb|EAW86642.1|_arachidonate_5lipoxygenase__isoform_CRA_a_
[Homo_sapiens]
gi|319443631|pdb|3O8Y|B_Chain_B__Stable5Lipoxygenase
gi|109150076|gb|AAI17663.1|_Zgc_136911_[Danio_rerio]
gi|63101274|gb|AAH95211.1|_Zgc_110251_[Danio_rerio]
gi|299469980|emb|CBN79157.1|_Lipoxygenase_[Ectocarpus_siliculosus]
gi|219924573|emb|CAQ87588.1|_valencene_oxygenase_[Pleurotus_sapidus]
gi|293330614|dbj|BAI99788.1|_lipoxygenase_[Pleurotus_ostreatus]
gi|70982632|ref|XP_746844.1|_lipoxygenase_[Aspergillus_fumigatus_Af293]
gi|347840630|emb|CCD55202.1|_similar_to_lipoxygenase_[Botryotinia_fuckeliana]
gi|347836078|emb|CCD50650.1|_similar_to_lipoxygenase_[Botryotinia_fuckeliana]
gi|322712613|gb|EFZ04186.1|_lipoxygenase_1_[Metarhizium_anisopliae_ARSEF_23]
gi|340905167|gb|EGS17535.1|_lipoxygenaselike_protein_[Chaetomium_
thermophilum_var._thermophilum_DSM_1495]
gi|350293481|gb|EGZ74566.1|_Lipoxigenase_[Neurospora_tetrasperma_FGSC_2509]
gi|56682225|gb|AAW21637.1|_lipoxygenase_[Gibberella_moniliformis]
gi|322706589|gb|EFY98169.1|_lipoxygenase_[Metarhizium_anisopliae_ARSEF_23]
gi|322700866|gb|EFY92618.1|_lipoxygenase_[Metarhizium_acridum_CQMa_102]
gi|320589636|gb|EFX02092.1|_arachidonate_15lipoxygenase_precursor_
[Grosmannia_clavigera_kw1407]
gi|17861374|gb|AAK81882.1|_manganese_lipoxygenase_[Gaeumannomyces_
graminis_var._avenae]
gi|70981869|ref|XP_746463.1|_arachidonate_5lipoxygenase_[Aspergillus_fumigatus_
Af293]

Chapter 4

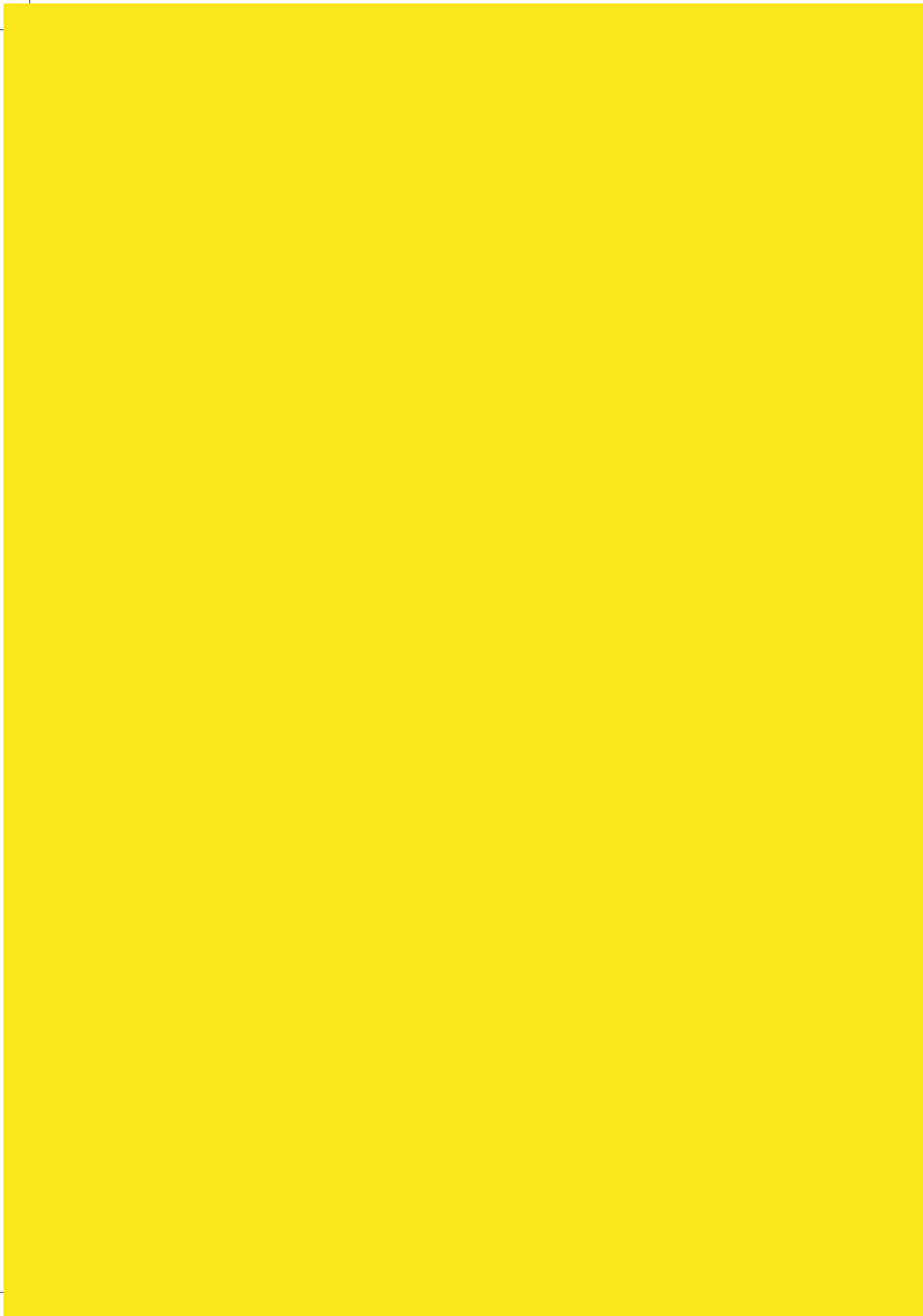# Methods for identifying lipoxygenase producing microorganisms on agar plates

Abstract

We developed plate assays for the screening of lipoxygenase-producing microorganisms on agar plates. Both potassium iodide-starch and indamine dye formation methods were effective for detecting soybean lipoxygenase activity on agar plates. A positive result was also achieved using the β-carotene bleaching method, but the sensitivity of this method was lower than the two other methods. The potassium iodide-starch and indamine dye formation methods were also applied for detecting lipoxygenase production by *Trichoderma reesei* and *Pichia pastoris* transformants expressing the lipoxygenase gene of the fungus *Gaeumannomyces graminis*. In both cases, lipoxygenase production in the transformants could be identified. The potassium iodide-starch method was successful for detection of the *G. graminis* lipoxygenase produced by *Aspergillus nidulans*. When *Escherichia coli* was grown on agar and soybean lipoxygenase was applied to the culture, lipoxygenase activity could clearly be detected by the indamine dye formation method. This suggests that the indamine dye method has potential for screening of metagenomic libraries in *E. coli* for lipoxygenase activity.

65

Introduction

Lipoxygenases (EC 1.13.11.12, EC 1.13.11.34 and EC 1.13.11.33) are non-heme, iron or manganese containing enzymes, that catalyze the oxidation of unsaturated fatty acids containing a 1-*cis*, 4-*cis*-pentadiene structure into fatty acid hydroperoxides. Linoleic acid, linolenic acid and arachidonic acid are the natural substrates of lipoxygenases (Su and Oliw 1998; Goldsmith et al. 2002).

Lipoxygenase-catalyzed oxidative coupling reactions are utilized extensively in bread making. The addition of lipoxygenase-rich soybean flour results in the production of fatty acid hydroperoxides, which bleach the pigments of wheat flours. Lipoxygenase activity also improves the mixing tolerance and rheological properties of the dough. (Frazier et al. 1973; Faubion & Hoseney 1981; Boussard et al. 2012). Furthermore, lipoxygenases have been used for bleaching dairy products, such as cheese, milk, butter oil, cream, and whey (de Roos et al. 2006). Alcohols and aldehydes have been produced industrially from the fatty acid hydroperoxides that are formed in the lipoxygenase-catalyzed reactions. These compounds are used for flavouring foods (Whitehead et al. 1995).

Lipoxygenases have been detected in plants, mammals, and in eukaryotic and prokaryotic microorganisms (Feussner & Wasternack 2002; Baysal & Demirdöven 2007; Hammarberg et al. 1993; Su & Oliw 1998; Koeduka et al. 2007; Vance et al. 2004). The recombinant production of secreted, stable lipoxygenases at high levels, however, has only been achieved by the expression of the lipoxygenase gene of the fungus *Gaeumannomyces graminis* in *Pichia pastoris* (Cristea et al. 2005).

Various assays to detect lipoxygenase activity have been developed (Axelrod et al. 1981; Williams et al. 1986; Suda et al. 1995; Villafuerte Romero & Barrett 1997; Anthon & Barrett 2001). The majority of these assays are based on identifying the formation of fatty acid hydroperoxides *via* indirect detection using colourimetry or direct detection using UV-spectroscopy. In most cases, however, plate assays for the screening of microorganisms for industrially interesting enzymatic activities are the method of choice because it allows high-throughput screening. We are unaware of any agar plate assays to screen microorganisms for the production of secreted lipoxygenases.

In this paper we present methods for detecting lipoxygenase activity in microorganisms. The tested organisms include *Trichoderma reesei*, *P. pastoris* and *Aspergillus nidulans* expressing the *G. graminis* lipoxygenase gene, as well as the native lipoxygenase producer *G. graminis* var. *triciti.*

Materials and methods

Cloning the *G. graminis* lipoxygenase gene

The gene encoding the *G. graminis* lipoxygenase AAK81882.1 was codon-optimized and synthesized by GenScript, a gene synthesis service (GenScript Corporation, Piscataway, USA), for expression in *P. pastoris*. To facilitate cloning, *Eco*RI and *Kpn*I restriction sites were added at the 5' and 3' ends of the fragment, respectively. The DNA fragment of the synthesized gene was ligated into the *Eco*RI and *Kpn*I sites of the vector pPICZαA (Invitrogen) bringing the expression under the control of the methanol inducible AOX1 promoter. The native signal sequences were replaced with the prepro sequence of the *Saccharomyces cerevisiae* α-factor secretion signal for secretion of the recombinant protein. *P. pastoris* wild type strain X-33 (Invitrogen) was transformed by using electroporation with the lipoxygenase expression vector that had been linearised with *Pme*I to target the integration into the AOX1 locus. The transformants were plated on YPDS plates containing 100 µl ml^{-1} Zeocin (Invitrogen)

and cultivated at 30 °C. Single-crossover recombination at the AOX1 locus was verified by PCR using 5′ and 3′ AOX1 primers.

For expression in *Trichoderma reesei* the *G. graminis* lipoxygenase-encoding gene AAK81882.1 was codon-optimized and synthesized by GeneArt (Regensburg, Germany). To facilitate cloning, *Sac*II and *Bam*HI restriction sites were added at the 5′ and 3′ ends of the fragment, respectively. The DNA fragment of the synthesized gene was ligated into the *Sac*II and *Bam*HI sites of the expression vector pAB100 containing the strong *cbhI*-promoter of *T. reesei*. The native signal sequence of the recombinant lipoxygenase was used for secretion. *T. reesei* strain RH32578 was transformed as described and transformants were screened for growth on plates with acetamide as sole nitrogen source (Penttilä et al. 1987). Resulting transformants were analyzed by PCR for integration of the lipoxygenase gene into the genome.

The gene encoding the *G. graminis* lipoxygenase AAK81882.1 was codon-optimized for expression in *Aspergillus niger* and synthesized by DNA 2.0 (Menlo Park, USA). For expression the promoter and secretion signal of the *xlnD* gene, from *Aspergillus niger* [GI: 74626559], replaced the native secretion signal of *G. graminis* (Van Peij et al. 1997). The *Xba*I and *Bam*HI restriction sites ensured the synthesized gene was incorporated into a pUC19 vector and was used to transform the *pyrA* strain *A. nidulans* WG505. The transformants were plated on MMS plates for four days at 37 °C (Kusters et al. 1991). Positive transformants were analyzed using PCR for verification of integration of the lipoxygenase gene into the genome.

Lipoxygenase plate assay methods

A 25 mM linoleic acid solution containing 14 mg ml^{-1} Tween 20 was used in the β-carotene bleaching assay. The indamine dye formation assay was prepared as previously described (Anthon & Barrett 2001). All incubations were carried out in the dark at room temperature.

β-carotene bleaching

The half-saturated solution of β-carotene in acetone used in the assay was prepared as follows. Five mg of β-carotene was dissolved in 5 ml of acetone. The suspension was mixed thoroughly and centrifuged for 10 min at 25 000 g. The supernatant was diluted with the same volume of acetone to give 50% saturation with respect to β-carotene.

The linoleic acid agarose solution contained 2.3 mM linoleic acid, 1% (w v⁻¹) low melting point agarose (Seaplaque) and 50 mM potassium phosphate buffer (pH 7.0). The linoleic acid agarose solution was mixed at a ratio of 39:5 (w v⁻¹) with half-saturated solution of β-carotene in acetone and applied as an overlay. This method is based on a previously described lipoxygenase assay of liquid samples (Villafuerte Romero & Barrett 1997).

Indamine dye formation

The indamine dye formation assay is based on the chemical reaction described by Ngo and Lenhoff (Ngo & Lenhoff 1980). A 10 mM DMAB [3-(dimethylamine) benzoic acid] stock solution buffered with 200 mM disodium phosphate pH 6.0; the 10 mM MBTH (3-Methyl-2-benzothiazolinone) stock solution and the 0.1 mg ml⁻¹ hematin stock solution buffered with 5 mM NaOH were prepared as previously described (Anthon & Barrett, 2001). An overlay of agarose with linoleic acid was used and the colouring reagents contained 2.3 mM linoleic acid, 1% (w v⁻¹) agarose, 50 mM K-phosphate buffer (pH 7.0), 4.6 mM DMAB, 0.1 mM MBTH, and 1 μg l⁻¹ hematin.

Potassium iodide (KI) - starch method

The potassium iodide assay described by Williams *et al.* was modified for detecting lipoxygenase activity of agar cultures (Williams et al. 1986). A linoleic acid solution was prepared by mixing linoleic acid (700 μl) and Tween 20 (700 μl) with 5 ml water. The resulting emulsion was clarified by adding 1 ml 1 M NaOH and diluted to a final volume of 25 ml. For detecting lipoxygenase activity, 100 μl of the linoleic acid solution was added into wells on agar containing 40 g l⁻¹ starch. After incubation, 100 μl of saturated potassium iodide solution and 75 μl of 75% (v v⁻¹) acetic acid were added into the wells and mixed.

Comparison of assay sensitivity

An ammonium sulfate suspension of type V soybean lipoxygenase (sLOX-1) (Sigma) was diluted with a 50 mM potassium phosphate buffer at pH 7.0. A lipoxygenase solution of 25 μl was applied to wells in LB-agar in amounts ranging from 8 ng to1 μg lipoxygenase per well. For the KI-starch method, starch was added to the LB-agar as described above. For comparison of the sensitivities, the plates were incubated at room temperature for 2 h and then were visually examined.

Cultivation of the microorganisms on agar plates for detection of lipoxygenase activity

P. pastoris transformants were grown for three days at 30°C on BMMY (Buffered Methanol Complex Medium, Invitrogen) agar. Inducer-methanol was added daily to the lid of the inverted plate to compensate for loss due to evaporation. *T. reesei* transformants were grown on potato dextrose agar at 28–30°C. For analysis using the KI-starch method, plates were supplemented with starch as described above. *A. nidulans* transformants were grown on 50 mM D-xylose minimal medium plates containing 1.2% agar and potato dextrose was omitted to prevent carbon catabolite repression (Pontecorvo et al. 1953). *G. graminis* var. *tritici* CBS 905.73 was grown on potato dextrose agar at 25°C. For the KI-starch analysis method, the medium was supplemented with starch as described above. Colouring reagents were applied as an agarose overlay for the indamine dye formation assay.

Effect of the linoleic acid oxidation products on the growth of *E. coli*

To study inhibitory effects of the reaction-products of lipoxygenase catalyzed oxidation, linoleic acid was dispersed by mechanical homogenization into LB-agar at a concentration of 0.4% (w v^{-1}). A 4 µl solution containing 0.5, 1.8 and 7.2 µg of soybean lipoxygenase (Sigma L-7395) in 50% (w v^{-1}) glycerol, 100 mM Na-phosphate buffer, pH 7.0 was applied to wells present in the agar. Plates were incubated for 30 min at room temperature. *E. coli* (XL1-blue) suspensions were spread on the plates after the incubation and cells were grown overnight.

Detection of lipoxygenase activity by the indamine dye formation method in the presence of *E. coli* cells

Wells were made in the LB-agar plates and each well was inoculated with 400 µl of *E. coli* (XL1-blue) suspension in LB medium. Cells were cultivated overnight at 37°C. A lawn on the plates sLOX-1 (Sigma) was diluted with a 50 mM potassium phosphate buffer, pH 7.0, and 25 µl of the lipoxygenase solution was applied into the agar wells at final amounts ranging from 8 ng to 1 µg. After the enzyme solutions had been absorbed, the indamine dye formation agarose was applied either as a single layer or sequentially as two layers of equal volume. In the latter case, the first layer contained 4.6 mM linoleic acid and 100 mM K-phosphate buffer pH 7.0 and the second layer contained 9.2 mM DMAB, 0.2 mM MBTH and 2 µg hematin. After the application of the first layer the plates were incubated overnight at room temperature before the

second layer was applied. Experiments both with and without 0.5 mM EDTA in the overlays were carried out.

Results

Comparison of assays for detection of soybean lipoxygenase

The formation of lipoxygenase-catalyzed hydroperoxides resulted in a violet-blue colour with the indamine dye formation method, a brown colour with the potassium iodide-starch method and bleached halos in the yellow-orange agarose background with the β-carotene bleaching method.

To compare the three methods, soybean lipoxygenase was applied on the agar plate and analyzed. As shown in Fig. 1, soybean lipoxygenase activity on linoleic acid was detectable with all three colourimetric methods. The sensitivities of the indamine dye formation and KI – starch methods were of the same order with the detection limits ranging from 63 and 130 ng soybean lipoxygenase. β-carotene bleaching was not used for the assays for lipoxygenase activity in further experiments; this method appeared to be the least sensitive of the three methods tested with the detection limit ranging from 0.5 μg to 1.0 μg of soybean lipoxygenase.
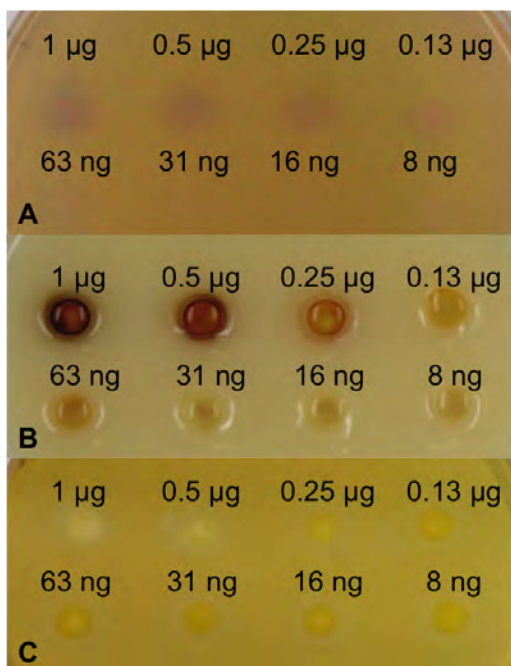
Figure 1: Comparison of sensitivities of (A) indamine dye formation, (B) KI-starch, and (C) β-carotene bleaching methods. Different concentrations of sLOX-1 were applied on the agar plates. Plates were incubated for two hours at room temperature.

Detection of lipoxygenase production by microbial transformants

P. pastoris, *T. reesei* and *A. nidulans* transformants with and without the lipoxygenase gene of *G. graminis* were grown on agar plates and were induced as described in Materials and Methods. Transformants were analyzed for lipoxygenase activity using the KI-starch and indamine colour formation methods. As shown in Fig. 2, both *P. pastoris* transformants with the lipoxygenase gene gave clear positive signals with both methods. For the assay using the KI-starch method it proved to be necessary to grow the *Pichia* transformants on plates composed of a layer without starch on top and a layer with it on the bottom. Wells were punched in the upper layer in the vicinity of the colonies and the reagents added. Possibly the starch used contained traces of free sugars that caused carbon catabolite repression of expression.
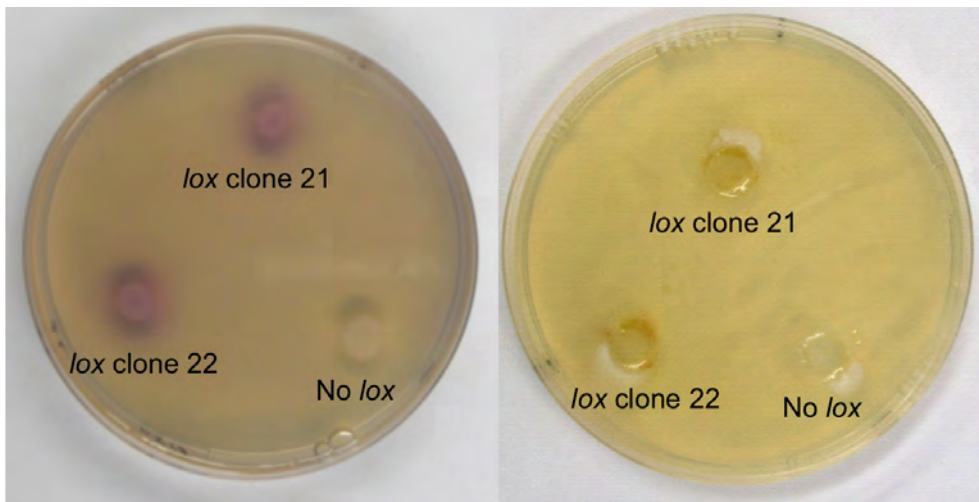
Figure 2: Lipoxygenase detection of *Pichia pastoris* transformants expressing the lipoxygenase of *Gaeumannomyces graminis* (A) analyzed by indamine dye formation method and (B) analyzed by KI-starch method.

As displayed in Fig. 3, there was a clear brown colour around the mycelium of *T. reesei*. This was not found for the wild type and indicates the plate assay can be used for this organism. For the plate assays using *A. nidulans,* potato dextrose was replaced by D-xylose to prevent inhibition of lipoxygenase by carbon catabolite respression. In these transformants the D-xylose inducible *xlnD* promoter (Van Peij et al. 1997) was used for the expression of the lipoxygenase. Results of the assay are displayed in Fig. 4. Positive transformants for lipoxygenase activity are visible as a brown colony. No positive transformant occurred with the *A. nidulans* wild type. The indamine colour assay was not successful for *A. nidulans* and is likely the result of the low enzyme expression level in this organism.
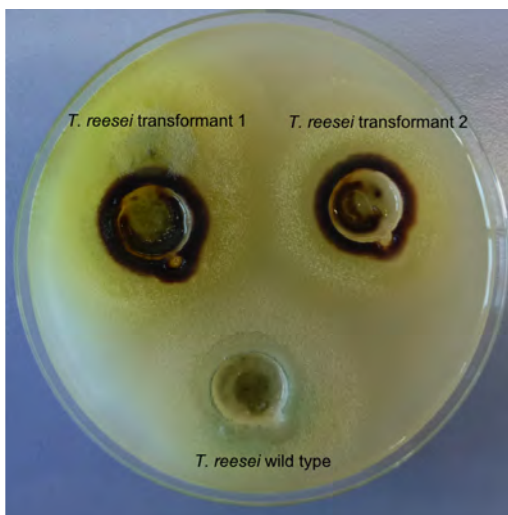
Figure 3: Detection of *Gaeumannomyces graminis* lipoxygenase activity in *Trichoderma reesei* transformants grown on plate using the KI staining method. Positive transformants show a brown colour around the mycelium.
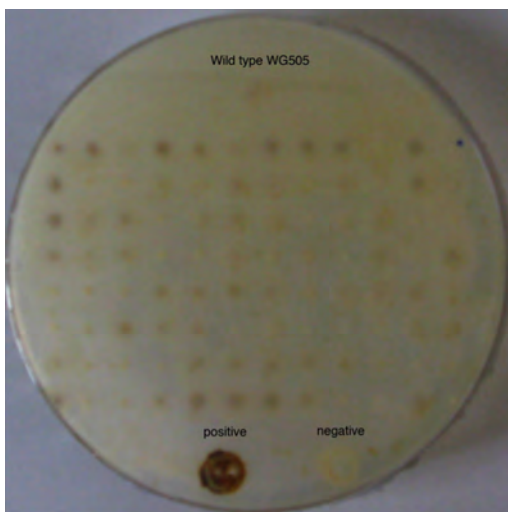
Figure 4: Detection of *Gaeumannomyces graminis* lipoxygenase activity in *Aspergillus nidulans* transformants using the KI-assay. Positive transformants are visualized as brown dots in the plate.

