IDENTIFICATION, CLONING AND HETEROLOGOUS EXPRESSION OF FUNGAL VANILLYL-ALCOHOL OXIDASES

BY

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DECLARATION

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Newlande van Rooyen (1998114274)

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This thesis is dedicated to my loving parents and my brother.

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LIST OF ABBREVIATIONS

%	Percentage
^{o}C	Degrees Celsius
4EPMH	4-Ethylphenol methylenehydroxylase
16S rRNA	Small subunit ribosomal ribose nucleic acid
BLAST	Basic local alignment search tool
Bp	base pairs
cDNA	Copy deoxyribose nucleic acid
DNA	Deoxyribose nucleic acid
E.coli	*Escherichia coli*
EUGH	Eugenol hydroxylase
EUGO	Eugenol oxidase
EV	Empty vector
FAD	Flavin adenine dinucleotide
FvVAO	*Fusarium verticillioides* vanillyl-alcohol oxidase
FvVAOpara	*Fusarium verticillioides* vanillyl-alcohol oxidase paralogue
FmVAO	*Fusarium moniliforme* vanillyl-alcohol oxidase
FmutVAO	*Fusarium moniliforme* vanillyl-alcohol oxidase containing several mutations
GC	Gas chromatography
HPLC	High performance liquid chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	kilo Dalton
LB	Luria Bertoni
µl	MicroLitre
µg	Microgram
M	Molar
mg	Milligram
min	Minute
ml	Millimeter

mM	Millimolar
Mr	Molecular mass
mRNA	Messenger ribo nucleic acid
NCBI	National Centre for Biotechnology Information
Nm	nanometer
OD	Optical density
PCR	Polymerase chain reaction
Psi	Pound per square inch
PCMH	*p*-Cresol methyl hydroxylase
PsVAO	*P. simplicissimum* vanillyl- alcohol oxidase
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
S	second
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TLC	Thin layer chromatography
TMADH	Trimethylamine dehydrogenase
Tris	2-Amino-2-(hydroxymethyl)-1, 3-propandiol
UV	Ultraviolet-visible
VAO	Vanillyl-alcohol oxidase
V	volume
W	weight
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranosidehosphate

CHAPTER 1

Flavoproteins responsible for side-chain hydroxylation of *p*-alkylphenols and *p*-allylphenols

A LITERATURE REVIEW

1.1 Introduction

Phenolics are naturally occurring compounds. After the carbohydrates, phenolic compounds constitute the second largest group of natural products. They are synthesised by plants and occur in conjugated forms as glucosides and esters and are also components of polymers such as lignins, tannins and melanins. Alkyl- and allylphenols include many compounds of which the cresols are the most common (Fig 1.1). Cresols occur in three different forms: *para*, *meta* and *ortho*. More complex alkyl- and allylphenols contain hydrocarbon tails, such as nonylphenol and eugenol. All angiosperms synthesise various alkylphenols. These include molecules such as chavicol and the eugenols as well as their derivatives (Koeduka *et al*., 2006). The monomeric compounds are volatile and some are toxic to insects as well as microbial life. Eugenol, as an example, is an essential ingredient of the essential oil of the clove tree (*Syzygium aromaticum*) and is regarded as a general acting antimicrobial and anti-animal toxin with analgesic properties for humans. As another example, some basil (*Ocimum basilicum*) varieties synthesize and accumulate eugenol, chavicol, or their methylated derivatives in the peltate glandular trichomes on the surface of their leaves. Plants often produce and store these compounds in their vegetative parts as defence against herbivores, parasitic bacteria and fungi. They are also sometimes emitted by flowers to attract pollinators. Isoeugenol, for instance, is one of three main volatiles emitted diurnally from the tube and corolla of the Petunia flower. Alkylphenols also occur as constituents or breakdown products of peptides, proteins and steroids (Enroth *et al*., 1998). As an example, *p*-cresol is formed from tyrosine by bacteria under anaerobic conditions. These conditions exist in the rumen or in the digestive tract of

non-ruminants and as a result, *p*-cresol is excreted by animals as a natural byproduct of metabolism. (Jones *et al.,* 1993; Enroth *et al*., 1998; Bergauer *et al*., 2005).