Effects of lipoxygenase reaction products on growth of *E. coli* and effects of *E. coli* cells on lipoxygenase activity

To study the effect of the oxidation products of linoleic acid on the growth of *E. coli*, soybean lipoxygenase was applied to linoleic acid LB-agar. After incubation, *E. coli* cells were grown overnight as a lawn on the plates. As shown in Fig. 5, clear growth-inhibiting zones could be detected on the plates around the soybean lipoxygenase (between 0.11 µg and 7.2 µg of the enzyme sample) application points.
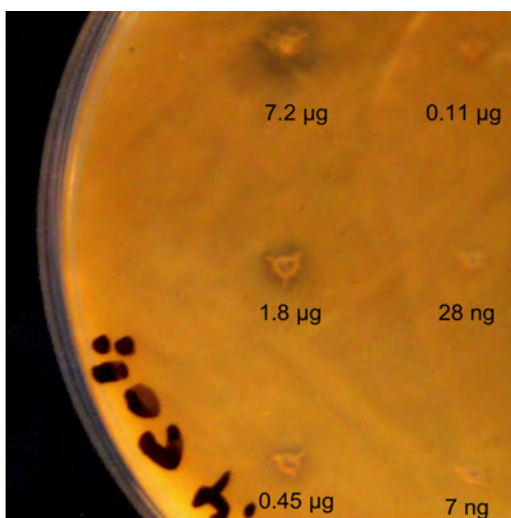
Figure 5: Overnight growth *Escherichia coli* on linoleic acid agar supplemented with sLOX-1. Plates were incubated for 30 min at room temperature in the presence of different amounts of the lipoxygenase and inoculated with the cell suspension.

73

The possibility that *E. coli* cells interfere with the lipoxygenase activity was investigated by applying soybean lipoxygenase on a lawn of growing cells. When all indamine dye formation reagents were added as a single layer, only 0.5 µg to 1 µg soybean lipoxygenase could be detected after five hours of incubation. When plates were incubated overnight, the blue indamine colour had disappeared. When linoleic acid agarose was added first, however, followed by an overnight incubation and addition of the colouring reagent agarose, the violet-blue colour was clearly visible between 63 ng and 130 ng of soybean lipoxygenase (Fig. 6). In both cases the addition of 0.5 M EDTA intensified the signal (data not shown).
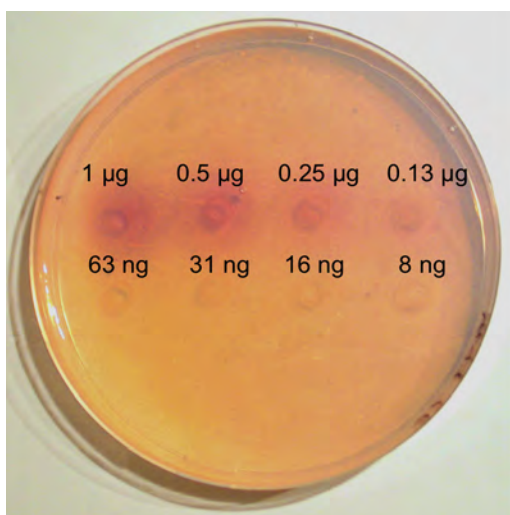
Figure 6: Detection of sLOX-1 applied on a lawn of *Escherichia coli* cells by indamine dye formation method. Substrate and colouring reagent agaroses were supplemented with 0.5 mM EDTA and applied sequentially.

Discussion

The methods used in the current work for identifying lipoxygenase-producing transformants on agar plates are based on detecting the presence of linoleic acid hydroperoxides formed in the lipoxygenase-catalyzed reaction. During the indamine dye formation method, 3-methyl-2-benzothiazolinone (MBTH) is coupled oxidatively with 3-(dimethylamino) benzoic acid (DMAB) in a hematin-catalyzed reaction. This results in the generation of the indamine dye (Anthon & Barrett 2001). In the potassium iodide starch method, the formed fatty acid hydroperoxides oxidize iodide to iodine, which then react with starch (Williams et al. 1986). β-Carotene can then be used for lipoxygenase detection because the fatty acid hydroperoxides cause it to bleach (Villafuerte Romero & Barrett 1997).

The indamine dye formation and KI-starch methods appeared to be more sensitive than the β-carotene bleaching method for detecting lipoxygenase activity. The poor sensitivity of the β-carotene bleaching method has been established in a study where several liquid assays for detecting lipoxygenase activity from vegetable extracts were compared (Villafuerte Romero & Barrett 1997).

The reaction products of lipoxygenase-catalyzed oxidation of linoleic acid appeared to be toxic for *E. coli* cells. It has been shown that fatty acid hydroperoxides and their degradation products (oxylipins) impair the growth of many microbial plant pathogens (Prost et al. 2005). If linoleic acid is present in the growth medium from the onset of the cultivation, lipoxygenase producers may not be able to survive.

74

Linoleic acid can also be spontaneously degraded *via* auto-oxidation during longer incubations (Seo et al. 1999). For these reasons, we chose to omit linoleic acid from the agar medium. Lipoxygenase positive *T. reesei* and *P. pastoris* transformants expressing the *G. graminis* lipoxygenase gene were clearly identified using either KI-starch or indamine dye formation methods. For *A. nidulans* transformants, only the KI-starch assay was successful. The indamine dye formation method did not show clear results for detecting lipoxygenase (data not shown).

A potential problem in screening *E. coli*-hosted metagenomic libraries is that the cells are able to use the lipoxygenase substrate, linoleic acid, for growth. This problem can, however, theoretically be overcome by disrupting genes encoding fatty acid transport and/or β-oxidation present in the *fad* regulon (DiRusso & Nyström 1998). As stated above, adding linoleic acid to the medium may not be feasible because positive transformants can produce toxic substances *via* the lipoxygenase catalyzed oxidation of the substrate.

Preferably tens of thousands of metagenomic library colonies would have to be screened to find lipoxygenase activity. Furthermore, it is typical for metagenomic expression libraries that the activities are low and may be detectable only after prolonged cultivations (Uchiyama & Miyazaki 2009). The use of the KI-starch method is problematic for the detection of lipoxygenase producing transformants of metagenomic libraries because the colour reaction takes place under very acidic conditions. Iodine is also a well-known microbicide (Vasudevan & Tandon 2010). We, therefore, selected the indamine dye formation method to investigate whether lipoxygenase activity can be detected in the presence of *E. coli* cells. If linoleic acid and the colouring reagents were applied as one layer on the *E. coli* culture supplemented with soybean lipoxygenase, the violet-blue colour was for unknown reasons only transiently visible. When the substrate agarose was applied first, however, followed by incubation and application of the colouring agarose, the soybean lipoxygenase catalyzed reaction was clearly detectable. EDTA intensified the signal, possibly because it chelates metal ions, which can catalyze hydroperoxide degradation.

75

In conclusion, both the KI-starch and the indamine dye formation methods can be used to detect lipoxygenase producing transformants of *P. pastoris* and *T. reesei*. These methods may also be suitable for other microbial hosts. An advantage of the indamine dye formation method is its possible use in identifing lipoxygenase positive transformants of *E. coli*-hosted metagenomic libraries. At present, however, detecting lipoxygenase production using natural isolates grown on agar remains a challenge.

Chapter 5

Heterologous expression of *Gaeumannomyces graminis* lipoxygenase in *Aspergillus nidulans*

Abstract

Aspergillus sp. express Ppo enzymes that produce oxylipins from polyunsaturated fatty acids. These oxylipins function as signal molecules in sporulation and influence the asexual-to-sexual ratio of *Aspergillus* sp. Another enzyme capable of synthesizing oxylipins is lipoxygenase. Lipoxygenase is hypothesized to be involved in quorum-sensing abilities and in the invasion of plant tissue by the fungus. *Aspergillus nidulans* and *Aspergillus niger* only contain *ppo* genes, whereas the human pathogenic *Aspergillus flavus* and *Aspergillus fumigatus* contain *ppo* in addition to lipoxygenase genes. In this study we introduced the *Gaeumannomyces graminis* lipoxygenase gene in *A. nidulans* WG505 to heterologously express the lipoxygenase protein.

The production of the *G. graminis* lipoxygenase induced phenotypic changes in *A. nidulans* transformants. The *G. graminis* LOX, however, could not be detected using SDS-PAGE or Western Blot analysis due to unspecific binding of polyclonal antibodies. Instead, the antibodies showed high homology for a ~125 kDa protein proposed to be PpoC. A proteomic analysis of an *A. nidulans* strain producing the *G. graminis* lipoxygenase revealed the presence of *G. graminis* lipoxygenase, PpoC, thioredoxin reductase, and aminopeptidase Y. These proteins were not detected in the *A. nidulans* wild type.

It is suggested that thioredoxin reductase protects *A. nidulans* against oxidative stress, which is induced by active *G. graminis* lipoxygenase. As a result the *G. graminis* lipoxygenase is degraded in the vacuole by aminopeptidase Y. Therefore, *A. nidulans* WG505 is not a suitable production host for heterologous production of *G. graminis* lipoxygenase.

79

Introduction

Aspergillus sp. contains *ppo* genes coding for dioxygenases that belong to the linoleate diol synthase (LDS) protein family. These dioxygenases produce a subset of oxylipins, called precocious sexual inducer (psi) factors. Psi-factor is a collective term for C18:1, C18:2 and C18:3 derived oxylipins (Gao et al. 2007). Oxylipins are signal molecules that are used by fungi to control sporulation (Brodhun & Feussner 2011). These oxylipins can be categorized into three groups depending on the position of hydroxyl group on the polyunsaturated fatty acid (PUFA): psiB (8'-hydroxy-PUFA), psiC (5',8'-dihydroxy-PUFA), or psiA, which has a δ-lactone ring at the 5' position of the psiC oxylipin (Tsitsigiannis et al. 2004). Depending on the PUFA, the oxylipin can be further categorized as α (18:2, linoleic acid), β (18:1, oleic acid), or γ (18:3, linolenic acid) (Tsitsigiannis & Keller 2007).

Amongst the *Aspergillus* sp., there are differences in the spectrum of ppo-genes that are present in the genome. The *Aspergillus nidulans* genome contains the *ppo* genes *ppoA*, *ppoB*, and *ppoC*, which code for enzymes synthesizing the oxylipins psiBα, psiBβ, and psiBβ, respectively (Brodhun et al. 2011). Although *Aspergillus niger* also contains three *ppo* genes, it lacks *ppoB* but contains *ppoD* (Wadman et al. 2009). In *A. nidulans,* PpoA and PpoC have antagonistic roles. PpoB upregulates the *ppoA* gene and, at the same time, represses *ppoC* (Tsitsigiannis & Keller 2005; Brodhun & Feussner 2011). Removing the *ppoA* and *ppoB* genes increases the ratio of asexual-to-sexual sporulation, and removing the *ppoC* gene decreases the ratio of asexual-to-sexual sporulation (Tsitsigiannis & Keller 2005). Developing conidia is preferred in asexual sporulation, whereas scleroatia is preferred in sexual reproduction. It has been speculated, however, that these sporulation phenotypes cannot be explained by the psiB-oxylipin-levels alone. Hence it is suggested that other oxylipins are also involved (Tsitsigiannis & Keller 2005).
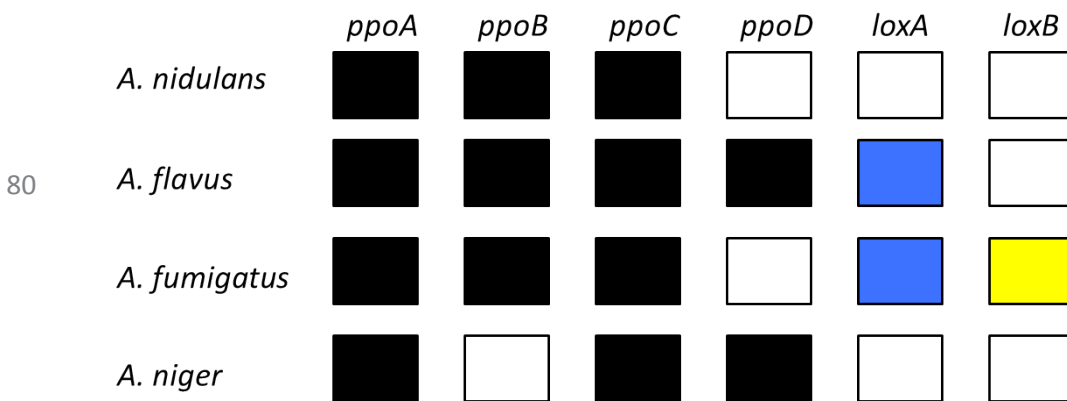
Figure 1: Distribution of *ppo* and *lox* genes amongst four different *Aspergillus* species. The black box indicates the presence of the *ppo* gene. The blue box indicates the presence of a *lox* gene coding for an intracellular LOX, and the yellow box represents a *lox* gene coding for an extracellular LOX. *Aspergillus nidulans* and *Aspergillus niger* have three different *ppo* genes. *Aspergillus flavus* has four different *ppo* genes and a *lox* gene. *Aspergillus fumigatus* has three different *ppo* genes and two *lox* genes.

Another enzyme capable of producing oxylipins is lipoxygenase (LOX). LOX is a non-heme, iron-containing or managese-containing dioxygenase that can be found in a wide variety of organisms, including fungi (Heshof et al. 2014a). Lipoxygenase is absent in *A. nidulans* and *A. niger,* but the human pathogens *Aspergillus flavus* and *Aspergillus fumigatus* contain both *ppo* genes and *lox* genes (Brown et al. 2008; Wadman et al. 2009; Affeldt et al. 2012). The distribution of *ppo* and *lox* genes in these *Aspergilli* is schematically shown in Fig. 1.

Studies have shown that LOX in *A. flavus* is involved in quorum-sensing and phenotypic differences are found when the gene is disrupted (Brown et al. 2008; Affeldt et al. 2012). The *Gaeumannomyces graminis* LOX is a secreted enzyme capable of producing 11*S*-HPODE and 13*R*-HPODE oxylipins and is hypothesized to be involved when invading plant tissue (Oliw 2002). Plant oxylipins 9*S*-HPODE and 13*S*-HPODE induce sporogenic effects in *A. nidulans* similar to those produced by the psi factor (Calvo et al. 1999). Both oxylipins cause decreased mycelial growth where 13*S*-HPODE in a 10-100 µM concentration also reduces mycotoxin production of aflatoxin and sterigmatocystin (Burow et al. 1997). In the present study we introduce the *G. graminis lox* gene in the *A. nidulans* WG505 expression host to verify whether *A. nidulans* is a suitable host for the heterologous expression of *G. graminis* LOX.

Materials and methods

Expression of *G. graminis* LOX in *A. nidulans*

Heterologous expression of *G. graminis* LOX in *A. nidulans* was performed according to the method published by Nyyssölä et al. (2012). The gene encoding the *G. graminis* LOX AAK81882.1 was codon-optimized for expression in *A. niger* and synthesized by DNA 2.0 (Menlo Park, USA) [GI: 676899629]. The *G. graminis* LOX was expressed under control of the promoter of the *A. niger xlnD* gene and using the secretion signal encoded by this gene [GI:74626559] that replaced the native secretion-signal of *G. graminis* (Van Peij et al. 1997, Van der Straat et al. 2014). With help of the *Xba*I and *Bam*HI restriction sites, the synthesized gene was incorporated into a pUC19 vector and was used to transform *A. nidulans* WG505, a *pyrA* derivative of *A. nidulans* WG096 (ATTC 48756) (Nyyssölä et al. 2012). The resulting *A. nidulans* GG-LOX transformants were plated on MMS plates and incubated for four days at 37°C (Kusters-van Someren et al. 1991).

81

We selected 10 positive *A. nidulans* GG-LOX transformants using the KI-assay according to the procedure published in Nyyssölä et al. (2012) and we used PCR to verify the integration of the *lox* gene into the genome. *A. nidulans* GG-LOX transformants were grown in 500 ml Erlenmeyer flasks for 48 hours at 37°C at 250 rpm in 100 ml MM + 50 mM D-xylose. The culture broth was separated from the mycelium using funnel filtration and both culture broth and mycelium were submerged into liquid nitrogen to freeze and preserve the materials. The culture broth and the mycelium were stored at -80°C for use in future research.

Growth and induction of *A. nidulans* wild type and *A. nidulans* GG-LOX

A. nidulans WG505 and an isogenic transformant expressing the *G. graminis* LOX (*A. nidulans* GG-LOX) were cultivated on agar plates containing complete medium (6.0 g/l NaNO$_3$, 1.5 g/l KH$_2$PO$_4$, 0.5 g/l KCl, 0.5 MgSO$_4$ · 7 H$_2$O, 2 g/l peptone, 1 g/l yeast extract, 1 g/l casamino acids, 0.3 g/l yeast ribonucleic acids, 2 ml/l vitamin solution, 1 ml/l Vishniac solution, 50 mM D-(+)xylose, 12% agar) (Pontecorvo et al. 1953). Liquid cultures of *A. nidulans* were grown at 37°C in 500 ml Erlenmeyers containing 100 ml complete medium without agar and inoculated with 10^6 spores/ml. Both media contained 50 mM D-xylose to induce the *xlnD* promotor and stimulate production of *G. graminis* LOX.

Mutagenesis of the *G. graminis* LOX

To test whether *G. graminis* LOX interferes with *A. nidulans* as an expression host, a double site-directed H306Q-H310E mutagenesis was performed resulting in inactive *A. nidulans* GG-LOX mutants. These mutations result in a catalytically inactive enzyme but do not affect protein expression levels (Cristea et al. 2005). For mutagenesis the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Amstelveen, The Netherlands) was used. The forward 5'-GTTCTACTCCCAAATGTACCAGGTGCTGTTCGAGACCATCCCGGAG-3' primer and the reverse 5'-CTCCGGGATGGTCTCGAACAGCACCTGGTACATTTGGGAGTAGAAC-3' primer were designed according to the protocol set out in the mutagenesis kit. Sequencing was used to identify positive transformants of this mutant protein (Baseclear, Leiden, The Netherlands).

Western blot analysis

Western blot analysis was performed on *A. nidulans* GG-LOX transformants 5, 7, and 9 to identify the presence of *G. graminis* LOX. Disrupted mycelium and 20 μl of culture broth was loaded and run on a 10% SDS-PAGE using a voltage of 100 V for 1 h and Tris-HEPES-SDS running buffer (Thermo Scientific PI28398). The gel was then rinsed with dH$_2$O and pre-soaked with CAPS-blot buffer. A nitrocellulose membrane was used to absorb the proteins from the SDS-PAGE and was blotted overnight at 70 mA. The membrane was washed for 30 min in TBST and afterwards it was blocked using TBST + 1% BSA for 30 min. Rabbit polyclonal antibody of *G. graminis* LOX (Eurogentec, Seraing, Belgium) was used in a 1/1000 dilution to detect LOX on the membrane. After 3x washes with TBST for 10 min, the membrane was incubated for 30 min using a 1/1000 dilution of secondary anti-rabbit peroxidase antibody

(Sigma-Aldrich Lot. A0545-1ML). After the final 3x 10 min wash step with TBST, the membrane was submitted to AP-detection. This was done by mixing two solvents: 60 mg of 4-chloro-1-naphtol to 20 ml methanol (A) and by adding 60 µl of 30% ice-cold H_2O_2 to 100 ml TBS (B). Prior to use solvents A and B were mixed and the membrane was added to this mixture. The reaction was stopped with dH_2O after 30 minutes of incubation.

mRNA isolation and identification of the *G. graminis* LOX

Mycelium from *A. nidulans* WG505 and *A. nidulans* GG-LOX transformant 5 was submerged in peqGOLD TriFast (peqLAB, De Meern, The Netherlands) and disrupted using glass beads and a MP FastPrep-24 beadbeater (MP Biomedicals, Eindhoven, The Netherlands). The RNA isolated was treated with DNase I and transcribed to cDNA using Omniscript RT enzyme (Qiagen, Venlo, The Netherlands). The resulting cDNA was submitted to PCR using the forward primer 5'-TGAGTTGCAGAACTGGATCG-3' and reverse primer 5'-GCAGAACGCCAGAAAACTTC-3' for detection of the *G. graminis lox* mRNA. cDNA from positive reactions were sequenced (Baseclear, Leiden, The Netherlands).

As a positive control the pyruvate kinase (*pkiA)* gene was amplified using the forward 5'-GCCAGTCTTGAACTGAACGC-3' primer and the reverse 5'-GCCAGATCTTGACGTTGAAGTC-3' primer (de Graaff et al. 1992). Amplified gDNA results in a 304 bp fragment whereas amplified cDNA results in a 204 bp fragment because of an intron in the fragment.

Immunoprecipitation using *G. graminis* LOX antibody and proteomics analysis

A. nidulans wild type and *A. nidulans* GG-LOX were grown with 50 mM D-xylose for 48 h and the culture broth and mycelium were separated by funnel filtration. Mycelium was disrupted using French press with a pressure of 1,000 psi (three times) and a cell-free extract was obtained using centrifugation. Samples of 5 ml from both the culture broth and cell-free extract were taken and incubated with 5 ml of polyclonal *G. graminis* LOX antibody (Eurogentec, Seraing, Belgium). The samples were incubated overnight at 4°C while being continuously stired (200 rpm).

Immunoprecipitation was performed using 100 µl Dynabeads Protein G Magnetic Beads (Life Technology, Bleiswijk, The Netherlands) and proteins were separated for 1 cm on a 10% SDS-PAGE at 100 V. Proteins underwent in-gel digestion using 100 ng trypsin/sample in 50 mM ammonium bicarbonate solution before being diluted (1:1 using 2% trifluoroacetic acid to acidify the solution). To purify the samples, the

peptides were bound to a reversed-phase C18 column followed by washing with 0.1% formic acid. Then proteins were eluted using 80% acetonitrile + 0.1% formic acid. Finally, samples were analyzed by LC-MS/MS (Radboud Proteomics Centre, Radboud University, Nijmegen, The Netherlands). MaxQuant software was used for data analysis (Cox & Mann 2008).

Cultivation of *A. nidulans*

A. nidulans wild type and *A. nidulans* GG-LOX transformant 5 were cultivated in a New Brunswick BioFlo® 310 (Eppendorf, Nijmegen, The Netherlands). The dissolved oxygen level was maintained at 20% *via* an agitation cascade from 300-1200 rpm using two Rushton impellers at an airflow of 1 vvm. The batch-phase was performed using 3 l of medium for 24 h (5 g/kg glucose, 0.5 g/kg KH_2PO_4, 0.5 g/kg $MgSO_4 \cdot 7 H_2O$, 4.0 g/kg $(NH_4)_2SO_4$, 1 g/kg yeast extract, 0.1 g/kg Struktol J673). When glucose was completely consumed, fermentation was fed 2 l of D-xylose medium at a speed of 0.35 g/min (75 g/kg D-xylose, 3.1 g/kg KH_2PO_4, 14.85 g/kg $(NH_4)_2SO_4$, 24.4 g/kg yeast extract). Samples of 5 ml were taken every 2 h to test for the presence of the *G. graminis* LOX in the culture broth *via* the ferrous oxidation-xylenol assay (FOX-assay) (Waslidge & Hayes 1995; DeLong et al. 2002).

Results

A. nidulans GG-LOX transformant selection

As shown in Fig. 2, 10 *A. nidulans* GG-LOX transformants were identified using the KI-assay procedure as positive for the production of *G. graminis* LOX. The culture broth of these positive transformants was submitted to a FOX-assay. The cofactor of active LOX binds to xylenol orange and results in a purple. This binding induces an orange to blue colour-shift. We measured light absorbance using spectrophotometry with a wavelength of 560 nm (see Fig. 3 for an overiew of the results).