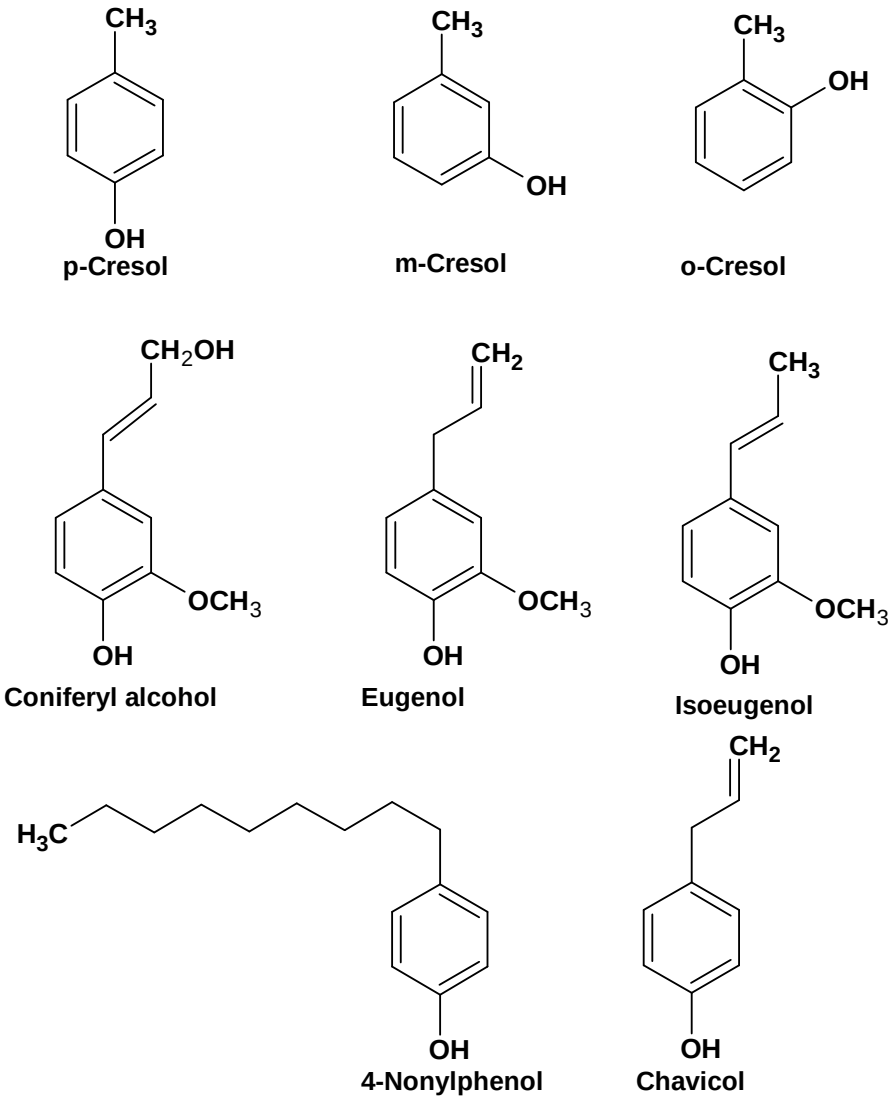

Fig 1.1: Examples of phenols and alkylphenols that occur in nature or originate from industry.

The phenol compounds that are produced from industrial processes contribute to the pool of naturally occurring phenolic compounds. They are considered industrial pollutants. Cresols are produced in large amounts by the petrochemical industry as components of resins, solvents, disinfectants and wood-preserving chemicals (Peters *et al*., 2007). Alkylphenols, such as nonylphenol, are raw materials used in the production of non-ionic surfactants. They are also pollutants that occur in the wastewater from the crude oil industry, ceramic and steel plants, from coal conversion processes and the phenol resin industry (Vallini *et al*., 1997).

Alkylphenols are, as mentioned above, toxic. When they accumulate in sediments as well as groundwater, they are especially toxic to marine and freshwater life. Nonylphenol has been shown to exhibit phytotoxic activity in plants (Corti *et al*., 1995; Vallini *et al*., 1997).

Many microorganisms have been reported to be able to degrade phenolic compounds. These include bacteria, yeasts, fungi and algae (Tsai *et al.*, 2005). The degradation of these compounds are often initiated by inducible flavoenzymes (Moonen *et al.*, 2002). These enzymes include flavoprotein monooxygenases that use NAD(P)H as the electron donor to activate and cleave a molecule of oxygen, to incorporate one oxygen atom into the substrate while the other is reduced to water, as well as flavoprotein oxidases that need no external cofactors and use only molecular oxygen as electron acceptor (Moonen *et al.*, 2002).

1.2 Flavoproteins

Enzymes can be divided into two major groups. The first group consists of enzymes that are capable of performing catalysis without the help of cofactors. Examples include enzymes such as hydrolases. These enzymes carry out catalysis by employing only the amino acids present in the polypeptide chain (Heuts *et al.,* 2009).

The second group consists of enzymes that require the help of one or more cofactors for catalysis. Cofactor-dependent enzymes make use of non-protein groups that may be

inorganic or organic in nature. Examples include inorganic compounds like Cu^+ or Fe-S clusters, or organic molecules such as $NADP^+$ or pyridoxal phosphate. These enzymes may also make use of a combination of more than one of these cofactors. Mitochondrial complex II, a succinate dehydrogenase, contains heme, flavin and three Fe-S clusters as cofactors (Heuts *et al*., 2009).

Cofactors are in most cases noncovalently linked and dissociate from the enzyme during catalysis and thereby act as coenzymes. Examples include $NADP^+$, coenzyme A and ubiquinone. Alternatively, the cofactor is noncovalently bound and dissociation from the enzyme is not required for catalysis. There are also cofactors in existence that, in contrast to the above-mentioned examples, are exclusively bound to the polypetide chain (e.g. lipoic acid and biotin). The covalently bound lipoyl-lysine and biotinyl-lysine function as swinging arms that shuttle intermediate compounds between the active sites of the respective enzyme complexes (Reche and Perham, 1999; Heuts *et al*., 2009). In some enzymes, amino acyl groups act as covalent cofactors, for example, in disulfide reductases redox cofactors are formed *in situ* from amino acyl groups (Xie and van der Donk, 2001; Argyrou and Blanchard, 2004; Heuts *et al*., 2009). Examples include topaquinone in serum amine oxidase, tryptophan tryptophylquinone in bacterial methylamine dehydrogenase, and cysteine tryptophylquinone in bacterial quino-cytochrome amine dehydrogenases. Topaquinone is made without an external catalyst, whereas the formation of tryptophan tryptophylquinone and cysteine tryptophylquinone requires external enzymes (McIntyre, 1998; Mure, 2004; Heuts *et al*., 2009). Heme and flavin cofactors are the only examples that can be either covalently or noncovalently bound to enzymes. Most flavoproteins contain a tightly but noncovalently bound flavin. It is however estimated that about 10% of all flavoproteins contain a covalently bound flavin (Heuts *et al.,* 2009).

Flavoproteins play a role in a variety of different biological processes that range from redox catalysis to DNA repair. The flavoprotein monooxygenases and flavoprotein oxidases are very important in the degradation of aromatic compounds. Two of the largest flavoprotein families are the glucose oxidase/methanol oxidase/cholesterol

oxidase (GMC) family and the vanillyl-alcohol oxidase (VAO) family. Each family has its own distinct protein fold for the binding of FAD (Heuts *et al*., 2009).