A. nidulans GG-LOX transformant 5 was selected for further research because it had the highest absorbance (0.164). After 48 hours of growth in a liquid culture, *A. nidulans* GG-LOX showed a different phenotype than both the wild type and the *A. nidulans* GG-LOX inactive mutant. As shown in Fig. 4, *A. nidulans* GG-LOX had a darker colour compared to *A. nidulans* wild type and the mutant. A problem, however, was that SDS-PAGE analysis was unable to detect the *G. graminis* LOX.
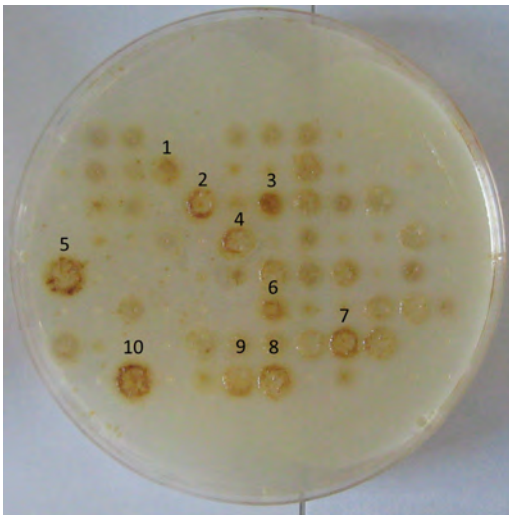
Figure 2: KI-assay performed on *Aspergillus nidulans* transformed with the *Gaeumannomyces graminis lox* gene. The ten transformants selected for further research are marked 1 to 10.
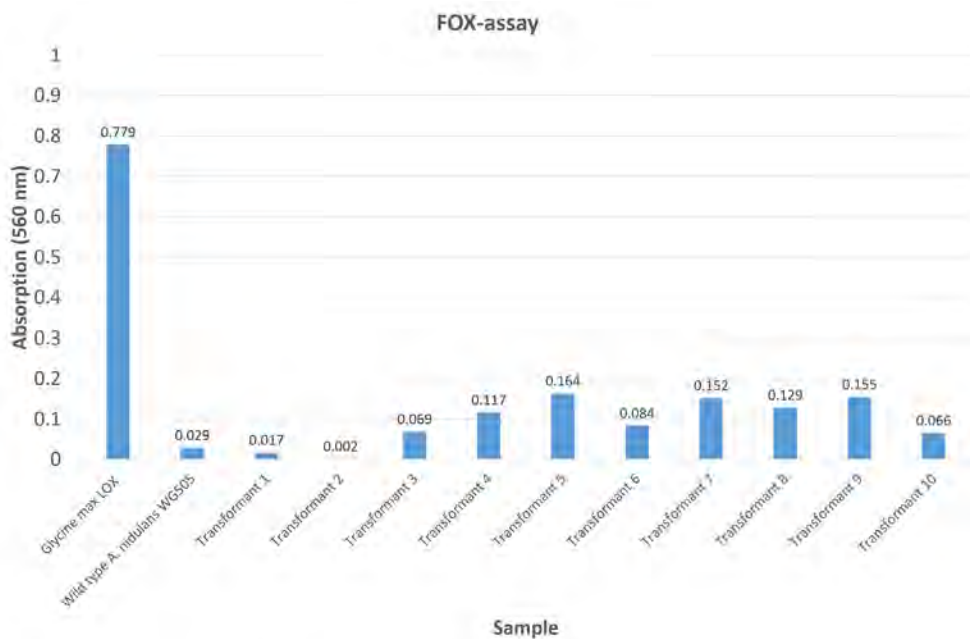
Figure 3: Analysis of culture broth from *Aspergillus nidulans* transformants carrying the *Gaeumannomyces graminis lox* gene using a FOX-assay (DeLong et al. 2002). *Glycine max* LOX was used as a positive control and the wild type *A. nidulans* WG505 was used as a negative control.

Figure 4: a) *Aspergillus nidulans* wild type WG505, b) GG-LOX transformant, and c) GG-LOX mutant grown in liquid media for 48 hours. All cultures were induced with 50 mM D-xylose. A clear phenotype is found, the transformant expressing *Gaeumannomyces graminis* LOX is darker than the wild type and the GG-LOX mutant. A dark colour indicates a clear phenotype.

SDS-PAGE and Western blot analysis

Based on the FOX-assay, three *A. nidulans* GG-LOX transformants giving the highest activities, 5, 7, and 9 (T_1-T_3), and three *A. nidulans* GG-LOX mutants transformants (M_1-M_3) were selected for an SDS-PAGE and Western blot analysis as shown in Fig. 5. The SDS-PAGE analysis revealed *A. nidulans* has two different protein band fingerprints in samples WT, T_2, T_3, M_2 and T_1, M_1, M_3 (Fig. 5a). This coincides with the single-band and double-band found in the Western blot analysis of the intracellular proteins of *A. nidulans* (Fig. 5d). The presence of the bands found in the wild type samples showed non-selective binding of polyclonal antibodies. Production of the *G. graminis* LOX was not detected in the culture broth or in the cell-free extract of the *A. nidulans* transformants (Fig. 5b and 5c). The mutant version of the *G. graminis* LOX was also not detected. Therefore a proteomic analysis was performed of the proteins isolated by immunoprecipitation using the polyclonal antibodies against *G. graminis* LOX.
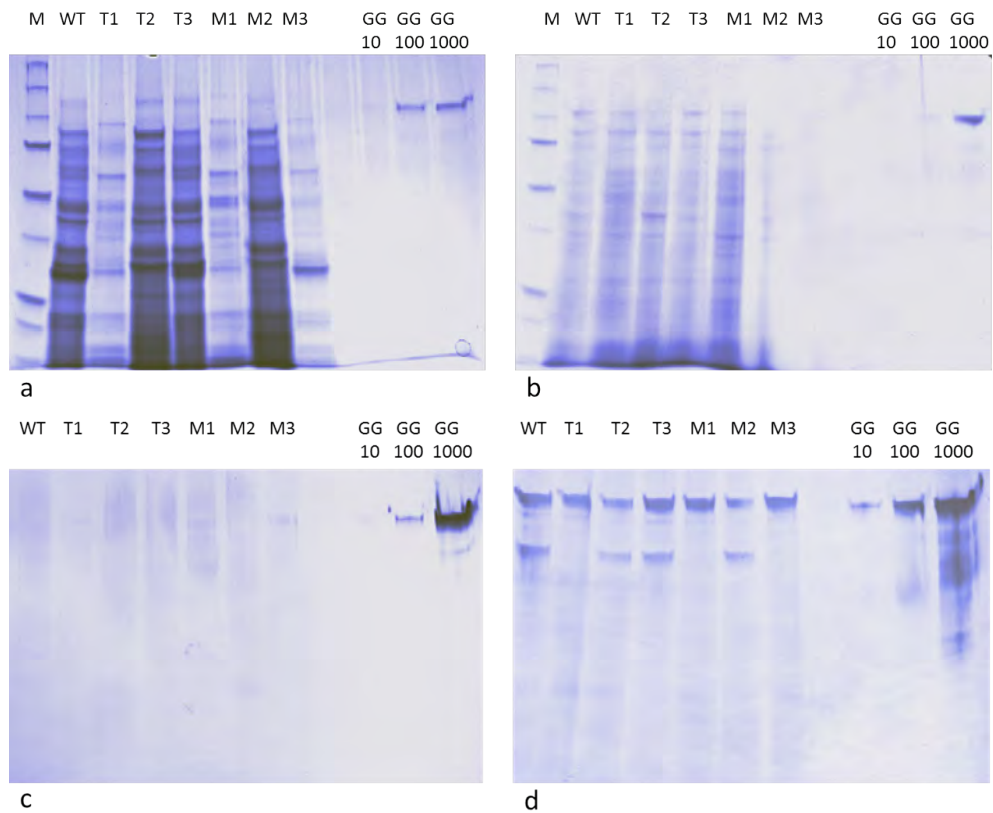
Figure 5: SDS-PAGE and Western blot analysis of *Gaeumannomyces graminis* LOX expression *Aspergillus nidulans*. Samples T1-T3 are transformants of *A. nidulans* carrying the *G. graminis lox* gene and M1-M3 are transformants of *A. nidulans* carrying the mutated *G. graminis lox* gene. As positive controls, 10 ng, 100 ng, and 1000 ng of the *G. graminis* LOX were applied. The wild type (WT) sample was used as a negative control. a) SDS-PAGE of the proteins in the culture broth of *A. nidulans*; b) SDS-PAGE of intracellular proteins of *A. nidulans*; c) Western blot of the proteins in the culture broth reacting to the antibodies of the *G. graminis* LOX; d) Western blot of intracellular proteins reacting with *G. graminis* LOX antibodies.

mRNA identification of the *G. graminis lox* gene

We isolated mRNA from *A. nidulans* GG-LOX and the wild type strain WG505 to confirm that the *G. graminis lox* gene was transcribed. This mRNA was then converted to cDNA and amplified using PCR with the aid of specific primers for the *G. graminis lox* gene. As expected, no product was obtained from the wild type strain WG505 but a PCR amplicon of the expected size was readily obtained from *A. nidulans* GG-LOX. Moreover, DNA sequence analysis of this 204 bp amplicon showed it corresponded with the *G. graminis lox* gene [GI: 676899629]. This indicates that the *G. graminis lox* gene was correctly transcribed in *A. nidulans* GG-LOX.

Comparative proteomics analysis of LOX immunoprecipitated fractions from *A. nidulans* WG505 and *A. nidulans* GG-LOX strains

Since phenotypical differences were found between the *A. nidulans* wild type and the *A. nidulans* GG-LOX, we confirmed mRNA was transcribed, but *G. graminis* LOX was not detected by SDS-PAGE and Western Blot analysis. Therefore, we performed a comparative proteomics analysis on *A. nidulans* wild type and *A. nidulans* GG-LOX transformant 5.

Immunoprecipitation was performed on both cell-free extract and culture broth. As displayed in Fig. 6, three different protein bands of ~67 kDa (1), ~54 kDa (2), and ~41 kDa (3) were found after immunoprecipitation in *A. nidulans* GG-LOX transformant 5. Cell-free extract and culture broth protein samples from *A. nidulans* wild type and *A. nidulans* GG-LOX (WT_{cfe}, WT_{cb}, T_{cfe}, and T_{cb}) were subjected to a proteomics analysis. Analysis of the T_{cfe} sample revealed three interesting findings. We identified a peptide with the TNVGVDLTYTPLDDK sequence, which corresponds to a part near the WLLAK-sequence of the *G. graminis* LOX. We identified aminopeptidase Y in the *A. nidulans* GG-LOX transformant by one peptide hit and identified the presence of thioredoxin reductase by four peptide hits in the GG-LOX transformant.

On the basis of the molecular weight, we suspect protein (1) is *G. graminis* LOX, (2) is aminopeptidase Y, and (3) corresponds to thioredoxin reductase (see Fig. 6). A final difference is found in the detection of PpoC with 24 different peptide hits. In the *A. nidulans* wild type this protein is present in the cell-free extract and the culture broth. The cell-free extract of the *A. nidulans* GG-LOX contained an amount of ~30% PpoC compared to the *A. nidulans* wild type and was not detected in the culture broth.
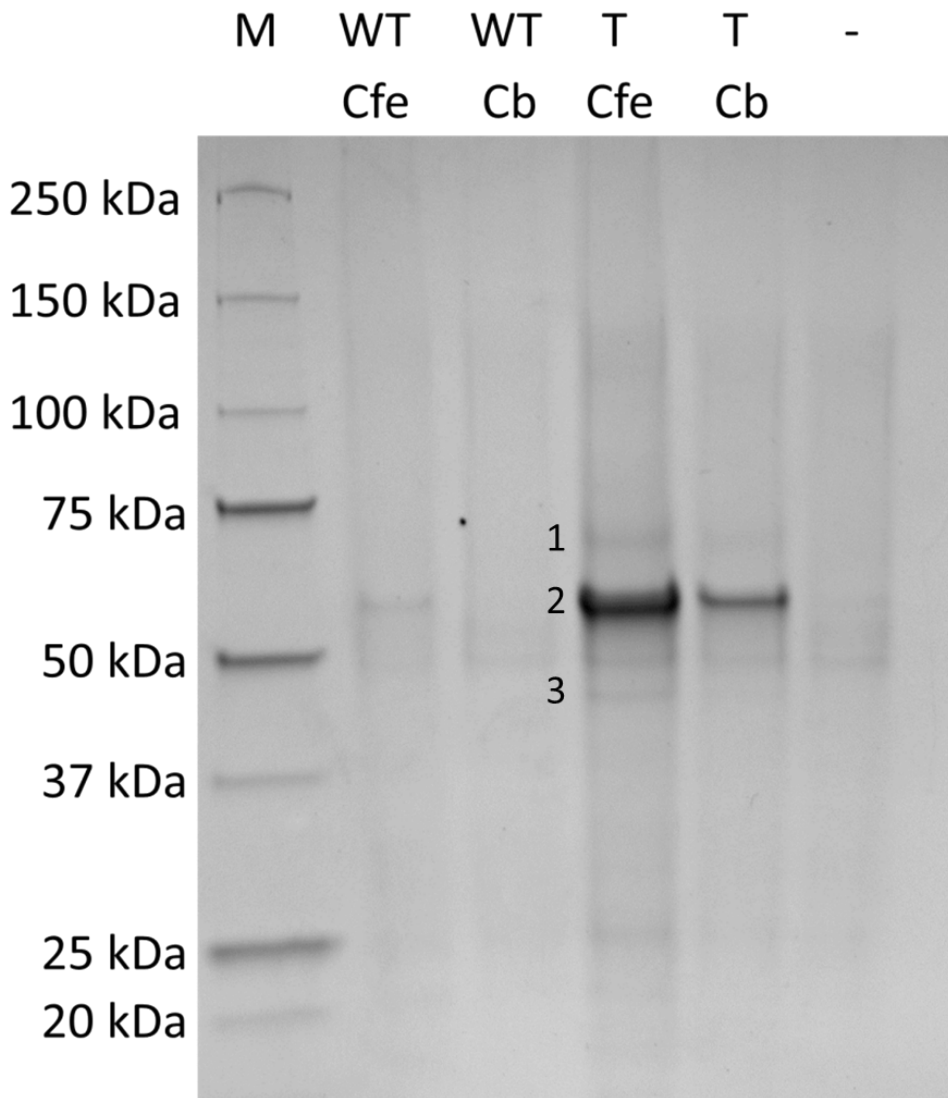
Figure 6: SDS-PAGE analysis of immunoprecipitated proteins in cell free extract (CFE) and culture broth (CB) of *Aspergillus nidulans* wild type (WT) and the transformant (T) using polyclonal antibodies against *Gaeumannomyces graminis* LOX. In sample T$_{cfe}$ protein bands are found at (1) ~67 kDa, (2) ~54 kDa, and (3) ~41 kDa. Proteomics analysis and the molecular weight of the proteins suggests these proteins corresponds to *G. graminis* LOX, aminopeptidase Y, and thioredoxin reductase respectively.

Cultivation of *A. nidulans*

To verify whether the uncontrolled pH, substrate consumption, and oxygen consumption in shake flasks were promoting the degradation of the *G. graminis* LOX, a three-to-five liter fed-batch fermentation was run. Culture broth samples were taken every two hours both during the batch phase and the fed-batch phase. Using the FOX assay, these samples were screened for the presence of *G. graminis* LOX. No *G. graminis* LOX, however, was detected. This implies stable culture conditions do not increase heterologous LOX production in *A. nidulans*.

Discussion

Phenotype differences in *A. nidulans* wild type and *A. nidulans* GG-LOX

A darker phenotype after 48 hours of growth was found in the *A. nidulans* GG-LOX compared to the *A. nidulans* wild type and the *A. nidulans* GG-LOX mutant. *A. nidulans* is capable of producing yellow, black, red, and green conidia and ascospores (Brown & Salvo 1994). Conidia formation can be induced by intrinsic signals or environmental stress (Adams et al. 1998). The dark pigmentation found in the *A. nidulans* GG-LOX could be a result of its coloured metabolites, however, the pigmentation also suggests the presence of conidia and, therefore, an asexual state of *A. nidulans*. The oxylipins produced by the *G. graminis* LOX might induce this phenotype. This phenotype and the presence of mRNA suggests an active *G. graminis* LOX in the *A. nidulans* GG-LOX transformant.

The *G. graminis* LOX itself, however, could not be detected using SDS-PAGE and Western Blot analysis. Western Blot analysis revealed non-specific binding of *G. graminis* LOX polyclonal antibodies as found in the *A. nidulans* wild type samples shown in Fig. 6d. The non-specific binding of the protein of ~125 kDa is proposed to be PpoC. PpoC was identified using proteomics analysis with 24 peptide hits. This suggests the polyclonal *G. graminis* LOX antibodies have an affinity for PpoC, which might be a reflection of a conserved epitope. One could speculate that *G. graminis* LOX has a repressing effect on the activity of *ppoC* as lower amounts of PpoC were detected in *A. nidulans* GG-LOX. The repressing and up-regulating functions of PpoB on *ppoA* and *ppoC* are in line with this hypothesis (Tsitsigiannis & Keller 2005). The Western Blot analysis, however, did not indicate less activity of the 125 kDa protein thought to be PpoC.

The presence of thioredoxin reductase, a defense-agent against oxidative damage, in *A. nidulans* GG-LOX suggests the *G. graminis* LOX oxylipins induce oxidative stress

to the host (Missall & Lodge 2004). In addition, the presence of aminopeptidase Y suggests the *G. graminis* LOX is degraded in the vacuole and would explain why the protein was not detected using SDS-PAGE analysis. Thioredoxin reductase and aminopeptidase Y, however, were found using *G. graminis* LOX polyclonal antibodies. It remains unclear how these proteins could be isolated using immunoprecipation. The non-specific binding of the antibodies, also found with PpoC, offers an explanation. Thioredoxin reductase was positive to four peptide hits and aminopeptidase to one peptide hit. Both proteins, however, were only found in the cell-free extract of the *A. nidulans* GG-LOX transformant.

Proposed effect of the *G. graminis* LOX in *A. nidulans*

It is unclear why phenotypic changes were observed. Our results suggest active *G. graminis* LOX is present and that the *G. graminis* LOX is degraded in the vacuoles. A previous study showed a concentration of 10-100 μM 9*S*-HPODE and 13*S*-HPODE had effect on mycelial growth (Burow et al. 1999). In another study, the effect of *A. flavus* LOX was tested on growth of the mycelium (Horowitz Brown et al. 2008). *A. flavus* LOX could not be detected but deletion of the *A. flavus lox* gene resulted in less conidia being produced. This suggests a small quantity of LOX can have an effect on *Aspergillus* development. This idea is in line with the phenotype found in the *A. nidulans* GG-LOX.

There are two explanations for the results of the *G. graminis* LOX in *A. nidulans*. The first explaination is that *G. graminis* LOX is active at the endoplasmatic reticulum and is degraded in the vacuole. If this is the case, it suggests the *G. graminis* LOX is active during transportation from the endoplasmic reticulum towards the Golgi apparatus or from the Golgi apparatus towards the vacuole. Since these transportation vesicles consist of fatty acids, it is possible these fatty acids are used by *G. graminis* LOX as a substrate to produce oxylipins. The second explanation is that *G. graminis* LOX is active in an early stage of growth before a defensive system is activated to inactivate *G. graminis* LOX. This hypothesis could be investigated in a future study by performing a time-lapsed comparative proteomics experiment.

In conclusion

Heterologous production of *G. graminis* LOX was successfully achieved in *P. pastoris* and *Trichoderma reesei* (Cristea et al. 2005; Nyysölä et al. 2012). A difference between *P. pastoris* and *T. reesei* compared to *Aspergillus* sp. is the presence of *ppo* genes. We hypothesize that the introduction of the *G. graminis* LOX disturbs the oxylipin

balance in *A. nidulans* resulting in a different phenotype. Thioredoxin reductase is then induced to neutralize the oxidative stress that is caused by the *G. graminis* LOX oxylipins. Finally, the *G. graminis* LOX is degraded in the vacuole by protease aminopeptidase Y. A fed-batch fermentation approach did also not induce protein production using the *A. nidulans* GG-LOX transformant. On the basis of these results we conclude that heterologous production of *G. graminis* LOX using *A. nidulans* as an expression system is not effective or efficient for industrial purposes.

Author's contribution

RH carried out the molecular cloning of *A. nidulans* GG-LOX, mRNA analysis, the Western blot experiments, the immunoprecipitation, the proteomics analysis, fermentation experiments, and drafted the manuscript. JPvS was responsible for the mutagenesis work and analysis presented in this paper. JATR conceived and performed the proteomics sample preparation and contributed to the manuscript. RH and LHdG designed the experiments participated to draft the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests**.**

Acknowledgements

93

Chapter 6

General discussion: what was and was not achieved and what are the implications for the use of lipoxygenase?
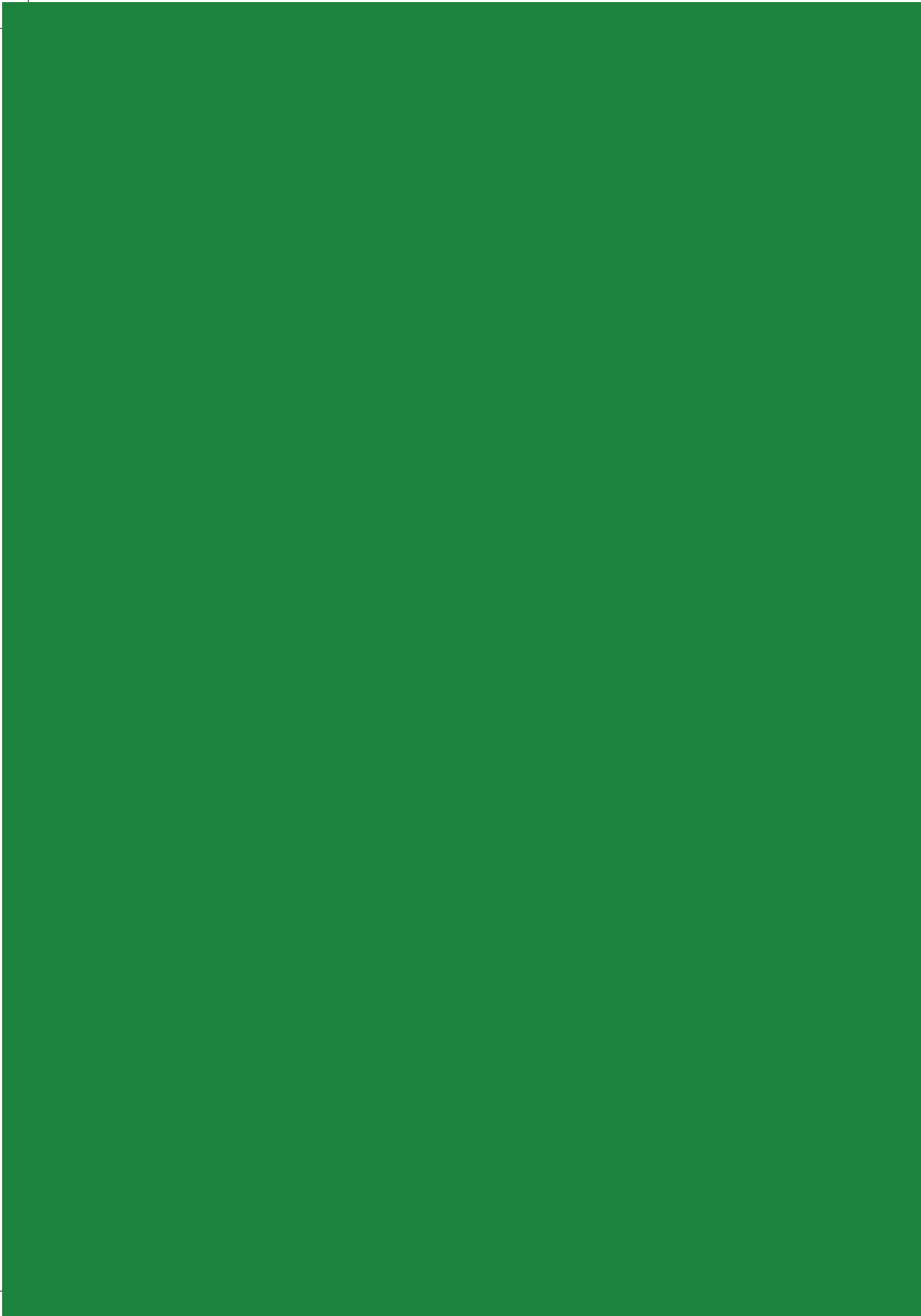
This thesis describes results obtained as part of a study on the biobased production of plastics using polyunsaturated fatty acids (PUFAs) and the enzyme lipoxygenase (LOX). By attaching a peroxide group to the PUFA with LOX, smaller building blocks can be produced. These building blocks can function as oleochemicals, which can then be used to produce polymers. In this discussion we summarize the main project results and discuss the industrial use of LOX-based products. For these processes, as written in **Chapter 2**, sufficient quantities of LOX as well as its substrates are necessary.