In general, flavoenzymes utilize two half-reactions in which the flavin will alternate between oxidized and reduced states. During the reductive half-reaction the flavin is reduced by an electron donor, which is the substrate in the case of flavoprotein oxidases and dehydrogenases. In flavoprotein oxidases and dehydrogenases the flavin is reoxidised during the oxidative half-reaction by an electron acceptor which can be oxygen or small molecules like $NAD(P)^+$, quinones or even redox-proteins. The overall catalytic cycle can occur through ternary complex formation or through a 'ping-pong' mechanism (Fig 1.2). In enzymes that function through the formation of a ternary complex, the electron acceptor reacts with the enzyme-product complex. In enzymes that function through a 'ping-pong' mechanism, the electron acceptor reacts with the free enzyme after release of the product (Mattevi, 2006; Moonen *et al*., 2002).

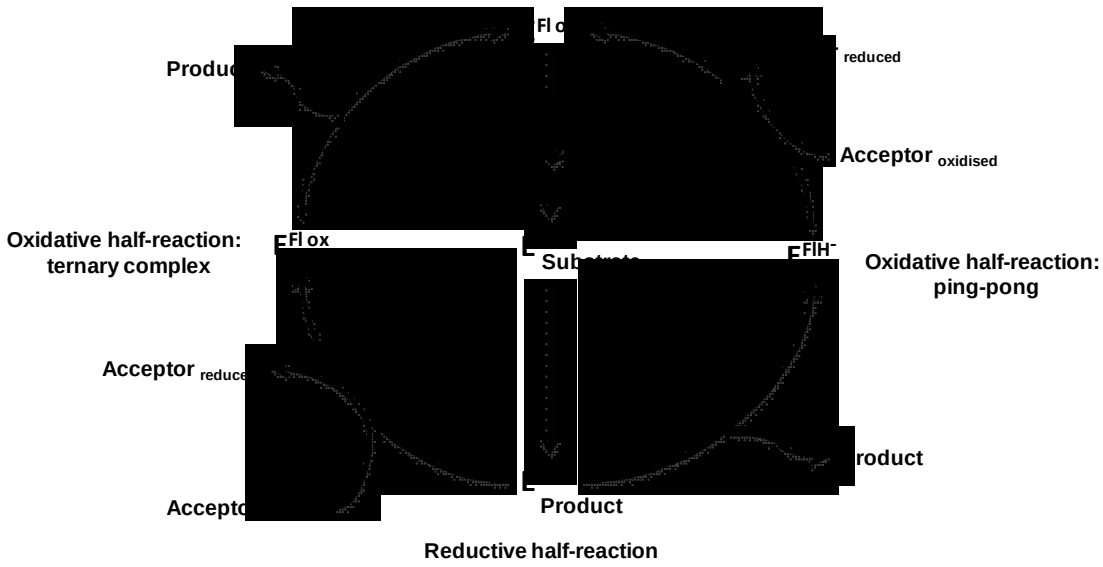

Fig 1.2: The catalytic cycle of flavoprotein oxidases/dehydrogenases where Fl indicates protein-bound flavin. The flavin oxidizes the substrate in the reductive half-reaction, whereas the reduced flavin is re-oxidized by an electron acceptor,

which is molecular oxygen in the case of the oxidases, in the oxidative half-reaction. Depending on the enzyme and the substrate, the overall reaction can follow a ternary complex (left) or ping-pong (right) mechanism (Taken from Mattevi, 2006; Moonen *et al.,* 2002).

The chemical versatility of the flavoenzymes stems from the ability of the reduced flavin to differentially react with molecular oxygen (Massey, 1994). Flavin-dependent monooxygenases activate oxygen by forming a C4α-(hydro)peroxide of the flavin which is employed to insert an oxygen atom into the substrate. Flavin-dependent oxidases use dioxygen as electron acceptor to produce hydrogen peroxide. Flavin-dependent dehydrogenases typically react slowly or not at all with oxygen. They make use of other electron acceptors instead. These can be either small molecules like $NAD(P)^+$, quinones or redox-proteins. The adjustable oxygen reactivity of flavoenzymes makes them useful catalysts in all types of organisms (Mattevi, 2006).