In this chapter we also discuss the choice of LOX and possible amino acid adaptations to optimize its biocatalytic properties. By using recently developed methods in bioinformatics, it is possible to define specific targets in the protein to synthetically improve its biocatalytic properties and make the enzyme more suitable for specific applications.

Evaluation of the approach

1. Choice of LOXs used in this thesis

This thesis was initiated by applying a bioinformatics approach to select LOXs from the kingdom of life as described in **Chapter 3**. Our main interest was in LOXs from fungal origin because we wanted to exploit *Aspergillus niger* as an expression host. From the dataset obtained, four different fungal LOXs were selected for further investigation: 1) extracellular *Gaeumannomyces graminis* LOX [GI:17861374], 2) extracellular *Aspergillus fumigatus* LOX [GI:70981869], 3) intracellular *A. fumigatus* LOX [GI: 70982632], and 4) intracellular *Pleurotus sapidus* LOX [GI:293330614]. The specific reasons to focus on these four different LOX enzymes are described below.

G. graminis LOX

G. graminis LOX was chosen to serve as a positive control and as a model enzyme in the production of oleochemicals. The production of this LOX had been achieved by using *Pichia pastoris* as an expression host (Cristea et al. 2005). As *P. pastoris* was used as an expression host by our partner at VTT, Finland, other partners explored the potential of other LOXs using *Trichoderma reesei* and *A. niger* as production hosts.

A. fumigatus LOX

Both *A. fumigatus* LOXs were chosen, since their expression in *A. niger* was expected to be relatively easy due to the close relationship between these organisms. Also, we intended to study the differences between an intracellular and extracellular LOX

97

from the same organism. Heterologous production and purification of these LOXs could be applied to research on its hypothesized pathogenic role in invading lung tissue (Latgé 1999). Due to the close relationship between extracellular *A. fumigatus* LOX and *G. graminis* LOX, we expected that expression levels and the function of *A. fumigatus* LOX would be comparable to *G. graminis* LOX. The effect of LOX in *Aspergillus* had to date not been investigated.

P. sapidus LOX

P. sapidus LOX is known for the conversion of (+)-valencene to (+)-nootkatone and is, so far, the only known LOX to convert a sesquiterpene. The substrate (+)-valencene is a flavour compound found in the peel of valencene oranges. This substrate can be converted using LOX to (+)-nootkatone, a grapefruit flavour compound. Grapefruit flavour can potentially be used in the food and cosmetics industry as a flavour compound. The (+)-nootkatone production process has been described in a patent application using LOX from soy flour (de Roos et al. 2006). Due to the unique use of the substrate, this LOX could be explored for conversion of other sesquiterpenes. Sesquiterpenes are a class of semiochemicals that function as signal molecules and pheromones. This type of compound can find its use in pest control (Norin 2007). The *P. sapidus* LOX was also chosen because it is a LOX of basidiomycete origin, which could reveal its function in this type of fungus.

2. Expression of LOXs

Expression of LOX in A. niger

All genes as described in the previous section were incorporated into a pUC19-based vector carrying the *xlnD* promoter and the *xlnD* terminator from *A. niger*. Variants of the genes carrying an N-terminal His-tag were also incorporated in this pUC19-based vector. The usefulness of this construct was shown by two colleagues in the expression of an *Aspergillus terreus cis*-aconitate decarboxylase and an *A. niger* galacturonic acid transporter (Van der Straat et al. 2014, Sloothaak et al. 2014). The pUC19-based vector carrying different *lox* genes were used to transform *A. niger* 872.11, a *pyrA* and *argB* deficient strain. Identifing transformant strains that expressed active LOX enzyme was completed by identifying the production of fatty acid hydroperoxides *via* the KI-plate assay as described in **Chapter 4**. None of the transformants, however, gave a positive reaction. The usefulness of the KI-assay was shown using *T. reesei* as an expression host. In this assay *T. reesei* transformants are grown on plates and transformants expressing active enzyme are identified by a brown halo around their

colony. The intensity of the halo also gives a first indication of expression levels of the enzyme by the host. The KI-assay can also be used for identification of LOX enzyme in culture broth as shown for *P. pastoris* transformants.

Another assay performed in this thesis is the ferric oxidation-xylenol orange (FOX) assay. This assay is based on the Fe^{2+} to Fe^{3+} conversion that occurs in presence of active LOX enzyme. The Fe^{3+} ion reacts with xylenol orange to form a blue-coloured complex that is measured at a wavelength of 560 nm (Waslidge & Hayes 1995; DeLong et al. 2002). The combination of the KI-assay and the FOX-assay gives a solid confirmation of LOX activity.

By using PCR, 15 out of 28 *P. sapidus lox* transformants were identified as positive for integration of the LOX expression construct into the genome of *A. niger* 872.11. On the basis of phenotypical differences between *A. niger* wild type and *A. niger* strains carrying the *P. sapidus lox* gene, transformant 28 was chosen for further investigation in LOX expression. The transformant was grown using D-xylose to induce LOX expression according to the methods described in **Chapter 4** and **Chapter 5**. Phenotypical differences were found and compared to wild type *A. niger* 872.11. The main difference was in regard to sporulation: the wild type strain had big black spores whereas *A. niger* transformants expressing *P. sapidus* LOX showed spores smaller in size but higher in number.

This phenotype can be explained by an increase of formed conidia, which form the basis of asexual sporulation. The role of the *P. sapidus* LOX on this phenotype was not further investigated. Culture broth was analyzed by SDS-PAGE analysis for the presence of LOX. Proteomics analysis was performed on the protein indicated by a red arrow in Fig. 1. This resulted in identification of aspergilliopepsin A-like aspartic endopeptidase [GI:145249508]. We could not validate, however, the presence of active LOX expression under study. In addition, both *A. fumigatus* LOXs were also not detected using the KI-assay and the FOX-assay. Due to negative results of the KI-assay and FOX-assay, the lack of presence of LOX, and the detection of aspergilliopepsin A-like aspartic endopeptidase in the *P. sapidus* LOX transformant, we concluded *A. niger* is not a suitable host for heterologous expression of LOXs. Therefore we abandoned *A. niger* as an expression host for the production of heterologous LOX.
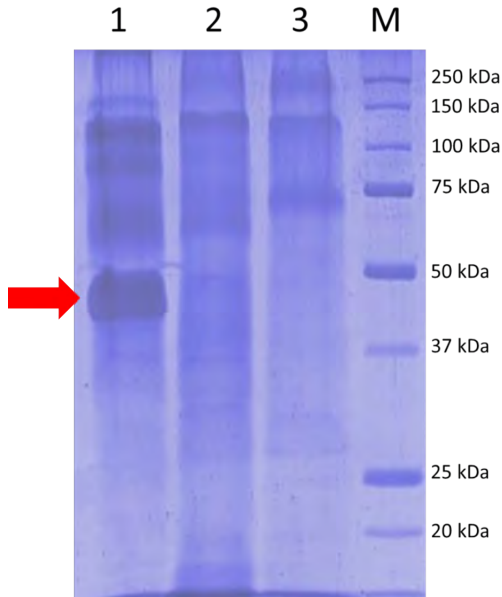
Figure 1: SDS-PAGE analysis of culture broth of *Aspergillus niger* transformant 28 carrying the *Pleurotus sapidus lox* gene (lane 1), *A. niger* 872.11 wild type (lane 2), *A. niger* carrying the empty vector (lane 3), and the marker (lane 4). The abundant protein with an approximate molecular weight of 40 kDa (red arrow) was recovered and submitted to mass spectrometry after trypsin digestion. This revealed the presence of aspergilliopepsin A-like aspartic endopeptidase.

Expression of LOX in Aspergillus nidulans

Due to the failure to express the selected LOXs in *A. niger*, we changed the expression host to *A. nidulans* WG312. *P. sapidus lox* was integrated into the genome of *A. nidulans* WG312. Its expression resulted in a phenotype. A white fluffy layer, found in 34 out of 88 transformants, grew over the transformants where spores were expected. Also, the mycelium colour turned pinkish. The KI-assay, however, showed negative results for all transformants. On the basis of these results, we discontinued using *A. nidulans* WG312 as an expression host.

We were able to use *A. nidulans* WG505, kindly provided by Dr. A. Debets, as an expression host in a follow-up study. The same *lox* genes as described in the previous section were used to transform this *A. nidulans* strain. The *G. graminis lox* was also used as our partners at VTT and AB Enzymes had successfully produced *G. graminis* LOX in *P. pastoris* and *T. reesei* as shown in **Chapter 4**. The strains transformed with the *G. graminis lox* gene gave the best initial results. KI-plate assays showed positive results and ten strains were selected as described in **Chapter 5**. From these ten transformants *A. nidulans* GG-LOX transformant 5 was chosen to investigate the outcome of the *G. graminis* LOX in *A. nidulans*. The LOX activity of the *A. nidulans* transformants, however, disappeared over time. This result raised the question about what happened to LOX in the expression host and whether the strain was genetically stable.

The steps undertaken in the analysis to identify the presence of active LOX are described in **Chapter 5**. mRNA of the LOX transformant was isolated to verify whether the gene was transcribed correctly, which was confirmed by sequencing of the resulting cDNA. We hypothesize that the production level of LOX is too low to be detected in SDS-PAGE analysis. Therefore *G. graminis* LOX produced in *T. reesei* was used to raise polyclonal antibodies for detection of LOX in Western blot analysis. The results are presented in **Chapter 5** from which it became clear that certain wild type proteins interfered with the *G. graminis* LOX antibodies. Two distinctive protein fingerprints in *A. nidulans* were found based on the performed SDS-PAGE analysis. These fingerprints correlated with the presence of one or two interfering protein bands found in Western Blot analysis. By using a comperative proteomics analysis we investigated intracellular proteins of an *A. nidulans* transformant carrying the *G. graminis lox* gene. Immunoprecipitation and proteomics analysis enabled the detection of aminopeptidase Y. The detection of proteases is in accordance with the results found using *A. niger* as an expression host. In addition, identification of thioredoxin reductase suggests *G. graminis* LOX is actively supressed. The hydroperoxides formed by *G. graminis* LOX may induce oxidative stress and in response thioredoxin reductase is expressed to minimize these effects.

Finally, the lesser detection of PpoC in the *A. nidulans* transformant compared to the wild type might be a result of the activity of the *G. graminis* LOX. The results led to the hypothesis that *G. graminis* LOX activity interfers with these Ppo enzymes and results in phenotypical differences. This interference then would result in genetical instability and selection of strains with deceased LOX activity. This would provide an explanation for the decrease in activity of *G. graminis* LOX in time in *A. nidulans* GG-LOX transformant strains.

To test whether the presence of active *G. graminis* LOX or the oxylipins generated by this LOX are lethal or induce phenotypical changes, *A. nidulans* and *A. niger* were grown on plates as shown in Fig. 2. *G. graminis* LOX was applied in a concentration of 1 mg/ml extracellularly to the medium with and without linoleic acid (LA). We found that LA has an effect on sporulation of both the *A. nidulans* transformant and *A. niger* transformant: the *A. nidulans* transformant has a darker mycelium colour compared to the wild type and the *A. niger* transformant shows a yellow halo when LA is added. Both these phenotypical changes are thought to be a result of active LOX produced by the transformants. Extracellular *G. graminis* LOX has no effect on the growth of *A. nidulans* and *A. niger* compared to their wild type. These results can be explained by a LOX inhibiting compound called nigerloxin, produced by *A. niger* in solid state

101

fermentation (Sekhar Rao et al. 2005). It could well be *A. nidulans* produces such a compound as well, although this has not been reported in literature or investigated in this work.

Finally, to exclude the effect of the activity of the expressed LOX, a fermenter experiment was performed in which the growth phase and biomass formation was separated from the enzyme expression and production phase. Both *A. niger* transformant 28 and *A. nidulans* transformant 5 were tested for production of LOX. As described in **Chapter 5** this separation of biomass formation and production phase had no effect on the heterologous production of LOX.
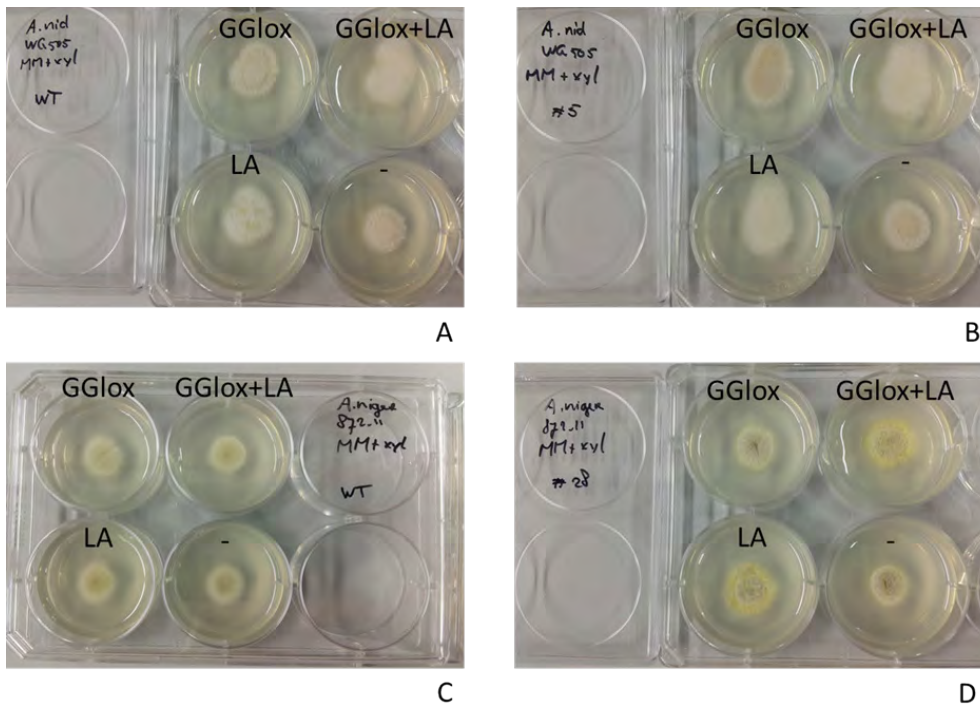
Figure 2: *Aspergillius nidulans* wild type (A) and *A. nidulans* carrying the *Gaeumannomyces graminis lox* gene (B) were grown in presence of 1 mg/ml of *G. graminis* LOX (GGlox) and/or 1 mg/ml linoleic acid (LA). This results shows linoleic acid has effect on growth of *A. nidulans*, whereas *G. graminis* LOX has no effect. The same phenomenon is seen for *Aspergillus niger* (C) and *A. niger* carrying the *P. sapidus lox* gene (D).

Expression of G. graminis and A. fumigatus LOX in P. pastoris

Heterologous expression and production of extracellular *G. graminis* LOX was shown to be successful using *P. pastoris* as an expression host (Cristea et al. 2005). On the basis of expression of this LOX in *P. pastoris*, we investigated the expression of the closely related extracellular *A. fumigatus* LOX in this host. Fermentation experiments of the wild type strain *P. pastoris* X-33, the *P. pastoris* strain carrying the *G. graminis lox* gene, and the *P. pastoris* carrying the *A. fumigatus lox* gene were performed simultaneously using 1.5 L Sartorius fermenters (Stratton et al. 1998). After the batch phase of ~30 h on glycerol an extra 8 h glycerol fed-batch was performed. This was done to generate extra biomass and simultaneously to perform the dissolved oxygen-spiking experiment. DO-spiking was used to determine the consumption rate of glycerol and to make sure the carbon source is depleted. The DO rose within a minute to 100% oxygen saturated in the culture broth when the carbon sourse was depleted because there was no need for oxygen consumption to metabolize the carbon source. We then added methanol to induce the AOX promoters in front of the *G. graminis* and the *A. fumigatus lox* genes. On the basis of the FOX-assay of the *G. graminis* transformant, we identified the production of *G. graminis* LOX after 12 h. Unlike the *G. graminis* LOX transformant, the *A. fumigatus* LOX transformant revealed no significant production levels. After 3 days of induction the fermentations were stopped because the positive control, *P. pastoris* producing *G. graminis* LOX, did not show increased production of *G. graminis* LOX.

103

The culture broth of *P. pastoris* carrying the *A. fumigatus lox* gene was obtained. Since a His-tag was added to the *A. fumigatus* LOX, purification was done *via* nickel affinity chromatography. This revealed a production of about 1 mg/l of *A. fumigatus* LOX. Despite the low production it was sufficient protein to analyze using Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) which metal cofactor is used by this protein. This technique requires pure protein that is burned in a plasma flame, which excites metals to a higher energy state. The metals decay to a lower energy state, which results in emission of light that is specific for the metal ion which is measured. Different metal ions can be identified due to their distinctive emission patterns. This revealed the presence of manganese and no presence of iron. We concluded, therefore, that *A. fumigatus* LOX uses manganese as a cofactor instead of iron. This is in line with the cofactor used of *G. graminis* LOX. Manganese is a more stable compound compared to iron and is therefore hypothesized to be a more suitable cofactor for extracellular LOX (Oliw 2002).

Aarhus University, Denmark, determined the oxylipins produced by *A. fumigatus* LOX as described in **Chapter 3**. This revealed high specificity for production of 13-(Z,E)-HPOD. The differences between the yields of the *G. graminis* LOX and *A. fumigatus* LOX using *P. pastoris* as an expression host suggested the cause is due to a difference in amino acid level. It might be *P. pastoris* is more effective in degrading *A. fumigatus* LOX during the fed-batch phase by proteases. This has, however, not been further investigated. Table 1 lists a summary of heterologously expressed LOXs as described in this discussion.

Table 1: overview of heterologous expression of different LOXs in different expression hosts.

	Escherichia coli	*Pichia pastoris*	*Aspergillus nidulans*	*Aspergillus niger*
Gaeuman-nomyces graminis LOX				
Vector	pCR2.1-TOPO	pPICZαA	pUC19	pUC19
Secretion signal	No	Yes	Yes	Yes
Secretion	No	Yes	No	No
Yield	Inactive protein	30 mg/l	Detected	N/A
Reference	Cristea et al. 2005	Cristea et al. 2005	Heshof et al. 2014b	This thesis
Aspergillus fumigatus LOX				
Vector	N/A	pPICZαA	pUC19	pUC19
Secretion signal	N/A	Yes	Yes	Yes
Secretion	N/A	Yes	No	No
Yield	N/A	1 mg/l	N/A	N/A
Reference	N/A	Heshof et al. 2014a	This thesis	This thesis
Pleurotus sapidus LOX				
Vector	pBAD/gIIIA	pPIC9K	pUC19	pUC19
Secretion signal	No	Yes	Yes	Yes
Secretion	No	No	No	No
Yield	60 mg/l	100-150 mg/l	N/A	N/A
Reference	Zelena et al. 2012	Kelle et al. 2014	This thesis	This thesis

105

Expression of LOX in Aspergillus oryzae and Aspergillus flavus

We recently found that the heterologous expression of LOX could be achieved using *Aspergillus oryzae* as an expression host. The production of *G. graminis* LOX and *M. salvinii* LOX using *A. oryzae* is described in a patent application by Novozymes (Sugio and Takagi 2002; Christensen et al. 2008). *A. oryzae* is considered to be a less sporulating and domesticated variant of *A. flavus* due to its use in oriental fermented foods (Kurtzman et al. 1986). A difference between these organisms is the lack of aflatoxins in *A. oryzae*, making it a non-pathogenic fungus (Chang & Ehrlich 2010). This non-pathogenicity makes the fungus suitable for industry. The function of LOX in sporulation has been shown in earlier research (Brown et al. 2008). Mutation of *lox* in *A. flavus* decreased the amount of conidia, which form the basis of asexual sporulation.

A. oryzae contains a hypothetical LOX [GI: 317147480], which is closely related to the hypothetical *A. flavus* LOX [GI: 220694095]. When we compare the *A. oryzae lox* gene to the *A. flavus lox* gene, two differences are found. The main difference is that unlike the *A. flavus* LOX, *A. oryzae* LOX contains a secretion signal, as predicted by SignalP 4.1. This is due to the different position of the start codon found in both *lox* genes. The start codon of the *A. flavus lox* gene is predicted to be 99 bp earlier in the genome compared to the *A. oryzae lox* gene (see Fig. 3) and means that an N-terminal secretion sequence is found in the *A. flavus lox* gene. Therefore, the *A. flavus lox* sequence is most likely a falsely annotated protein-coding gene. The second difference between the genes is that despite the identical sequence of the two genes, the *A. oryzae lox* sequence contains an intron that is not identified as an intron in *A. flavus lox*. As illustrated in Fig. 3, a premature stop codon emerges because of this intron and as a result, *A. oryzae* LOX lacks a significant part of the C-terminus including the catalytically important C-terminal isoleucine or valine.

The DNA sequences of *A. oryzae lox* gene and the *A. flavus lox* gene are, however, almost identical. The question is whether the intron found in *A. oryzae* is due to the difference in DNA sequence upstream of this intron or is due to the intron being wrongly predicted. According to Novozymes' patents, however, there is no LOX activity present using their untransformed strain. On the basis of the sporulation phenotype and the DNA sequence of the *lox* gene in *A. oryzae*, we hypothesize LOX is catalytically inactive in *A. oryzae*. This hypothesis can be tested by the heterologous expression of *A. flavus* LOX in *A. oryzae* to verify whether conidia formation increases. Such an increase would be in line with the results found in **Chapter 5**, where phenotype differences were found between the wild type *A. nidulans* and the *A. nidulans* carrying the *G. graminis lox* gene.

Aspergillus oryzae

Aspergillus flavus

Figure 3: Comparison of *Aspergillus oryzae lox* and *Aspergillus flavus lox*. In green are predicted introns, in red predicted exons. The high similarity between the LOXs shows the close relationship between these organisms. The additional N-terminal amino acids found in *A. flavus* disrupt the putative secretion signal present in *A. oryzae* as predicted by SignalP 4.1. The corrected start position of *A. flavus lox* is indicated with a red arrow. In addition, in *A. oryzae lox* an intron is detected, which is not detected as an intron in *A. flavus lox* (red boxes). This leads to a misinterpreted translated protein sequence resulting in a premature stop codon.

How to make lipoxygenase-derived products feasible in a biobased economy

1. Biobased economy

Our society is moving towards a biobased economy in which not only first, but also second, third, and fourth generation of biomasses are used as a resource by industry. The first generation consists of food crops, the second generation includes waste products from food and the third and fourth generations include the conversion of CO_2 to sugars by non-food photosynthetic organisms, such as algae and fast-growing plants (i.e poplars). These third and fourth generations are considered CO_2-neutral because the production organisms use CO_2 as a carbon source. The difference between the two generations is that the fourth generation produces biofuels using genetically engineered organisms (Vanholme et al. 2013; Lee & Lavoie 2012; Demirbas 2009).

One of the major drawbacks of first-generation biomass is deforestation in using food for fuels. For instance, the demand in Argan oil boomed in 1999 for use as a food source, cosmetics, and for medical purposes and this demand threatens the endemic forest in Morocco (Lybbert et al. 2011). Another example is the deforestation of the Amazon due to pasture. Afterwards this land is used for soybean production known for its high linoleic acid content (Barona et al. 2010; Chowdhury et al. 2007). The second generation of biomass is better suited for industry, since valuable compounds are available in waste biomass (Schieber et al. 2001). A drawback of using plant-based waste material from first-generation and second-generation biomass is the green degradation using enzymes due to the robustness of the plant cell wall, which consists of hard-to-degrade compounds such as hemicellulose, cellulose, pectin, and lignin (Vanholme et al. 2013). Since fungi are specialists in degrading plant cell wall material, the use of fungi in a biobased economy is essential.

2. Potential role of lipoxygenase

LOX may have a valuable potential in a biobased economy in many different applications (**Chapter 2**). A relatively unexplored use of LOXs is in the production of polymers. These polymers can be used as a building block for paints, plastics, and lubricants. Modern plastics are generally made from petroleum although the amount of bioplastics being produced is rising (Gironi & Piemonte 2011). LOXs have specificity for attaching the peroxide group to its specific substrate: polyunsaturated fatty acids (PUFAs). The PUFAs used as a LOX substrate are mainly linoleic acid (C18:2)

and arachidonic acid (C20:4) (Andreou et al. 2009). In this way LOXs are classified as 5-, 8-, 9-, 10-, 11-, 12-, 13-, and 15-LOXs with the number referring to the PUFA carbon atom the peroxide group is attached to. *Via* the acid or radical degradation route, and depending on the PUFA and LOX used, many different polymers can be formed. Fig. 4 shows the possible polymers that can be formed by the *G. graminis* 13-LOX using the acid or radical degradation pathway (**Chapter 2**).
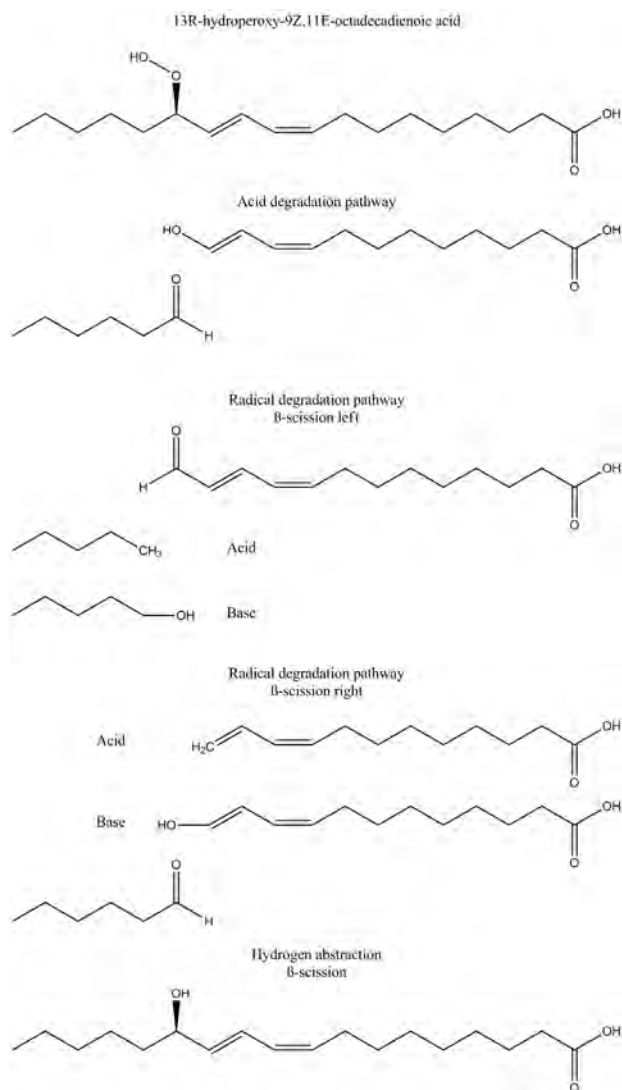
Figure 4: Different polymers that can be formed using the *Gaeumannomyces graminis* 13-LOX. The acid degradation pathway delivers two polymers, whereas in the radical degradation pathway β-scission and the acidic or alkaloid environment determine the oxylipins that are formed.

3. Oil production in microorganisms

One of the hurdles in making LOX attractive for industry is the supply of PUFAs. One approach is to produce PUFAs with the use of microorganisms. The filamentous fungus *Mortierella alpina* is capable of producing higher fatty acids such as arachidonic acid (C20:4). *M. alpina* includes many different strains and *M. alpina* strain 1S-4 is an industrialized producer of arachidonic acid with a yield of 13 g/l when produced in a 10.000 l fermenter (Higashiyama et al. 2002). The research in oil-producing microorganisms, however, is mainly focussed on yeasts. Yeasts usually have a duplication time of less than 1 hour and the production is relatively easy to scale up (Ageitos et al. 2011). Yeast is called an oily yeast when 20% of its dry weight is composed of lipids. To date, only 5% of yeasts have been reported to accumulate more than 25% lipids (Beopoulos et al. 2009) and *Cryptococcus curvatus*, *Lipomyces starkeyi*, *Rhodosporidium turoloides*, *Rhodosporodium glutinis*, and *Yarrowia lipolytica* are all oily yeasts feasible for use in oil production of industrial proportions.

A drawback to using yeasts, however, is extracting oil from the cell. Yeasts have thick cell walls and, to date, there is no feasible method to obtain the oils from the cells (Jacob 1992, Ageitos et al. 2011). Another drawback of yeasts is that oleic acid (C18:1) is the main product of fatty acid synthesis whereas linoleic acid (C18:2) and arachidonic acid (C20:4) are desired as a substrate for LOX (Ageitos et al. 2011). The use of desaturases overcomes this problem.

Desaturases are able to convert (un)saturated fatty acids to (higher) unsaturated fatty acids. Heterologous expression of the *Mucor rouxii* Δ^{12}-desaturase in *Saccharomyces cerevisiae* results in the accumulation of linoleic acid in the cell (Passorn et al. 1999). Another enzyme involved in fatty acid synthesis is elongase. Elongase adds two carbons to fatty acids generating higher fatty acids (Haslam & Kunst 2013). Overexpression of this gene in *M. alpina* pushes the fatty acid synthesis towards production of higher fatty acids (Takeno et al. 2005). A combination of the correct desturases and elongases makes it theoretically possible to produce higher fatty acids such as arachidonic acid (C20:4) from palmitic acid (C16:0). In this manner, specific PUFAs needed for LOXs are created in a biobased way and therefore do not compete with first-generation biomass.

4. Current lipoxygenase expression systems

There are many different expression hosts, such as bacteria *Escherichia coli* and *Bacillus subtilis*, budding yeasts *P. pastoris* and *S. cerevisiae*, and filamentous fungi *A. oryzae* and *T. reesei*. In **Chapter 2,** we showed that all these expression hosts are suitable for the heterologous production of LOXs and in Chapter 4 we developed the use of plate assays to screen developed libraries to rapidly detect the highest producing clone. The success of one specific heterologously expressed LOX, however, does not imply the successful expression of another LOXs in that particular host. An example of this is the expression of the *G. graminis* LOX and the *A. fumigatus* LOX in *P. pastoris*. This uni-cellular budding yeast is used as an expression host due to its high cell-density cultures and relatively easy transformation methods. The alcohol oxidase (AOX) promoter used is strong and uses toxic methanol as an inducer as well as a carbon source. The *G. graminis* LOX expressed in *P. pastoris* resulted in the production of 30 mg/l, where the *A. fumigatus* LOX was expressed in an amount of 1 mg/l (Cristea et al. 2005, **Chapter 3**). These two different LOXs are closely related and there is no explanation for the different expression levels.

Similar problems have been encountered in the heterologous expression of *P. sapidus* LOX in *P. pastoris* where the secretion signal did not function properly. It is suspected the LOX is relocated towards membranes inside the cell due to its predicted N-terminal β-barrel (Zelena et al. 2014). However, the *A. fumigatus* LOX does not contain this putative N-terminal β-barrel but does contain a secretion signal. Also, the biological role of the to be expressed LOX is important in the choice of expression host. The *G. graminis* LOX is hypothesized to be involved in programmed cell death and the production of developmental signal compounds (Oliw 2002). Expression of *G. graminis* LOX in *A. nidulans* resulted in a phenotype and the production of aminopeptidase Y. Thus, *A. nidulans* is not a suitable host for heterologous expression of LOXs (**Chapter 5**).

The use of a bacterium such as *E. coli* as an expression host has pros and cons. The pros are that *E. coli* is easy to transform and grows rapidly. The cons are the formation of inclusion bodies and the lack of protein glycosylation. Cristea *et al.* discovered the *G. graminis* LOX does not need to be glycosylated to be active. Heterologous expression in *E. coli*, however, results in inclusion bodies, resulting in inactive LOX (Cristea et al. 2005). The highest reported heterologous expression of a LOX, however, was produced in *E. coli* (Lu et al. 2013). In this study, the authors successfully expressed the *Pseudomonas aeruginosa* LOX with its endogenous secretion signal. Due to the extracellular production of the *P. aeruginosa* LOX, the costs for down-

111

stream processing could be drastically decreased, making it a potential LOX for production in industrial proportions.

5. Expression of lipoxygenase in *Aspergillus*

Aspergillus is a popular expression host used by industry for the production of chemicals, such as citric acid, and for the heterologous expression of enzymes (Meyer et al. 2010; Papagianni 2007; Lubertozzi & Keasling 2009). Furthermore, these organisms are capable of using various sugars found in plant waste material (e.g. C5-sugar xylose) and this makes them suitable for use in a biobased economy (de Vries & Visser 2001).

Expression of LOX in *Aspergillus* grown on plant waste material could result in a one-step procedure in the production of fatty acid hydroperoxides. **Chapter 5** describes the use of *A. nidulans* as an expression host for LOX, which did not result in the desired one-step procedure. The same phenomenom is seen using *A. niger* as an expression host. Other *Aspergillus* species, such as *A. fumigatus, A oryzae, and A. flavus*, naturally produce LOXs according to their genomes. Therefore, it is hypothesized these organisms can be used in the heterologous production of LOXs. The main drawback of using these organisms is their pathogenicity. The most suitable host of these three organisms would be *A. oryzae*.

It is a debated point whether this organism has lost is pathogenicity over time due to its extensive use in biotechnology by mankind (Kurtzman et al. 1986). Its close phylogenetic relationship to *A. flavus*, however, might suggest otherwise. On the basis of genomic data of *A. oryzae lox* compared to the *A. flavus lox*, we hypothesize the *A. oryzae* LOX has lost its functionality by point mutations. These point mutations induce a premature stop codon and result in inactive *A. oryzae* LOX. The use of *A. oryzae* in heterologous LOX expression has been proven (Sugio et al. 2002; Christensen et al. 2008). The question remains whether this positive expression is due to a highly adapted industrial strain.

6. Perspectives

The *G. graminis* fungus, also called the "take-all" fungus due to its devastation on agricultural crops, infects root cells of young wheat plants and reduces the plant's nitrogen uptake (Macdonald et al. 2012). The fungus secretes a LOX thought to interfere with the plant's signalling pathways in programmed cell death (Oliw 2002). This LOX is designed by nature to be active extracellular; it is strongly glycosylated, it has a broad pH range, it uses manganese instead of iron as a cofactor, and it is stable

to heat. This type of enzyme is favoured by industry, because it does not need to be renewed frequently.

It is logical to search for a suitable LOX in the fungal kingdom because fungi are capable of degrading plant waste material and are suitable expression hosts. A novel group of fungal LOXs was identified and resulted in eight different fungal species that naturally secrete LOXs (**Chapter 3**). Based on this comparison, the secreted LOX from *A. fumigatus* was selected to be heterologously expressed in *P. pastoris*. Although the expression level of the *A. fumigatus* LOX was low, six other fungi house possible LOX candidates for heterologous expression. Also, due to the rapid developments in genome sequencing, more LOXs are being identified which could result in more suitable candidates.

It would be favourable to construct a LOX that can be used to produce multiple oxylipins because LOXs require specific substrates and make specific oxylipins. Through 3D-structures and bioinformatics modelling, this is within our grasp. Many modifications can be made to increase the potential of LOX use. A "perfect" LOX has to be a) small, b) stable, c) active in a broad pH spectrum, d) able to use all PUFAs, and e) enantiomeric specific. An overview of these adaptations is given in Table 2. Many of these specifications are found in secreted fungal LOXs and these may form the basis of a perfect LOX.

Table 2: Overview of different adaptations that can be used to create a perfect LOX.

Adaptation	Technique	Natural example	Synthetic example	Reference
Small size	β-barrel deletion	*Gaeumannomyces graminis* LOX	*Mus musculus* 5-LOX	Walther et al. 2011
Stability	Glycosylation	*Gaeumannomyces graminis* LOX	N/A	Oliw 2002
pH spectrum	Mutagenesis	*Glycine max* L1	*Homo sapiens* 15-LOX	Walther et al. 2009
Binding pocket depth	Mutagenesis	All LOXs	*Orytolagus cuniculus*	Borngräber et al. 1999
Use of all PUFAs	Mutagenesis	*Pseudomonas aeruginosa* LOX	N/A	Jisaka et al. 1999
Enantiomeric specific	Mutagenesis	All LOXs	*Homo sapiens* 15-LOX	Coffa et al. 2005

Regarding LOX size, secreted LOX enzymes are small since they lack the N-terminal β-barrel and this makes so-called "mini-LOXs" (Zheng et al. 2008). As less amino acids are needed, this lowers the cellular burden and might speed up production. For industrial polymer production LOX is not needed to bind to a membrane. Deletion of the N-terminal β-barrel often results in decreased activity, which makes intracellular LOXs less favourable for modification (Walther et al. 2011).

In terms of LOX stability, the secreted fungal *G. graminis* LOX is glycosylated and this makes the LOX more stable and soluble (Oliw 2002). To enhance protein thermostability, two adaptations can be made: including a larger proportion of salt bridges by a higher frequency of arginine and avoiding proline, cysteine, and histidine in α-helices (Kumar et al. 2000).

The pH level effects how the PUFA enters the substrate-binding pocket because pH is involved in the charge of the carboxyl group of the PUFA. Certain amino acids in the binding pocket of LOX can be altered to broaden the pH range to increase generation of specific oxylipins (Hornung et al. 2008; Walther et al. 2009). The LOX can also be changed to favour the "head" or "tail" entrance of the PUFA into the binding pocket of the LOX, which is important for the oxylipin formation (Jisaka et al. 2000). To adapt a LOX favouring a certain PUFA, the depth of the substrate-binding pocket has to be altered. In this way the LOX will react with a different double bond in the PUFA. An example of such an adaptation is the conversion of a 12-LOX to a 15-LOX in a rabbit LOX, in which the catalytic site is active at the 15th carbon position of the PUFA instead of the 12th carbon (Borngräber et al. 1999). With these changes to the binding pocket, it is possible to create a LOX that uses a variety of PUFAs. For example, the *P. aeruginosa* LOX is known to use oleic acid, linoleic acid, and arachidonic acid as a substrate (Lu X et al. 2013; Vance et al. 2004).

The final modification that can be made to increase the use of LOX is manipluating the PUFA's enantiomeric specificity of the peroxide group from *S* to *R*. This can be achieved by altering an alanine to a glycine at the Coffa-Brash site. This is due to the larger size of alanine pushing the PUFA in an *S*-enantiomeric position in the binding pocket (Coffa et al. 2005). This forces the LOX to generate specific oxylipins. These specific oxylipins can be used for medical purposes by signalling cells to follow a certain metabolic pathway (Joo & Oh 2012).

For all these modifications it is recommended to obtain 3D structures of the secreted fungal LOXs. Fig. 5 shows two 3D protein models of the *G. graminis* LOX using the UCSF Chimera modelling tool (Sali & Blundell 1993). The 3D structures of the *Glycine*

max LOX and the *P. aeruginosa* LOX were used as reference models. In these models the native secretion signal of the *G. graminis* LOX was deleted from the amino acid sequence. The accuracy of the models is based on a zDOPE value calculated by the tool. The lower the score, the better the prediction. The *G. graminis* LOX based on the *G. max* LOX scores a zDOPE value of 0.29 whereas the model based on the *P. aeruginosa* LOX scores a zDOPE value of 0.03. The main differences between the models are found in the N-terminus. The *P. aeruginosa* LOX and the *G. graminis* LOX are both naturally secreted enzymes lacking the N-terminal β-barrel. The lower zDOPE value and the lack of the β-barrel suggest the *G. graminis* LOX looks more like the *P. aeruginosa* LOX than the *G. max* LOX. Recently, crystals of the *G. graminis* LOX have been obtained, but the structure remains unsolved (Wennman et al. 2014). Production and purification of the *A. fumigatus* LOX can deliver a piece of the puzzle in unravelling the 3D structure of secreted fungal LOXs.
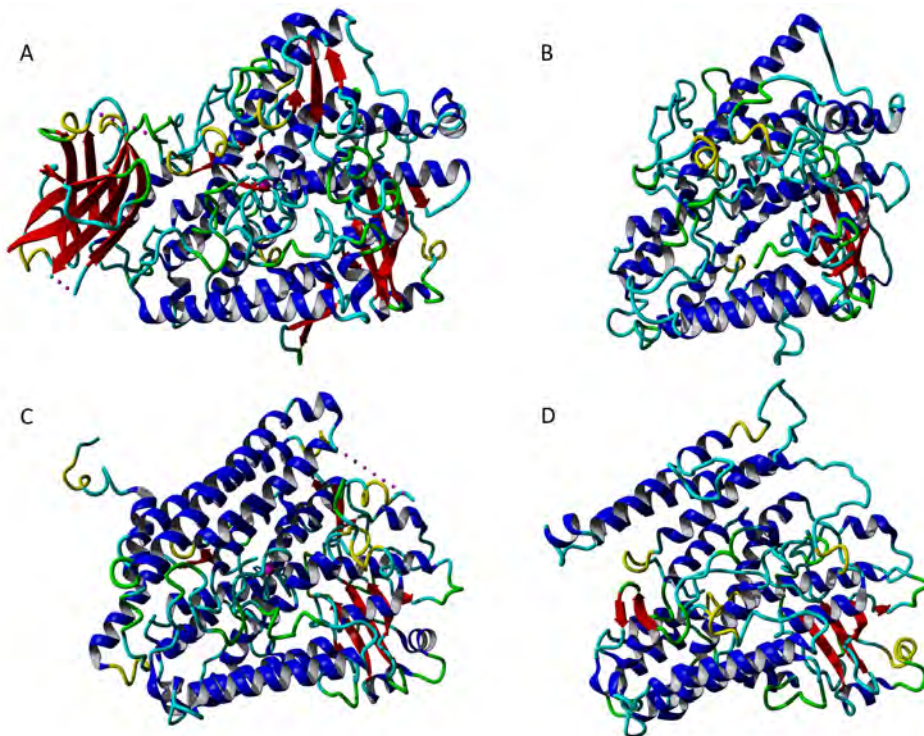
115

Figure 5: 3D protein models of *Gaeumannomyces graminis* LOX using *Glycine max* LOX (A) and *Pseudomonas aeruginosa* LOX (C) as reference models for the UCSF Chimera modelling tool. Both *G. graminis* LOX models (B and D) lack the N-terminal β-barrel. The *G. graminis* LOX has 23.8% sequence homology compared to the *G. max* LOX and a zDOPE-value of 0.29, whereas compared to the *P. aeruginosa* LOX is has 24.3% sequence homology and a zDOPE value of 0.03. A lower zDOPE value results in a more accurate model.

7. In conclusion

Although challenges remain in making LOX-derived products viable for industry, LOX-derived products have enourmous potentional for use in a biobased economy. One of the challenges is the supply of PUFAs for the LOX. This could, for example, be overcome by the production of microbial oil. Another challenge is the relative low yield of heterologous LOX production using various expression hosts. This could be overcome by understanding the biological role of LOX and anticipating what the effect might be on the production host. With the use of bioinformatics techniques, suitable candidates for heterologous expression could be identified and a more suitable LOX could be synthetically engineered. If these challenges could be meet, LOX could become a game-changing enzyme in a biobased economy.

117

Bibliography

Adams TH, Wieser JK, Yu JH (1998) Asexual sporulation in *Aspergillus nidulans*. Microbiol Mol Biol 62(1): 35-54.

Adriaanse AJ, Convents D, Smith IE, Verhagen JJ (2000) Bleaching composition. GB 2372261 A

Affeldt KJ, Brodhagen M, Keller NP (2012) *Aspergillus* oxylipin signaling and quorum sensing pathways depend on G protein-coupled receptors. Toxins 4(9): 695-717. doi: 10.3390/toxins4090695

Ageitos JM, Vallejo JA, Veiga-Crespo P, Villa TG (2011) Oily yeasts as oleaginous cell factories. Appl Microbiol Biotechnol 90: 1219-1227

Amselem J, Cuomo CA, van Kan JA, Viaud M, Benito EP, Couloux A, Coutinho PM, de Vries RP, Dyer PS, Fillinger S, Fournier E, Gout L, Hahn M, Kohn L, Lapalu N, Plummer KM, Pradier JM, Quévillon E, Sharon A, Simon A, ten Have A, Tudzynski B, Tudzynski P, Wincker P, Andrew M, Anthouard V, Beever RE, Beffa R, Benoit I, Bouzid O, Brault B, Chen Z, Choquer M, Collémare J, Cotton P, Danchin EG, Da Silva C, Gautier A, Giraud C, Giraud T, Gonzalez C, Grossetete S, Güldener U, Henrissat B, Howlett BJ, Kodira C, Kretschmer M, Lappartient A, Leroch M, Levis C, Mauceli E, Neuvéglise C, Oeser B, Pearson M, Poulain J, Poussereau N, Quesneville H, Rascle C, Schumacher J, Ségurens B, Sexton A, Silva E, Sirven C, Soanes DM, Talbot NJ, Templeton M, Yandava C, Yarden O, Zeng Q, Rollins JA, Lebrun MH, Dickman M (2011) Genomic analysis of the necrotrophic fungal pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea*. PLoS Genet 7(8): e1002230

Andreou A, Brodhun F, Feussner I (2009) Biosynthesis of oxylipins in non-mammals. Prog Lipid Res 48 (3-4): 148-170. doi: 10.1016/j.plipres.2009.02.002

Andreou A, Goebel C, Hamberg M, Feussner I (2010) A bisallylic mini-lipoxygenase from cyanobacterium *Cyanothece* sp that has an iron as cofactor. J Biol Chem 285(19): 14178-14186

Anthon GE, Barrett DM (2001) Colorimetric method for the determination of lipoxygenase activity. J Agric Food Chem 49: 32–37

Axelrod B, Cheesbrough TM, Laakso S (1981) Lipoxygenase from soybeans. Methods Enzymol 71: 441–451

Banni S, Carta G, Contini MS, Angioni E, Deiana M, Dessi MA, Melis MP, Corongiu FP (1996) Characterization of conjugated diene fatty acids in milk, dairy products, and lamb tissues. J Nutr Biochem 7(3): 150-155

Barona E, Ramankutty N, Hyman G, Coomes OT (2010) The role of pasture and soybean in deforestation of the Brazilian Amazon. Environ Res Lett 5: 024002

Baysal T, Demirdöven A (2007) Lipoxygenase in fruits and vegetables: A review. Enzyme Microb Tech 40 (4): 491-496

Beopoulos A, Cescut J, Haddouche R, Uribelarrea JL, Molina-Jouve C, Nicaud JM (2009) *Yarrowia lipolytica* as a model for bio-oil production. Prog Lipid Res 48: 375-387

Blanksby SJ, Ellison GB, Bierbaum VM, Kato S (2002) Direct evidence for base-mediated decomposition of alkyl hydroperoxides (ROOH) in the gas phase. J Am Chem Soc 124(13): 3196-3197

Blee E (2002) Impact of phyto-oxylipins in plant defense. Trends Plant Sci 7(7): 315-321

Borngräber S, Browner M, Gillmor S, Gerth C, Anton M, Fletterick R, Kuhn H (1999) Shape and specificity in mammalian 15-lipoxygenase active site. The functional interplay of sequence determinants for the reaction specificity. J Biol Chem 274: 37345-37350

Borngräber S, Kuban RJ, Anton M, Kuhn H (1996) Phenylalanine 353 is a primary determinant for the positional specificity of mammalian 15-lipoxygenases. J Mol Biol 264: 1145-1153

Boussard A, Cordella CBY, Rakotozafy L, Moulin G, Buche F, Potus J, Nicolas J (2012) Use of chemometric tools to estimate the effect of the addition of yeast, glucose-oxidase, soybean or hors bean flours to wheat flour on biochemical bread dough characteristics. Chemom and Intel Lab Systems 113: 68-77

Brash AR (1999) Lipoxygenases: Occurrence, functions, catalysis, and acquisition of substrate. J Biol Chem 274(34): 23679-23682

Brodhun F, Cristobal-Sarramian A, Zabel S, Newie J, Hamberg M, Feussner I (2013) An iron 13S-lipoxygenase with an alpha-linolenic acid specific hydroperoxidase activity from *Fusarium oxysporum*. PloS one 8(5): e64919

Brodhun F, Feussner I (2011) Oxylipins in fungi. FEBS J 278(7): 1047-1063. doi: 10.1111/j.1742-4658.2011.08027.x

Brown DW, Salvo JJ (1994) Isolation and characterization of sexual spore pigments from *Aspergillus nidulans*. Appl Environ Microbiol 60(3): 979-983

Brown SH, Zarnowski R, Sharpee WC, Keller NP (2008) Morphological transitions governed by density dependence and lipoxygenase activity in *Aspergillus flavus*. Appl Environ Microbiol 74(18): 5674-5685. doi: 10.1128/AEM.00565-08

Buchhaupt M, Guder JC, Etschmann MM, Schrader J (2012) Synthesis of green note aroma compounds by biotransformation of fatty acids using yeast cells coexpressing lipoxygenase and hydroperoxide lyase. Appl Microbiol Biotechnol 93(1): 159-168

Burow GB, Nesbitt TC, Dunlap J, Keller NP (1997) Seed lipoxygenase products modulate *Aspergillus* mycotoxin biosynthesis. Mol Plant Microbe In 10(3): 380-387

Calco AM, Hinze LL Gardner HW, Keller NP (1999) Sporogenic effect of polyunsaturated fatty acids on development of *Aspergillus* spp. Appl Environ Microbiol 65(8): 3668-3673

Casey R, Hughes RK (2004) Recombinant lipoxygenases and oxylipin metabolism in relation to food quality. Food Biotechnol 18(2): 135-170

Cass BJ, Schade F, Robinson CW, Thompson JE, Legge RL (2000) Production of tomato flavor volatiles from a crude enzyme preparation using a hollow-fiber reactor. Biotechnol Bioeng 67(3): 372-377

Cato L, Halmos AL, Small DM (2006) Measurement of lipoxygenase in Australian white wheat flour: the effect of lipoxygenase on the quality properties of white salted noodles. J Sci Food Agric 86(11): 1670-1678

Cenis JL (1992) Rapid extraction of fungal DNA for PCR amplification. Nucleic Acids Res 20 (9): 2380

Chahinian H, Sias B, Carriere F (2000) The C-terminal domain of pancreatic lipase: functional and structural analogies with C2 domains. Curr Protein Pept Sci 1(1): 91-103

Chang PK, Ehrlich KC (2010) What does genetic diversity of *Aspergillus flavus* tell us about *Aspergillus oryzae*? Int J Food Micriobiol 138(3): 189-199

Christensen S, Sugio A, Takagi S, Ostergaard L, Oliw EH (2008) A polynucleotide encoding a lipoxygenase and its use for recombinant production of a lipoxygenase; obtaining a lipoxygenase by screening a DNA library with specific probes. US Patent US 7456001 B2

Choi J, Chon JK, Kim S, Shin W (2008) Conformational flexibility in mammalian 15S-lipoxygenase: reinterpretation of the crystallographic data. Proteins 70(3): 1023-1032

Chowdhury K, Banu L, Khan S, Latif A (2007) Studies on the fatty acid composition of edible oil. Bangladesh J Sci Ind Res. 42: 311-316

Coffa G, Brash AR (2004) A single active site residue directs oxygenation stereospecificity in lipoxygenases: stereocontrol is linked to the position of oxygenation. Proc Natl Acad Sci USA 101 (44): 15579-15584

Coffa G, Schneider C, Brash AR (2005) A comprehensive model of positional and stereo control in lipoxygenases. Biochem Biophys Res Commun 338(1): 87-92

Cox J, Mann M (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol 26(12): 1367-1372. doi: 10.1038/nbt.1511

Cristea M, Engstrom A, Su C, Hörnsten L, Oliw EH (2005) Expression of manganese lipoxygenase in *Pichia pastoris* and site-directed mutagenesis of putative metal ligands. Arch Biochem Biophys 434 (1): 201-211. doi: 10.1016/j.abb.2004.10.026

Cristea M, Oliw EH (2006) A G316A mutation of manganese lipoxygenase augments hydroperoxide isomerase activity - Mechanism of biosynthesis of epoxyalcohols. J Biol Chem 281 (26): 17612-17623. doi: 10.1074/jbc.M510311200

Cristea M, Oliw EH (2007) On the singular, dual, and multiple positional specificity of manganese lipoxygenase and its G316A mutant. J Lipid Res 48 (4): 890-903. doi: 10.1194/jlr.M600505-JLR200

Dahlén B, Nizankowska E, Szczeklik A, Zetterström O, Bochenek G, Kumlin M, Mastalerz L, Pinis G, Swanson LJ, Boodhoo TI, Wright S, Dubé LM, Dahlén SE (1998) Benefits from adding the 5-lipoxygenase inhibitor zileuton to conventional therapy in aspirin-intolerant asthmatics. Am J Respir Crit Care Med 157(1): 1187-1194

Dean RA, Talbot NJ, Ebbole DJ, Farman ML, Mitchell TK, Orbach MJ, Thon M, Kulkarni R, Xu JR, Pan H, Read ND, Lee YH, Carbone I, Brown D, Oh YY, Donofio N, Jeong JS, Soanes DM, Djonovic S, Kolomiets E, Rehmever C, Li W, Harding M, Kim S, Lebrun MH, Bohnert H, Coughlan S, Butler J, Calvo S, Ma LJ, Nicol R, Purcell S, Nusbaum C, Galagan JE, Birren BW (2005) The genome sequence of the rice blast fungus *Magnaporthe grisea*. Nature 434(7036): 980-986

DeLong JM, Prange RK, Hodges DM, Forney CF, Bishop MC, Quilliam M (2002) Using a modified ferrous oxidation-xylenol orange (FOX) assay for detection of lipid hydroperoxides in plant tissue. J Agr Food Chem 50(2): 248–254

Demirbas MF (2009) Biorefineries for biofuel upgrading: A critical review. Appl Energ 86: S151-S161

Di Venere A, Salucci ML, van Zadelhoff G, Veldink G, Mei G, Rosato N, Finazzo-Agro A, Maccarrone M (2003) Structure-to-function relationship of mini-lipoxygenase, a 60-kDA fragment of soybean lipoxygenase-1 with lower stability but higher enzymatic activity. J Biol Chem 278(20): 18281-18288

DiRusso CC, Nyström T (1998) The fats of *Escherichia coli* during infancy and old age: regulation by global regulators, alarmones and lipid intermediates. Mol Microbiol 27: 1–8

Dubé E, Shareck F, Hurtubise Y, Beauregard M, Daneault C (2008) Enzyme-based approaches for pitch control in thermomechanical pulping of softwood and pitch removal in process water. J Chem Technol Biotechnol 83(9): 1261-1266

Duroudier NP, Tulah AS, Sayers I (2009) Leukotriene pathway genetics and pharmacogenetics in allergy. Allergy 64 (6): 823-839. doi: 10.1111/j.1398-9995.2009.02015.x

Edgar RC (2004) MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics 5: 1-19. doi: 11310.1186/1471-2105-5-113

Eek P, Jaerving R, Jaerving I, Gilbert NC, Newcomer ME, Samel N (2012) Structure of a calcium-dependent 11R-lipoxygenase suggests a mechanism for Ca2+ regulation. J Biol Chem 287(26): 22377-22386

Emanuelsson O, Brunak S, von Heijne G, Nielsen H (2007) Locating proteins in the cell using TargetP, SignalP and related tools. Nat Protoc 2 (4): 953-971. doi: 10.1038/nprot.2007.131

Emek SC, Akerlund H-E, Erlanson-Albertsson C, Albertsson P-A (2013) Pancreatic lipase-colipase binds strongly to the thylakoid membrane surface. J Sci Food Agric 93(9): 2254-2258

FAO. Pulp and paper capacities. Survey 2012-2017, 2013; ISSN 0255-7665

Faubion JM, Hoseney RC (1981) Lipoxygenase: its biochemistry and role in breadmaking. Cereal Chem 58(3): 175-180

Feussner I, Kuhn H, Wasternack C (2001) Lipoxygenase-dependent degradation of storage lipids. Trends Plant Sci 6(6): 268-273

Feussner I, Wasternack C (2002) The lipoxygenase pathway. Annu Rev Plant Biol 53: 275–297

Frankel EN, Gardner HW. 1989. Effect of alpha-tocopherol on the volatile thermal decomposition products of methyl linoleate hydroperoxides. Lipids 24(7): 603-608

Frankel, EN (2005) Lipid oxidation Second Edition, The Oily Press, Bridgwater, England, 470pp

Frazier PJ, Brimblecombe FA, Daniels NWR, Russell Eggitt PW (1977) The effect of lipoxygenase action on the mechanical development of doughs from fat-extracted and reconstituted wheat flours. J Sci Food Agric 28(3): 247-254

Frazier PJ, Leigh-Dugmore FA, Daniels NWR, Russell Eggitt PW, Coppock JBM (1973) The effect of lipoxygenase action on the mechanical development of wheat flour doughs. J Sci Fd Agric 24: 421–436

Furusawa M, Hashimoto T, Noma Y, Asakawa Y (2005) Highly efficient production of nootkatone, the grapefruit aroma from valencene, by biotransformation. Chem Pharm Bull 53(11): 1513-1514

Gao X, Shim WB, Göbel C, Kunze S, Feussner I, Meeley R, Balint-Kurti P, Kolomiets M (2007) Disruption of a maize 9-lipoxygenase results in increased resistance to fungal pathogens and reduced levels of contamination with mycotoxin fumonisin. Mol Plant Microbe In 20(8): 922-933

Gao X, Starr J, Goebel C, Engelberth J, Feussner I, Tumlinson J, Kolomiets M (2008) Maize 9-lipoxygenase ZmLOX3 controls development, root-specific expression of defense genes, and resistance to root-knot nematodes. Mol Plant Microbe In 21(1): 98-109

124

Gardner HW (1989) Oxygen radical chemistry of polyunsaturated fatty acids. Free Radic Biol Med 7(1): 65-86

Gardner HW (1991) Recent investigations into the lipoxygenase pathway of plants. Biochim Biophys Acta 1084(3): 221-239

Gardner HW, Nelson EC, Tjarks LW, England RE (1984) Acid-catalyzed transformation of 13(S)-hydroperoxylinoleic acid into epoxyhydroxyoctadecenoic and trihydroxyoctadecenoic acids. Chem Phys Lipids 35(2): 87-101

Garreta A, Val-Moraes SP, García-Fernández Q, Busquets M, Juan C, Oliver A, Ortiz A, Gaffney BJ, Fita I, Manresa À, Carpena X (2013) Structure and interaction with phospholipids of a prokaryotic lipoxygenase from *Pseudomonas aeruginosa*. FASEB J 27(12): 4811-4821

Gerhardt B, Fischer K, Balkenhohl TJ, Pohnert G, Kuhn H, Wasternack C, Feussner I (2005) Lipoxygenase-mediated metabolism of storage lipids in germinating sunflower cotyledons and beta-oxidation of (9Z,11E,13S)-13-hydroxy-octadeca-9,11-dienoic acid by the cotyledonary glyoxysomes. Planta 220(6): 919-930

Gigot C, Ongena M, Fauconnier M-L, Wathelet J-P, Du Jardin P, Thonart P (2010) The lipoxygenase metabolic pathway in plants: potential for industrial production of natural green leaf volatiles. Biotech Agron Soc Environ 14(3): 451-460

Gilbert NC, Bartlett SG, Waight MT, Neau DB, Boeglin WE, Brash AR, Newcomer ME (2011) The Structure of Human 5-Lipoxygenase. Science 331(6014): 217-219

Gillmor SA, Villasenor A, Fletterick R, Sigal E, Browner MF (1997) The structure of mammalian 15-lipoxygenase reveals similarity to the lipases and the determinants of substrate specificity. Nat Struct Biol 4(12): 1003-1009

Gironi F, Piemonte V (2011) Bioplastics and petroleum-based plastics: strengths and weaknesses. Energ Source Part A 33: 1949-1959

Goldman BS, Nierman WC, Kaiser D, Slater SC, Durkin AS, Eisen J, Ronning CM, Barbazuk WB, Blanchard M, Field C, Halling C, Hinkle G, Iartchuk O, Kim HS, Mackenzie C, Madupu R, Miller N, Shvartsbeyn A, Sullivan SA, Vaudin M, Wiegand R, Kaplan HB (2006) Evolution of sensory complexity recorded in a myxobacterial genome. Proc Natl Acad Sci USA 103 (41): 15200-15205. doi: 10.1073/pnas.0607335103

Goldsmith CR, Jonas RT, Stack TDP (2002) C-H bond activation by a ferric methoxide complex: modeling the rate-determining step in the mechanism of lipoxygenase. J Am Chem Soc 124: 83–96

van Gorkum R, Bouwman E (2005) The oxidative drying of alkyd paint catalysed by metal complexes. Coord Chem Rev 249(17-18): 1709–1728

de Graaff LH, van den Broeck H, Visser J (1992) Isolation and characterization of the *Aspergillus niger* pyruvate kinase gene. Curr Genet 22(1): 21-27

Gutiérrez A, Río J, Martínez A (2009) Microbial and enzymatic control of pitch in the pulp and paper industry. Appl Microbiol Biotechnol 82(6): 1005-1018

Haefliger OP, Sulzer JW (2007) Rapid LC-UV-ESI-MS method to investigate the industrial preparation of polyunsaturated fatty acid hydroperoxides in real-time. Chromatographia 65 (7-8): 435-442

Hamberg M, Gotthammar B (1973) A new reaction of unsaturated fatty acid hydroperoxides: formation of 11-hydroxy-12,13-epoxy-9-octadecenoic acid from 13-hydroperoxy-9,11-octadecadienoic acid. Lipids 8(12): 737–744

Hamberg M, Sanz A, Rodriguez MJ, Calvo AP, Castresana C (2003) Activation of the fatty acid α-dioxygenase pathway during bacterial infection of tobacco leaves - Formation of oxylipins protecting against cell death. J Biol Chem 278 (51): 51796-51805. doi: 10.1074/jbc.M310514200

Hamberg M, Su C, Oliw E (1998) Manganese lipoxygenase - Discovery of a bis-allylic hydroperoxide as product and intermediate in a lipoxygenase reaction. J Biol Chem 273 (21): 13080-13088

Hammarberg T, ZhangY-Y LB, Rådmark O, Samuelsson B (1993) Iron content of human 5-lipoxygenase, effects of mutations regarding conserved histidine residues. J Biol Chem 268: 2535–41

Hammond VJ, Morgan AH, Lauder S, Thomas CP, Brown S, Freeman BA, Lloyd CM, Davies J, Bush A, Levonen AL, Kansanen E, Villacorta L, Chen YE, Porter N, Garcia-Diaz YM, Schopfer FJ, O'Donnell VB (2012) Novel keto-phospholipids are generated by monocytes and macrophages, detected in cystic fibrosis, and activate peroxisome proliferator-activated receptor-gamma. J Biol Chem 287(50): 41651-41666

Hansen J, Garreta A, Benincasa M, Fusté MC, Busquets M, Manresa A (2013) Bacterial lipoxygenases, a new subfamily of enzymes? A phylogenetic approach. Appl Microbiol Biot 97 (11): 4737-4747. doi: 10.1007/s00253-013-4887-9

Haslam TM, Kunst L (2013) Extending the story of very-long-chain fatty acid elongation. Plant Sci 210: 93-107

Hayes DG. (2004) Enzyme-catalyzed modification of oilseed materials to produce eco-friendly products. J Am Oil Chem Soc 81(12): 1077-1103

Heshof R, Jylhä S, Haarmann T, Jørgensen AL, Dalsgaard TK, de Graaff LH (2014a) A novel class of fungal lipoxygenases. Appl Microbiol Biotechnol 98(3): 1261-1270 doi: 10.1007/s00253-013-5392-x

Heshof R, van Schayck JP, Tamayo-Ramos JA, de Graaff LH (2014b) Heterologous expression of *Gaeumannomyces graminis* lipoxygenase in *Aspergillus nidulans*. AMB Express 4(1): 65. doi: 10.1186/s13568-014-0065-4

Higashiyama K, Fujikawa S, Park E, Shimizu S (2002) Production of arachidonic acid by *Mortierella* fungi. Biotechnol Bioproc Eng 7: 252-262

Hilbers MP, Finazzi-Agrò A, Veldink GA, Vliegenthart JF (1996) Purification and characterization of a lentil seedling lipoxygenase expressed in *E. coli*: implications for the mechanism of oxodiene formation by lipoxygenases. Int J Biochem Cell Biol 28(7): 751-760

Hirota N, Kaneko T, Ito K, Takeda K (2006) Mapping a factor controlling the thermostability of seed lipoxygenase-1 in barley. Plant Breeding 125(3): 231-235

Hörnsten L, Su C, Osbourn AE, Hellman U, Oliw EH (2002) Cloning of the manganese lipoxygenase gene reveals homology with the lipoxygenase gene family. Eur J Biochem 269(11): 2690-2697

Hornung E, Kunze S, Liavonchanka A, Zimmermann G, Kuhn D, Fritsche K, Renz A, Kuhn H, Feussner I (2008) Identification of an amino acid determinant of pH regiospecificity in a seed lipoxygenase from *Momordica charantia*. Phytochemistry 69: 2774-2780.

Horowitz Brown S, Zarnowski R, Sharpee WC, Keller NP (2008) Morphological transitions governed by density dependence and lipoxygenase activity in *Aspergillus flavus*. Appl Environ Microbiol 74(18): 5674-5685

Hoseney RC, Rao H, Faubion J, Sidhu JS (1980) Mixograph studies IV. The mechanism by which lipoxygenase increases mixing tolerance. Cereal Chem 57(3): 163-166

Howe GA, Jander G (2008) Plant immunity to insect herbivores. Annu Rev Plant Biol 59: 41-66

Howe K, Bateman A, Durbin R (2002) QuickTree: building huge Neighbour-Joining trees of protein sequences. Bioinformatics 18 (11): 1546-1547. doi: 10.1093/bioinformatics/18.11.1546

Huang FC, Schwab W (2011) Cloning and characterization of a 9-lipoxygenase gene induced by pathogen attack from *Nicotiana benthamiana* for biotechnological application. BMC Biotechnol 11: 30

Hughes RK, De Domenico S, Santino A (2009) Plant cytochrome CYP74 family: biochemical features, endocellular localisation, activation mechanism in plant defence and improvements for industrial applications. Chembiochem 10(4): 1122-1133

Hughes RK, Wu ZC, Robinson DS, Hardy D, West SI, Fairhurst SA, Casey R (1998) Characterization of authentic recombinant pea-seed lipoxygenases with distinct properties and reaction mechanisms. Biochem J 333(1): 33-43

Ivanov I, Heydeck D, Hofheinz K, Roffeis J, O'Donnell VB, Kuhn H, Walther M (2010) Molecular enzymology of lipoxygenases. Arch Biochem Biophys 503 (2): 161-174. doi: 10.1016/j.abb.2010.08.016

Izumi T, Hoshiko S, Radmark O, Samuelsson B (1990) Cloning of the cDNA for human 12-lipoxygenase. Proc Natl Acad Sci USA 87 (19): 7477-7481

Jacob Z (1992) Yeast Lipids: Extraction, quality analysis, and acceptability. Crit Rev Biotechnol 12: 463-491

Jansen C, Hofheinz K, Vogel R, Roffeis J, Anton M, Reddanna P, Kuhn H, Walther M (2011) Stereocontrol of arachidonic acid oxygenation by vertebrate lipoxygenases: newly cloned zebrafish lipoxygenase 1 does not follow the ala-versus-gly concept. J Biol Chem 286 (43): 37804-37812. doi: 10.1074/jbc.M111.259242

Jensen AB, Poca E, Rigaud M, Freyssinet G, Pages M (1997) Molecular characterization of L2 lipoxygenase from maize embryos. Plant Mol Biol 33 (4): 605-614

Jiang WG, Douglas-Jones A, Mansel RE (2003) Levels of expression of lipoxygenases and cyclooxygenase-2 in human breast cancer. Prostag Leukotr Ess 69(4): 275-281

Jisaka M, Kim RB, Boeglin WE, Brash AR (2000) Identification of amino acid determinants of the positional specificity of mouse 8S-lipoxygenase and human 15S-lipoxygenase-2. J Biol Chem 275: 1287-1293

Jisaka M, Kim RB, Boeglin WE, Nanney LB, Brash AR (1997) Molecular cloning and functional expression of a phorbol ester-inducible 8S-lipoxygenase from mouse skin. J Biol Chem 272 (39): 24410-24416. doi: 10.1074/jbc.272.39.24410

Junqueira RM, Rocha F, Moreira MA, Castro IA (2007) Effect of proofing time and wheat flour strength on bleaching, sensory characteristics, and volume of French breads with added soybean lipoxygenase. Cereal Chem 84(5): 443-449

Kandouz M, Nie DT, Pidgeon GP, Krishnamoorthy S, Maddipati KR, Honn KV (2003) Platelet-type 12-lipoxygenase activates NF-kappa B in prostate cancer cells. Prostag Oth Lipid M 71(3-4): 189-204

Kang EJ, Campbell RE, Bastian E, Drake MA (2010) Invited review: annatto usage and bleaching in dairy foods. J Dairy Sci 93(9): 3891-3901

Kelle S, Zelena K, Krings U, Linke D, Berger RG (2014) Expression of soluble recombinant lipoxygenase from *Pleurotus sapidus* in *Pichia pastoris*. Protein Expression Purif 95: 233-239

Kharasch MS, Fono A, Nudenberg W (1950) The chemistry of hydroperoxides I. The acid-catalyzed decomposition of α,α-dimethylbenzyl (α-cumyl) hydroperoxide. J Org Chem 15(4): 748–752

Knust B, Von Wettstein D. (1992) Expression and secretion of pea-seed lipoxygenase isoenzymes in *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 37(3): 342-351

Koeduka T, Kajiwara T, Matsui K (2007) Cloning of lipoxygenase genes from a cyanobacterium, *Nostoc punctiforme*, and its expression in *Eschelichia coli*. Curr Microbiol 54(4): 315-319

Konovalova A, Petters T, Søgaard-Andersen L (2010) Extracellular biology of *Myxococcus xanthus*. FEMS Microbiol Rev 34 (2): 89-106. doi: 10.1111/j.1574-6976.2009.00194.x

Kuhn H, Saam J, Eibach S, Holzhutter HG, Ivanov I, Walther M (2005) Structural biology of mammalian lipoxygenasese enzymatic consequences of targeted alterations of the protein structure. Biochem Biophys Res Commun 338(1): 93-101

Kumar S, Chung-Jung T, Nussinov R (2000) Factors enhancing protein stability. Protein Eng 13: 179-191

Kuo JM, Hwang A, Hsu HH, Pan BS (1996) Preliminary identification of lipoxygenase in algae (Enteromorpha intestinalis) for aroma formation. J Agr Food Chem 44 (8): 2073-2077. doi: 10.1021/jf950774c

Kusters-van Someren MA, Harmsen JAM, Kester HCM, Visser J (1991) Structure of the *Aspergillus niger* pelA gene and its expression in *Aspergillus niger* and *Aspergillus nidulans*. Curr Genet 20(4): 293–299

Latgé JP (1999) *Aspergillus fumigatus* and aspergillosis. Clin Microbiol Rev 12(2): 310-350

Lagarde M, Chen P, Véricel E, Guichardant M (2010) Fatty acid-derived lipid mediators and blood platelet aggregation. Prostag Leukotr Ess 82 (4-6): 227-230. doi: 10.1016/j.plefa.2010.02.017

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007) Clustal W and clustal X version 2.0. Bioinformatics 23 (21): 2947-2948. doi: 10.1093/bioinformatics/btm404

Lee RA, Lavoie JM (2013) From first- to third-generation biofuels: Challenges of producing a commodity from a biomass of increasing complexity. Anim Front 3: 6-11

Lewis RA, Austen KF, Soberman RJ (1990) Leukotrienes and other products of the 5-lipoxygenase pathway. Biochemistry and relation to pathobiology in human diseases. New Engl J Med 323(10): 645-655

Lu X, Zhang J, Liu S, Zhang D, Xu Z, Wu J, Li J, Du G, Chen J (2013) Overproduction, purification, and characterization of extracellular lipoxygenase of *Pseudomonas aeruginosa* in *Escherichia coli*. Appl Microbiol Biotechnol 97(13): 5793-5800

Lubertozzi D, Keasling JD (2009) Developing *Aspergillus* as a host for heterolougus expression. Biotechnol Adv 27(1): 53-75

Lybbert TJ, Aboudrare A, Chaloud D, Magnan N, Nash M (2011) Booming markets for Moroccan argan oil appear to benefit some rural households while threatening the endemic argan forest. Proc Natl Acad Sci USA 108: 13963-13968

Macdonald A, Gutteridge R (2012) Effects of take-all (*Gaeumannomyces graminis* var. *tritici*) on crop N uptake and residual mineral N in soil at harvest of winter wheat. Plant Soil 350: 253-260

Madzak C, Otterbein L, Chamkha M, Moukha S, Asther M, Gaillardin C, Beckerich JM (2005) Heterologous production of a laccase from the basidiomycete *Pycnoporus cinnabarinus* in the dimorphic yeast *Yarrowia lipolytica*. FEMS Yeast Res 5 (6-7): 635-646

Márczy JS, Németh AS, Samu Z, Háger-Veress A, Szajáni B (2002) Production of hexanal from hydrolyzed sunflower oil by lipoxygenase and hydroperoxid lyase enzymes. Biotechnol Lett 24(20): 1673-1675

Marques G, Molina S, Babot ED, Lund H, del Rio JC, Gutierrez A (2011) Exploring the potential of fungal manganese-containing lipoxygenase for pitch control and pulp delignification. Bioresour Technol 102(2): 1338-1343

Maurer SC, Schmid RD (2005) Biocatalysts for the epoxidation and hydrolylation of fatty acids and fatty alcohols. In: Handbook of industrial biocatalysis, C.T. Hou (ed.) Taylor & Francis 4-4

McAlexander MA, Myers AC, Undem BJ (1998) Inhibition of 5-lipoxygenase diminishes neurally evoked tachykinergic contraction of guinea pig isolated airway. J Pharmacol Exp Ther 285(2): 602-607

Mène-Saffrané L, Esquerré-Tugayé MT, Fournier J (2003) Constitutive expression of an inducible lipoxygenase in transgenic tobacco decreases susceptibility to *Phytophthora parasitica* var. *nicotianae*. Mol Breed 12(4): 271-282

Metzger JO, Bornscheuer U (2006) Lipids as renewable resources: current state of chemical and biotechnological conversion and diversification. Appl Microbiol Biotechnol 71(1): 13-22

Meyer V, Wu B, Ram AF (2011) *Aspergillus* as a multi-purpose cell factory: current status and perspectives. Biotechnol Lett 33(3): 469-476

Minor W, Steczko J, Stec B, Otwinowski Z, Bolin JT, Walter R, Axelrod B (1996) Crystal structure of soybean lipoxygenase L-l at 1.4 angstrom resolution. Biochemistry 35(33): 10687-10701

Missall TA, Lodge JK (2005) Thioredoxin reductase is essential for viability in the fungal pathogen *Cryptococcus neoformans*. Eukaryot Cell 4(2): 487-489

Mita G, Fasano P, De Domenico S, Perrone G, Epifani F, Iannacone R, Santino A (2007) 9-Lipoxygenase metabolism is involved in the almond/*Aspergillus carbonarius* interaction. J Exp Bot 58(7): 1803-1811

Mosblech A, Feussner I, Heilmann I (2009) Oxylipins: Structurally diverse metabolites from fatty acid oxidation. Plant Physiol Biochem 47(6): 511-517

Muller B, Dean C, Schmidt C, Kuhn JC (1998) Process for the preparation of nootkatone. US Patent US5847226 A

Nakamura M, Matsumoto T, Noguchi M, Yamashita I, Noma M (1990) Expression of a cDNA encoding human 5-lipoxygenase under control of the STA1 promoter in *Saccharomyces cerevisiae*. Gene 89(2): 231-237

Nemchenko A, Kunze S, Feussner I, Kolomiets M (2006) Duplicate maize 13-lipoxygenase genes are differentially regulated by circadian rhythm, cold stress, wounding, pathogen infection, and hormonal treatments. J Exp Bot 57(14): 3767-3779

Ngo TT, Lenhoff HM (1980) A sensitive and versatile chromogenic assay for peroxidase and peroxidase-coupled reactions. Anal Biochem 105: 389–397

Nierman WC, Pain A, Anderson MJ, Wortman JR, Kim HS, Arroyo J, Berriman M, Abe K, Archer DB, Bermejo C, Bennett J, Bowyer P, Chen D, Collins M, Coulsen R, Davies R, Dyer PS, Farman M, Fedorova N, Feldblyum TV, Fischer R, Fosker N, Fraser A, García JL, García MJ, Goble A, Goldman GH, Gomi K, Griffith-Jones S, Gwilliam R, Haas B, Haas H, Harris D, Horiuchi H, Huang J, Humphray S, Jiménez J, Keller N, Khouri H, Kitamoto K, Kobayashi T, Konzack S, Kulkarni R, Kumagai T, Lafon A, Latgé JP, Li W, Lord A, Lu C, Majoros WH, May GS, Miller BL, Mohamoud Y, Molina M, Monod M, Mouya I, Mulligan S, Murphy L, O'Neil S, Paulsen I, Peñalva MA, Pertea M, Price C, Pritchard BL, Quail MA, Rabbinowitsch E, Rawlins N, Rajandream MA, Reichard U, Renauld H, Robson GD, Rodriguez de Córdoba S, Rodríguez-Peña JM, Ronning CM, Rutter S, Salzberg SL, Sanchez M, Sánchez-Ferrero JC, Saunders D, Seeger K, Squares R, Squares S, Takeuchi M, Tekaia F, Turner G, Vazquez de Aldana CR, Weidman J, White O, Woodward J, Yu JH, Fraser C Galagan JE, Asai K, Machida M, Hall N, Barrell B, Denning DW (2005) Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. Nature 438(7071): 1151-1156

Nishikawa N, Yamada K, Matsutani S, Higo M, Kigawa H, Inagaki T (1995) Structures of ozonolysis products of methyl oleate obtained in a carboxylic acid medium. J Am Oil Chem Soc 72(6): 735–740

Noordermeer MA, van der Goot W, van Kooij AJ, Veldsink JW, Veldink GA, Vliegenthart JFG (2002) Development of a biocatalytic process for the production of C6-aldehydes from vegetable oils by soybean lipoxygenase and recombinant hydroperoxide lyase. J Agric Food Chem 50(15): 4270-4274

Norin T (2007) Semiochemicals for insect pest management. Pure Appl Chem 79(12) 2129-2136

Nothaft H, Szymanski CM (2010) Protein glycosylation in bacteria: sweeter than ever. Nat Rev Microbiol 8(11): 765-778

Nyyssölä A, Heshof R, Haarmann T, Eidner J, Westerholm-Parvinen A, Langfelder K, Kruus K, de Graaff LH, Buchert J (2012) Methods for identifying lipoxygenase producing microorganisms on agar plates. AMB Express 2(1): 17. doi: 10.1186/2191-0855-2-17

Oldham ML, Brash AR, Newcomer ME (2005) Insights from the X-ray crystal structure of coral 8R-lipoxygenase: calcium activation via a C2-like domain and a structural basis of product chirality. J Biol Chem 280(47): 39545-39552

Oliw EH (2002) Plant and fungal lipoxygenases. Prostag Oth Lipid M 68-69: 313-323

Omar MN, Moynihan H, Hamilton R (2003) Scaling-up the production of 13S-hydroxy-9Z,11E-octadecadienoic acid (13S-HODE) through chemoenzymatic technique. Bull Korean Chem Soc 24(3): 397-399

Page RDM (1996) TreeView: An application to display phylogenetic trees on personal computers. Comput Appl Biosci 12 (4): 357-358

Papagianni M (2007) Advances in citric acid fermentation by *Aspergillus niger*: biochemical aspects, membrane transport and modeling. Biotechnol Adv 25(3): 244-263

Passorn S, Laoteng K, Rachadawong S, Tanticharoen M, Cheevadhanarak S (1999) Heterologous expression of Mucor rouxii delta(12)-desaturase gene in Saccharomyces cerevisiae. Biochem Biophys Res Commun 263: 47-51

van Peij NN, Brinkmann J, Vrsanská M, Visser J, de Graaff LH (1997) beta-Xylosidase activity, encoded by *xlnD*, is essential for complete hydrolysis of xylan by *Aspergillus niger* but not for induction of the xylanolytic enzyme spectrum. Eur J Biochem 245(1): 164-173

Penttilä M, Nevalainen H, Ratto M, Salminen E, Knowles J (1987) A versatile transformation system for the cellulolytic filamentous fungus *Trichoderma reesei*. Gene 61 (2): 155-164. doi: 10.1016/0378-1119(87)90110-7

Permyakova MD, Trufanov VA (2011) Effect of soybean lipoxygenase on baking properties of wheat flour. Appl Biochem Microbiol 47(3): 315-320

Peters-Golden M (1998) Cell biology of the 5-lipoxygenase pathway. Am J Respir Crit Care Med 157(6): S227-S232

Pidgeon GP, Lysaght J, Krishnamoorthy S, Reynolds JV, O'Byrne K, Nie D, Honn KV (2007) Lipoxygenase metabolism: roles in tumor progression and survival. Cancer Metast Rev 26(3-4): 503-524

Plewako H, Holmberg K, Oancea I, Rak S. 2006. Increased expression of lipoxygenase enzymes during pollen season in nasal biopsies of pollen-allergic patients. Allergy 61(6): 725-730

Pontecorvo G, Roper JA, Hemmons LM, Macdonald KD, Bufton AW (1953) The genetics of *Aspergillus nidulans*. Adv Genet 5: 141–238

Prost I, Dhondt S, Rothe G, Vicente J, Rodriguez MJ, Kift N, Carbonne F, Griffiths G, Esquerré-Tugayé MT, Rosahl S, Castresana C, Hamberg M, Fournier J (2005) Evaluation of the antimicrobial activities of plant oxylipins supports their involvement in defense against pathogens. Plant Physiol 139(4): 1902-1913

Rancé I, Fournier J, Esquerré-Tugayé MT (1998) The incompatible interaction between *Phytophthora parasitica* var. *nicotianae* race 0 and tobacco is suppressed in transgenic plants expressing antisense lipoxygenase sequences. Proc Natl Acad Sci USA 95(11): 6554-6559

Reinbothe C, Springer A, Samol I, Reinbothe S (2009) Plant oxylipins: role of jasmonic acid during programmed cell death, defence and leaf senescence. FEBS J 276(17): 4666-4681

Reynolds JC, Last DJ, McGillen M, Nijs A, Horn AB, Percival C, Carpenter LJ, Lewis AC (2006) Structural analysis of oligomeric molecules formed from the reaction products of oleic acid ozonolysis. Environ Sci Technol 40(21): 6674-6681

Riccioni G, Zanasi A, Vitulano N, Mancini B, D'Orazio N (2009) Leukotrienes in atherosclerosis: new target insights and future therapy perspectives. Mediators Inflamm 2009: 737282

Robinson DS, Wu Z, Domoney C, Casey R (1995) Lipoxygenases and the quality of foods. Food Chem 54(1): 33-43

de Roos AL, van Dijk AA, Folkertsma B (2006) Bleaching of dairy products. US Patent US 2006/0127533 A1

Sali A, Blundell TL (1993) Comparative protein modelling by satisfaction of spatial restraints. J Mol Bio 234: 779-815

Salmon S, Shi C, Liu J (2004) Treatment of fabrics, fibers or yarns. PCT Publication WO 2004/05 9074 A1

Santano E, Pinto MD, Macias P (2002) Xenobiotic oxidation by hydroperoxidase activity of lipoxygenase immobilized by adsorption on controlled pore glass. Enzyme Microb Technol 30(5): 639-646

Sapan CV, Lundblad RL, Price NC (1999) Colorimetric protein assay techniques. Biotechnol Appl Bioc 29: 99-108

Schieber A, Stintzing FC, Carle R (2001) By-products of plant food processing as a source of functional compounds - recent developments. Trends Food Sci Technol 12: 401-413

Schwarz K, Walther M, Anton M, Gerth C, Feussner I, Kuhn H (2001) Structural basis for lipoxygenase specificity - Conversion of the human leukocyte 5-lipoxygenase to a 15-lipoxygenating enzyme species by site-directed mutagenesis. J Biol Chem 276 (1): 773-779

Sekhar Rao KC, Karanth NG, Sattur AP (2005) Production of nigerloxin, an enzyme inhibitor and a free radical scavenger, by *Aspergillus niger* using solid state fermentation. Process Biochem 40(7): 2517-2522

Seo H-S, Endo Y, Fujimoto K (1999) Kinetics for the autoxidation of conjugated linoleic acid. Biosci Biotechnol Biochem 63: 2009–2010

Serhan CN, Arita M, Hong S, Gotlinger K (2004) Resolvins, docosatrienes, and neuroprotectins, novel omega-3-derived mediators, and their endogenous aspirin-triggered epimers. Lipids 39(11): 1125-1132

Shetty RP, Endy D, Knight TF (2008) Engineering BioBrick vectors from BioBrick parts. J Biol Eng 2(5)

Shirano Y, Shibata D (1990) Low temperature cultivation of Escherichia coli carrying a rice lipoxygenase L-2 cDNA produces a soluble and active enzyme at a high level. FEBS Lett 271(1-2): 128-130

Shureiqi I, Lippman SA (2001) Lipoxygenase modulation to reverse carcinogenesis. Cancer Res 61(17): 6307-6312

Skrzypczak-Jankun E, Amzel LM, Kroa BA, Funk MO (1997) Structure of soybean lipoxygenase L3 and a comparison with its L1 isoenzyme. Proteins 29(1): 15-31

Sloane DL, Leung R, Craik CS, Sigal E (1991) A primary determinant for lipoxygenase positional specificity. Nature 354(6349): 149-152

Sloothaak J, Schilder M, Schaap PJ, de Graaff LH (2014) Overexpression of the *Aspergillus niger* GatA transporter leads to preferential use of D-galacturonic acid over D-xylose. AMB Express 4(1): 66. doi: 10.1186/s13568-014-0066-3Steczko J, Donoho GA, Dixon JE, Sugimoto T, Axelrod B (1991) Effect of ethanol and low-temperature culture on expression of soybean lipoxygenase L-1 in *Escherichia coli*. Protein Expr Purif 2(2-3): 221-227

van der Straat L, Vernooij M, Lammers M, van den Berg W, Schonewille T, Cordewener J, van der Meer I, Koops A, de Graaff LH (2014) Expression of the *Aspergillus terreus* itaconic acid biosynthesis cluster in *Aspergillus niger*. Microb Cell Fact 13(11). doi: 10.1186/1475-2859-13-11

Stratton J, Chiruvolu V, Meagher M (1998) High cell-density fermentation. Methods Mol Biol 103: 107-120

Su C, Oliw EH (1998) Manganese lipoxygenase - Purification and characterization. J Biol Chem 273 (21): 13072-13079

Suda I, Hajika M, Nishiba Y, Furuta S, Igita K (1995) Simple and rapid method for the selective detection of individual lipoxygenase isozymes in soybean seeds. J Agric Food Chem 43: 742–747

Sugio A, Takagi S (2002) Lipoxygenase. PCT Publication WO 02/06114 A1

Takeno S, Sakuradani E, Murata S, Inohara-Ochiai M, Kawashima H, Ashikari T, Shimizu S (2005) Molecular evidence that the rate-limiting step for the biosynthesis of arachidonic acid in *Mortierella alpina* is at the level of an elongase. Lipids 40: 25-30

Tang S, Bhatia B, Maldonado CJ, Yang P, Newman RA, Liu J, Chandra D, Traag J, Klein RD, Fischer SM, Chopra D, Shen J, Zhau HE, Chung LW, Tang DG (2002) Evidence that arachidonate 15-lipoxygenase 2 is a negative cell cycle regulator in normal prostate epithelial cells. J Biol Chem 277(18): 16189-16201

Terp N, Göbel C, Brandt A, Feussner I (2006) Lipoxygenases during *Brassica napus* seed germination. Phytochem 67 (18): 2030-2040. doi: 10.1016/j.phytochem.2006.06.023

Toledo L, Masgrau L, Lluch JM, Gonzalez-Lafont A (2011) Substrate binding to mammalian 15-lipoxygenase. J Comput Aid Mol Des 25(9): 825-835

Tomchick DR, Phan P, Cymborowski M, Minor W, Holman TR (2001) Structural and functional characterization of second-coordination sphere mutants of soybean lipoxpgenase-1. Biochem 40 (25): 7509-7517

Tsitsigiannis DI, Keller NP (2007) Oxylipins as developmental and host-fungal communication signals. Trends Microbiol 15 (3): 109-118

Tsitsigiannis DI, Kowieski TM, Zarnowski R, Keller NP (2005) Three putative oxylipin biosynthetic genes integrate sexual and asexual development in *Aspergillus nidulans*. Microbiology 151(6): 1809-1821. doi: 10.1099/mic.0.27880-0

Tsitsigiannis DI, Kunze S, Willis DK, Feussner I, Keller NP (2005) *Aspergillus* infection inhibits the expression of peanut 13S-HPODE-forming seed lipoxygenases. Mol Plant Microbe In 18(10): 1081-1089

Tsitsigiannis DI, Zarnowski R, Keller NP (2004) The lipid body protein, PpoA, coordinates sexual and asexual sporulation in *Aspergillus nidulans*. J Biol Chem 279(12): 11344-11353

Uchiyama T, Miyazaki K (2009) Functional metagenomics for enzyme discovery: challenges to efficient screening. Curr Opin Biotechnol 20: 616–622

Vance RE, Hong S, Gronert K, Serhan CN, Mekalanos JJ (2004) The opportunistic pathogen *Pseudomonas aeruginosa* carries a secretable arachidonate 15-lipoxygenase. Proc Natl Acad Sci USA America 101(7): 2135-2139

Vanholme B, Desmet T, Ronsse F, Rabaey K, Van Breusegem F, De Mey M, Soetaert W, Boerjan W (2013) Towards a carbon-negative sustainable bio-based economy. Front Plant Sci 4: 174

133

Vasudevan P, Tandon M (2010) Antimicrobial properties of iodine based products. J Sci Ind Res 69: 376–383

Vellosillo T, Aguilera V, Marcos R, Bartsch M, Vicente J, Cascon T, Hamberg M, Castresana C (2013) Defense activated by 9-lipoxygenase-derived oxylipins requires specific mitochondrial proteins. Plant Physiol 161(2): 617-627

Vidal-Mas J, Busquets M, Manresa A (2005) Cloning and expression of a lipoxygenase from *Pseudomonas aeruginosa* 42A2. Anton Leeuw Int J G 87(3): 245-251

Villafuerte Romero M, Barrett DM (1997) Rapid methods for lipoxygenase assay in sweet corn. J Food Sci 62: 696–700

Villaverde JJ, Santos SAO, Simoes MMQ, Neto CP, Domingues MRM, Silvestre AJD (2011) Analysis of linoleic acid hydroperoxides generated by biomimetic and enzymatic systems through an integrated methodology. Ind Crop Prod 34(3): 1474-1481

Villaverde JJ, Santos SAO, Maciel E, Simoes MMQ, Neto CP, Domingues MRM, Silvestre AJD (2012) Formation of oligomeric alkenylperoxides during the oxidation of unsaturated fatty acids: an electrospray ionization tandem mass spectrometry study. J Mass Spectrom 47(2): 163-172

Villaverde JJ, van der Vlist V, Santos SAO, Haarmann T, Langfelder K, Pirttimaa M, Nyyssölä A, Jylha S, Tamminen T, Kruus K. de Graaff LH, Neto CP, Simoes MMQ, Domingues MRM, Silvestre AJD, Eidner J, Buchert J (2013a). Hydroperoxide production from linoleic acid by heterologous *Gaeumannomyces graminis tritici* lipoxygenase: optimization and scale-up. Chem Eng J 217: 82-90

Villaverde JJ, Santos SAO, Haarmann T, Neto CP, Simoes MMQ, Domingues MRM, Silvestre AJD (2013b). Cloned *Pseudomonas aeruginosa* lipoxygenase as efficient approach for the clean conversion of linoleic acid into valuable hydroperoxides. Chem Eng J 231: 519-525

de Vries RP, Visser J (2001) *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. Microbiol Mol Biol Rev 65(4): 497-522

Waché Y, Bosser-De Ratuld A, Belin JM (2006) Dispersion of β-carotene in processes of production of β-ionone by cooxidation using enzyme-generated reactive oxygen species. Process Biochem 41(11): 2337-2341

Wadman MW, de Vries RP, Kalkhove SI, Velding GA, Vliegenthart JF (2009) Characterization of oxylipins and dioxygenase genes in the asexual fungus *Aspergillus niger*. BMC Microbiol 9(59). doi: 10.1186/1471-2180-9-59

Walther M, Anton M, Wiedmann M, Fletterick R, Kuhn H (2002) The N-terminal domain of the reticulocyte-type 15-lipoxygenase is not essential for enzymatic activity but contains determinants for membrane binding. J Biol Chem 277(30): 27360-27366.

Walther M, Hofheinz K, Vogel R, Roffeis J, Kuehn H (2011) The N-terminal beta-barrel domain of mammalian lipoxygenases including mouse 5-lipoxygenase is not essential for catalytic activity and membrane binding but exhibits regulatory functions. Arch Biochem and Biophys 516(1): 1-9

Walther M, Roffeis J, Jansen C, Anton M, Ivanov I, Kühn H (2009) Structural basis for pH-dependent alterations of reaction specificity of vertebrate lipoxygenase isoforms. Biochim Biophys Acta 1791: 827-835

Waslidge NB, Hayes DJ (1995) A colorimetric method for the determination of lipoxygenase activety suitable for use in high throughput assay format. Anal Biochem 231(1): 354-358

Wennman A, Oliw EH (2013) Secretion of two novel enzymes, manganese 9S-lipoxygenase and epoxy alcohol synthase, by the rice pathogen *Magnaporthe salvinii*. J Lipid Res 54(3): 762-775. doi: 10.1194/jlr.M033787

Wennman A, Oliw EH, Karkehabadi S (2014) Crystallization and preliminary crystallographic analysis of manganese lipoxygenase. Acta Crystallogr F Struct Biol Commun 70: 522-525

Whitehead IM, Muller BL, Dean C (1995) Industrial use of soybean lipoxygenase for the production of natural green note flavour compounds. Cereal Foods World 40: 193–197

Williams DC, Lim MH, Chen AO, Pangborn RM, Whitaker JR (1986) Blanching of vegetables for freezing – which indicator enzyme to choose. Food Technol 40: 130–140

Xu S, Mueser TC, Marnett LJ, Funk MO Jr (2012) Crystal structure of 12-lipoxygenaseatalytic-domain-inhibitor complex identifies a substrate-binding channel for catalysis. Structure 20(9): 1490-1497

Yoshimoto T, Yoshitaka T (2002) Arachidonate 12-lipoxygenases. Prostag Oth Lipid M 68: 245-262

Zelena K, Krings U, Berger RG (2012) Functional expression of a valencene dioxygenase from *Pleurotus sapidus* in *E. coli*. Bioresour Technol 108: 231-239

Zhang C, Tao T, Ying Q, Zhang D, Lu F, Bie X, Lu Z (2012) Extracellular production of lipoxygenase from Anabaena sp. PCC 7120 in Bacillus subtilis and its effect on wheat protein. Appl Microbiol Biotechnol 94(4): 949-958

Zhang X, Nguyen D, Paice MG, Tsang A, Renaud S (2007) Degradation of wood extractives in thermo-mechanical pulp by soybean lipoxygenase. Enzyme Microb Technol 40(4): 866-873

Zheng Y, Boeglin WE, Schneider C, Brash AR. (2008) A 49-kDa mini-lipoxygenase from *Anabaena* sp PCC 7120 retains catalytically complete functionality. J Biol Chem 283(8): 5138-5147

Zoia L, Perazzini R, Crestini C, Argyrpoulos DS (2011) Understanding the radical mechanism of lipoxygenases using 31P NMR spin trapping. Bioorg Med Chem 19(9): 3022-3028

Summary
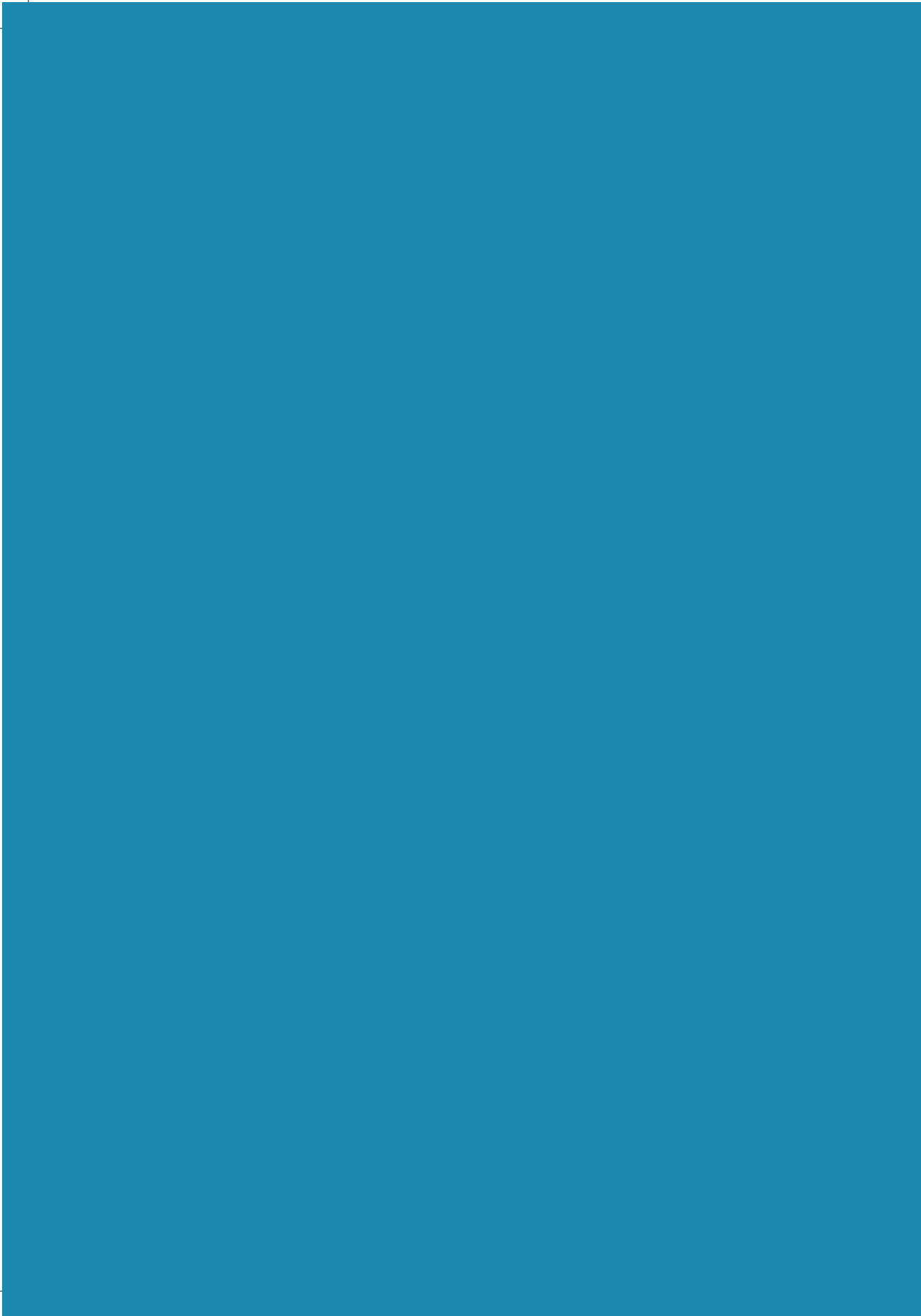
Many challenges lie ahead in using LOXs as tools in industrial oleochemistry. One of these challenges is the supply of PUFAs. Although we are moving towards a biobased economy where second and third generation biomass is taking a leading role, it is still faster and cheaper to use first generation biomass. Industrialization of microbial oils is a good alternative to supply the demand of PUFAs. Another challenge is the production of heterologous LOX in sufficient quantities. Since the last decade this problem is being tackled and more research is being done in heterologous expression of LOXs. The LOX with the highest potential so far is the secreted *Pseudomonas aeruginosa* LOX produced in *Escherichia coli*. During this thesis research different *lox* genes were tried for heterologous production of LOX using different *Aspergillus niger* and *Aspergillus nidulans* strains as expression hosts. These LOXs were identified as discussed in **Chapter 3** and **Chapter 6**. Unfortunately, heterologous production in sufficient quantities was unsuccessful using these expression hosts as discussed in **Chapter 5** and **Chapter 6**. Since production of *Gaeumannomyces graminis* LOX was successful in *Trichoderma reesei*, as discussed in **Chapter 4**, the production of polymers used for bioplastics could be demonstrated in this ERA-NOEL project anyway. Therefore this thesis shifted its focus on resolving the question of the difficulties in the heterologous expression of LOX in different *Aspergillus* species. **Chapter 5** is the result of a systematic approach to analyze different aspects of *G. graminis* LOX expression in *A. nidulans*. **Chapter 2** shows that heterologous expression of extracellular fungal LOX can be performed using *T. reesei* and *Pichia pastoris* as production hosts, and *E. coli* can be used for the production of intracellular LOXs of plant, mammal, bacterial, and fungal origin. As shown in **Chapter 2**, *E. coli* is not very efficient in the production of heterologous LOX due to the formation of inclusion bodies and low induction temperature necessary for production. The use of *Aspergillus oryzae* can be exploited further in the heterologous production of LOXs. Due to the choice of using *A. niger* and *A. nidulans* as expression hosts, this expression host was not exploited for its potential. The last challenge is to synthetically engineer LOX to broaden its use in industry. In this way more building blocks for chemicals can be synthetically produced and more products based on LOX origin can be made. Therefore, LOX can be a world-wide game-changing enzyme in a biobased economy as its use can decrease the demand for petroleum-based products.

Appendices

Nederlandse samenvatting
Dankwoord
About the author
List of publications
Overview training activities

Nederlandse samenvatting

De maatschappij beweegt zich richting een biobased economy; een economie waarin chemicaliën, materialen en energie wordt gemaakt uit biomassa in plaats van fossiele brandstoffen. Voor deze transitie is het noodzakelijk innovatieve oplossingen te bedenken om biomassa zo efficiënt mogelijk te gebruiken. Een voorbeeld van een transitie naar een biobased economy is het produceren van plastic. Plastic is een van de meeste gebruikte stoffen in ons huishouden. Hoewel het meeste nog steeds van petroleum gemaakt wordt, komt er ook meer biologisch (afbreekbaar) plastic op de markt.

Een van de biobased oplossingen in de productie van plastic is het gebruik van enzymen in de industrie. Enzymen voor industriële toepassingen worden geïsoleerd uit bijvoorbeeld planten en dieren. Maar ze kunnen ook geproduceerd worden door genetisch gemodificeerde micro-organismen, zoals bacteriën en schimmels. Deze gastheren zijn vele malen efficiënter in het produceren van enzymen dan via de natuurlijke weg. Schimmels leven van dood organisch materiaal, zoals plantafval, en is bedoeld om deze biomassa om te zetten voor energie en de groei van de schimmel. Deze energie en bouwstoffen kunnen tevens gebruikt worden voor het produceren van enzymen.

143

Lipoxygenase is een enzym dat allerlei op onverzadigde vetzuur gebaseerde producten kan maken, zoals lijm, coatings en plastics. Een aantal andere toepassingen is het verwijderen van deeltjes tijdens papierproductie, bleekmiddel voor brood en productie van smaakstoffen. Dit doet het enzym door peroxide, een OOH-groep, aan het onverzadigde vetzuur te koppelen. Hierdoor wordt het onverzadigde vetzuur reactief en kan het uiteen vallen in kleinere moleculen door chemische reacties. Deze moleculen kunnen gebruikt worden als bouwstof voor plastics. Door de locatie van de dubbele binding in onverzadigde vetzuren, zoals in de welbekende omega 3 vetzuren uit vis en de omega 6 vetzuren uit plantaardige olie, kunnen er verschillende bouwmoleculen gemaakt worden wat ander soort plastic oplevert. Echter, voor deze toepassingen tot uitvoering gebracht kunnen worden, is het noodzakelijk om het enzym in grote hoeveelheden te kunnen produceren. De biologische functie en toepassingen van lipoxygenase alsmede de productie van plastic met behulp van dit enzym is beschreven in **Hoofdstuk 2**.

Voordat productie van lipoxygenase in een schimmel kan plaatsvinden, moet er eerst een aantal keuzes gemaakt worden. Er moet een goede gastheer gekozen worden voor de productie van het enzym en er moet bepaald worden welk lipoxygenase

enzym er gebruikt gaat worden. In **Hoofdstuk 3** is onderzocht welke lipoxygenases interessant zijn voor de industrie. Met behulp van bioinformatische technieken is er een nieuwe groep lipoxygenases ontdekt die bestaan in schimmels. Deze groep lipoxygenases worden uitgescheiden door schimmels, wat ze interessant maakt voor de industrie. Dit omdat deze enzymen stabiel zijn, aangezien ze hun functie buiten de cel hebben en opgewassen moeten zijn tegen deze vijandelijke omgeving. In dit proefschrift is de lipoxygenase van de schimmel *Gaeumannomyces graminis* als model gebruikt. Dit enzym komt voor in de nieuw ontdekte groep enzymen.

Hoofdstuk 4 beschrijft een aantal technieken waarmee we snel en goedkoop de juiste gastheer kunnen kiezen voor productie. Deze technieken zijn toegepast op de schimmels *Trichoderma reesei*, *Pichia pastoris* en *Aspergillus nidulans*; allen zijn bekende schimmels in de industrie voor de productie van enzymen en chemische stoffen. Voor deze schimmels is aangetoond dat de "kalium-iodide" techniek toegepast kan worden.

Omdat het enzym niet goed geproduceerd wordt in *A. nidulans,* is er onderzocht wat de oorzaak hiervan is. Dit onderzoek wordt beschreven in **Hoofdstuk 5**. *A. nidulans* dat lipoxygenase produceert, verandert het fenotype van de schimmel; de schimmel krijgt een andere kleur. Er is aangetoond dat het lipoxygenase-DNA in de schimmel aanwezig is en geschreven wordt naar mRNA. Daarna is gekeken of het enzym vertaald wordt en waar het enzym dan is. Door in de cellen van de schimmel te kijken is er ontdekt dat er een kleine hoeveelheid lipoxygenase geproduceerd wordt en in de cel zit. Ook werden er proteases gedetecteerd. Proteases zijn enzymen die andere enzymen afbreken en deze hierdoor onschadelijk maken. Daarom is er geconcludeerd dat *A. nidulans* geen goed productiesysteem is voor lipoxygenase.

Tot slot wordt er in de **Discussie** beschreven hoe we verder kunnen gaan om dit enzym in een biobased economy door te laten dringen. Een aantal knelpunten zal moeten worden opgelost, zoals de hoeveelheid lipoxygenase die geproduceerd kan worden. Deze hoeveelheid zal omhoog moeten om het proces economisch rendabel te maken voor de industrie. De meest succesvolle techniek tot dusver is de productie van de bacteriële lipoxygenase van *Pseudomonas aeruginosa* in de bacterie *Escherichia coli*. Het is bekend dat dit enzym meerdere vetzuren kan bewerken, wat het interessant maakt voor de industrie. Echter, de mogelijkheden van bacteriële lipoxygenases zullen geëxploiteerd moeten worden. Ook zijn de benodigde stoffen, de onverzadigde vetzuren, niet in overmaat aanwezig. Het is niet de bedoeling dat deze techniek moet concurreren met de voedselindustrie. De vetzuren zullen dus niet uit planten, maar op een andere manier verkregen moeten worden. Een oplossing

is gebruik maken van de vetproducerende schimmels als *Yarrowia lipolytica* en *Mortierella alpina*. Deze schimmels kunnen in grote hoeveelheden onverzadigde vetzuren produceren die gebruikt kunnen worden in de lipoxygenase-gebaseerde biobased economy.

Er zal nog een aantal obstakels overwonnen moeten worden, maar dit proefschrift levert een proof-of-principle dat lipoxygenase gebruikt kan worden in een biobased economy. De volgende stap is meer onderzoek doen naar andere lipoxygenases en deze enzymen integreren in een biobased economy.

145

Dankwoord

En dan nu het gedeelte wat de meeste mensen waarschijnlijk wèl zullen lezen. Ik had deze uitdaging voor geen goud willen missen. Als ik denk hoe ik binnenkwam en hoe ik nu wegloop; een wereld van verschil. Dat is niet mogelijk geweest zonder de hulp van vele mensen. Aan iedereen die ik vergeten ben: jullie worden bedankt!

Allereerst wil ik mijn moeder bedanken. Ik citeer: "Je moet mij als eerste bedanken, want zonder mij was dit helemaal niet mogelijk geweest". Om deze reden wil ik ook mijn vader bedanken. En uiteraard ook mijn zus. Het heeft me altijd goed gedaan wanneer jullie zeiden: "We snappen er niets van, maar je hebt mooie plaatjes". Bedankt voor al jullie steun! Het is niet altijd makkelijk dat jullie aan de andere kant van Nederland wonen, maar vanaf nu heb ik eindelijk meer tijd om met jullie door te brengen.

Ik wil graag mijn dagelijks begeleider Leo de Graaff bedanken voor al zijn steun en wijze raad tijdens mijn PhD. Leo, we zijn allebei eigenwijze mensen, waarbij je volgens mij vaak dacht: "(zucht) Je komt er nog wel achter". Ik ben erg blij dat je me de kans hebt gegeven om soms het wiel opnieuw uit te vinden, maar me aan de lijn hield wanneer ik als een dolle hond mijn focus verloor op wat belangrijk is. Zonder jou was dit resultaat er nooit gekomen. Ik heb zeer genoten van onze samenwerking en tripjes door Europa. Je hebt me goed geleerd hoe de wetenschappelijke wereld er achter de schermen uitziet, iets wat ik nu nodig heb voor mijn nieuwe baan. Hopelijk komen we elkaar nog tegen in deze kleine schimmelwereld. Hoewel jij nooit meer met lipoxygenases wilt werken, gooi ik de handdoek nog niet in de ring. Hoezo eigenwijs? Het zal wel iets Zeeuws zijn.

Willem de Vos, bedankt voor je hulp in het bewerkstelligen van dit proefschrift. Je hebt me als een raket door de laatste loodjes heen geloodst. Ook je ideeën en commentaar op dit proefschrift heeft me de motivatie gegeven om op mijn vakantiedagen toch weer om 8u 's ochtends achter de computer te zitten om het af te schrijven. De gesprekken waren altijd erg fijn. Je hoeft maar een halve zin te zeggen, of je hebt al precies door wat er moet gebeuren.

I would like to thank all the people that helped me during this European project: Thomas Haarmann, Trine Dalsgaard, Kim Langfelder, Juan José Villaverde, Armando Silvestre, Antti Nyyssölä, Sirpa Jylhä, and Johanna Buchert. I had a pleasant

cooperation with you all during this project. I hope you will all do well in the future and hopefully we meet again.

Ook Anja en Carolien wil ik graag bedanken voor het aanhoren van mijn klaagzangen en het altijd snel willen helpen met formulierverzoekjes. Ik heb er groot respect voor hoe jullie de bende op de vakgroepen op rolletjes laten lopen. Zonder jullie zou alles in het water vallen. Hopelijk houden jullie al die maffe wetenschappers op de afdeling nog lang in het gareel.

Laura, jou wil ik ook nadrukkelijk bedanken. Het viel me soms niet mee om op te moeten boksen tegen een collega, waarbij zowat alles in één keer lukt met haar magische handjes. Maar ik heb het altijd als zeer prettig ervaren hoe wij konden discussiëren over alledaagse (PhD) problemen en zaken. Ik ben er van overtuigd dat je het ver gaat schoppen in de wetenschappelijke wereld. Ik kan nog veel leren van jouw organisatievermogen, zowel digitaal als met studenten. Ik wens je heel veel succes met de laatste loodjes van je proefschrift! Hopelijk ligt er in de toekomst nog een mooie samenwerking op ons te wachten.

Juanan, what a crazy world we live in. Years ago I met you in Spain where a weird unexperienced Dutchy suddenly lived in your house. A few months later you were living in the Netherlands and you worked in the same office as I did. Talking about a small world. I want to thank you for your daily help in the lab and your always critical view on my scientific experiments. Also I want to thank you for your help in the final chapter of this thesis. I am very happy we published a paper together. To me this is the cherry on the pie of the years I worked with you. I wish you the best of luck in finding a new exciting job, if you did not already find one.

Dorett and Jasper, to me you will always be "the new students". I want to thank you for the good discussions in the office, fun in the lab, and your help during some of my experiments. I think you both will do fine during your PhD and I wish you the best luck in the future. Together with Laura and Juanan, you guys are a good team!

Tom Schonewille, jou wil ik graag bedanken voor je hulp in het lab. Ik waardeer je enorme enthousiasme voor wetenschap, waardoor je een belangrijke toevoeging bent voor het team. Hopelijk kunnen we in de toekomst nog brainstormen over alledaagse problemen die optreden tijdens fermentatieprocessen. Maar niet op dinsdag, want dan heb je papa-dag.

In het laatste jaar verhuisde ik op geheel vrijwillige basis van kantoor. En wat een keuze wat dat! Philippe en Detmer, toen ik bij jullie op de kamer kwam, ging het ineens

allemaal een stuk beter met mijn onderzoek. Nu zouden we allemaal graag willen concluderen dat dat door de verhuizing komt, maar dat zou een overenthousiaste conclusie zijn. Desalniettemin wil ik jullie bedanken voor jullie leuke gesprekken en fijne schwung in het kantoor. Philippe, heb je het 5S-systeem al doorgevoerd? En Detmer, als je nog leuke schimmels opduikt uit de diepzee, bel me. Voor jou is het afval, voor mij een schatkamer.

Daaraan gekoppeld wil ik kamer 0.022 bedanken. Het was altijd leuk televisiekijken hoe Elleke moest samenwerken met twee losgeslagen mannen: Tijn en Tom. Het klinkt bijna als Sjors en Sjimmie. Elleke, ik wens je veel succes met het afronden van je PhD. Ik lees graag het resultaat, want ik vind dat je een erg leuk onderwerp hebt. Tom, ik weet zeker dat ik je nog tegenkom in de toekomst. Het fermentatiewereldje is een kleine, maar ik ben erg blij dat daar iemand is die graag een goed biertje drinkt. Tijn, je bent en blijft een vervelende Ajacied. Tegen jou wil ik zeggen "hand in hand", want je poot werkt niet echt mee. Hopelijk heb je van me geleerd dat je altijd achter je club moet blijven staan en niet halverwege het seizoen uit protest opgeeft. Maar ja, je kunt nu eenmaal niet de aard van het beestje veranderen.

Sven, Martin, Teun, Peer, Bas, Michael, Bram, and Edze. I have had a very good time with you guys during my time at Microbiology and especially the PhD-trip in China and Japan. You made this trip unforgettable for me. The days in Shanghai were the best, where Sven almost did not arrive and the big party in the club was an eye-opener for me.

Christel, Youri, Sanne en Paul, jullie hebben me enorm geholpen in het lab. Ik hoop dat ik jullie wat lab-hacks heb kunnen leren en deze nuttig blijken in jullie toekomst. Het is niet altijd makkelijk geweest om dit lastige onderwerp te kunnen vertalen naar lesgeven. Nu zijn jullie zowat allemaal PhD-studenten met waarschijnlijk je eigen frustraties. Of niet natuurlijk. Al met al bedankt voor jullie hulp!

Nieuwe collega's bij het HAN BioCentre in Nijmegen, jullie wil ik graag bedanken voor jullie steun en begrip bij het afschrijven van dit proefschrift. Het heeft even geduurd, maar vanaf nu kan ik de volledige 100% geven. We gaan samen het HAN BioCentre eens goed op de kaart zetten!

Anne-Lotte, Carlien, Dirk-Jan, Janneke, Jenny, Katelijne, Martijn en Matthijs, bedankt voor het heerlijk los kunnen laten van de PhD-sleur, het naar huis dragen van een door neurotoxine besmette niet nader te noemen persoon, en de uien. Ik vind het allemaal maar kut. Maar dat krijg je ervan als je bij Dolly zit. Bèèèh!

149

Agnar, Bragi, Freki, Hodbrod en Nokkvi, wat een prachtige, volledig onbezonnen tijden hebben we tot nu toe gehad. De vele verhalen maken ze onbeschrijflijk, maar het toppunt was de bolderkarvakantie en de overnachting in een pas gegierd veld. Voor iedereen onbegrijpelijk, voor ons een gratis slaapplaats. Door de jaren heen zijn we een geoliede machine geworden, soms met grote frustraties, maar altijd opgelost. ODIN ODIN ODIN!

Marjolein Helder, jou wil ik graag bedanken voor je hulp in de laatste weken. Je hebt aardig wat werkdruk weggenomen door mijn papers na te lezen en je hebt me nog wat geleerd ook! Had ik dit maar eerder geweten.

Ook Dagmar Schröder - Walters wil ik graag bedanken voor het in elkaar zetten van dit boekje. Ook jij hebt me heel veel werk uit handen genomen en een prachtig resultaat afgeleverd. Ik zal je zeker aanbevelen bij iedereen die iets wil laten ontwerpen.

En als laatste wil ik graag Nanda bedanken, mijn dagelijkse steun en toeverlaat. Het zijn een paar aparte jaren geweest, waarbij ik niet altijd volledig aanwezig kon zijn. Ongelooflijk hoe je altijd het geduld en begrip kon opbrengen in deze tijden. Al jaren roep ik "Ja, na mijn PhD". Na deze zin is het dan eindelijk zover.

Ruud Heshof

151

152

About the author

Ruud Heshof was born on the 9th of June 1984 in Tholen, The Netherlands. He obtained his Gymnasium VWO diploma at RSG 't Rijks in Bergen op Zoom in 2002. Afterwards he started his study in Molecular Life Sciences at Wageningen University. After two master theses in Biochemistry and Microbiology he finished his study by an internship in València, Spain. He then graduated in the specializations Medical Research and Biological Chemistry. During his study he got fascinated by microbiology and enzymology and he wanted to continue in the field in heterologous expression of enzymes. In September 2009 he started his PhD-research focussing on heterologous expression of enzymes for industry using fungal expression hosts under the supervision of Dr. L. H. de Graaff, Prof. W. M. de Vos, and Prof. V. A. P. Martins dos Santos. He is currently working as a project leader in fermentation technology at HAN BioCentre in Nijmegen, The Netherlands.

153

List of publications

Chapter 2

Heshof R, de Graaff LH, Villaverde JJ, Silvestre AJD, Haarmann T, Dalsgaard TK, Buchert J (2015) Industrial potential of lipoxygenases. Crit Rev Biotech (Epub ahead of print) doi: 10.3109/07388551.2015.1004520

Chapter 3

Heshof R, Jylhä S, Haarmann T, Jørgensen AL, Dalsgaard TK, de Graaff LH (2014) A novel class of fungal lipoxygenases. Appl Microbiol Biotechnol 98(3): 1261-1270 doi: 10.1007/s00253-013-5392-x

Chapter 4

Nyyssölä A*, Heshof R*, Haarmann T, Eidner J, Westerholm-Parvinen A, Langfelder K, Kruus K, de Graaff L, Buchert J (2012) Methods for identifying lipoxygenase producing microorganisms on agar plates. AMB Express 2(1): 17 doi: 10.1186/2191-0855-2-17

Chapter 5

Heshof R, van Schayck JP, Tamayo-Ramos JA, de Graaff LH (2014) Heterologous expression of *Gaeumannomyces graminis* lipoxygenase in *Aspergillus nidulans*. AMB Express 4(1): 65 doi: 10.1186/s13568-014-0065-4

* Contributed equally

Overview of completed training activities

Discipline specific activities:
26th Fungal Genomics Conference. 2011. Asilomar, United States of America.
Practical training AB-enzymes. 2011. Darmstadt, Germany.
NBC-14 congress speaker. 2012. Ede, The Netherlands.
BCF Career Event Speaker. 2013. Amsterdam, The Netherlands.
Project meetings ERA-IB. 2009-2013. Aarhus University, Denmark; University of
Aveiro, Portugal; VTT, Finland; AB Enzymes, Germany; Fraunhofer Institute, Germany.
Wageningen University, The Netherlands.

General activities:
PhD competence assessment
Scientific publishing
Scientific writing
Project & Time management

Optional activities:
Advanced Bioinformatics, Wageningen University
Fungal System Biology group meetings (weekly)
Systems and Synthetic Biology seminars (monthly)
Organizing PhD excursion MIB
Participation PhD trip to China and Japan
Preparation of PhD Project Proposal
Laboratory of Microbiology PhD/PostDoc meetings (biweekly)