1.3 Flavoenzymes capable of oxidising 4-alkylphenols and 4- allylphenols

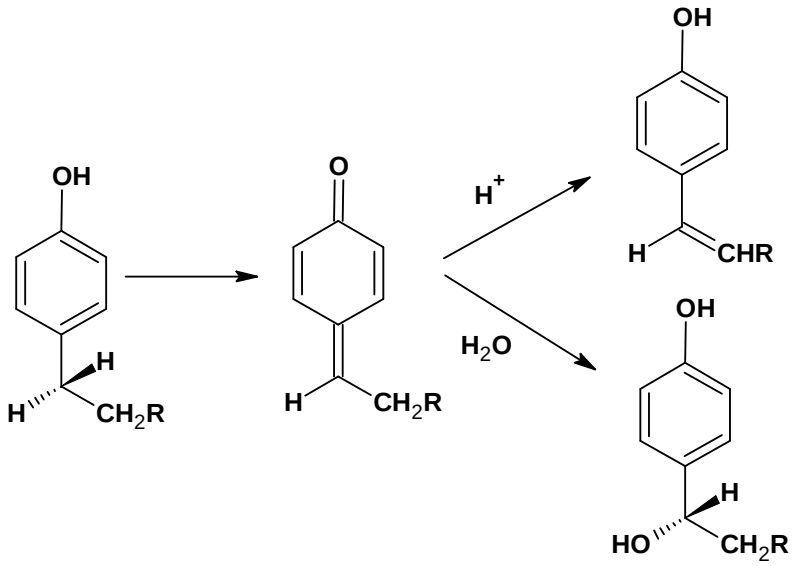

Fig 1.3: Two half-reactions of the flavoenzyme catalyzed oxidation of alkylphenols (Taken from van den Heuvel *et al*., 2000c)**.**

A small number of flavoprotein oxidases and dehydrogenases react with 4-alkylphenols and 4-allylphenols. Catalysis involves two half-reactions in which first the flavin cofactor is reduced by the substrate and subsequently the reduced flavin is reoxidised by an electron acceptor (Moonen *et al*., 2002). The protein-bound quinone methide either reacts with water to yield the (R)-enantiomer of the alcohol or is rearranged to yield the alkene (Fig 1.3) (van den Heuvel *et al*., 2000c). Flavoenzymes capable of accepting alkylphenols as substrates can be subdivided into two groups: the flavoprotein oxidases capable of using oxygen as electron acceptor and the flavoprotein dehydrogenases that use cytochrome *c* or azurine as electron acceptor.

1.3.1 Flavoenzymes that use oxygen as electron acceptor

1.3.1.1 Vanillyl-alcohol oxidase

Vanillyl-alcohol oxidase (VAO) is a flavoprotein that was first isolated from *Penicillium simplicissimum*, a non-lignolytic fungus that is capable of utilizing a wide range of aromatic compounds, including veratryl alcohol and vanillyl alcohol as sole sources of carbon (de Jong *et al*., 1992). It was found that when this organism was grown on veratryl alcohol, an intracellular H_2O_2-generating oxidase was induced that did not act on the veratryl alcohol but catalysed the oxidation of vanillyl alcohol to vanillin (de Jong *et al*., 1992; van den Heuvel *et al*., 2001b). Induction of VAO was highest during the growth phase of *P*. *simplicissimum*. In addition to VAO, an intracellular catalase was also induced (Fraaije *et al*., 1997). Isoeugenol had an inhibitory effect on the production of VAO when added to media containing veratryl alcohol, but had no effect when added to media containing anisyl alcohol (Fraaije *et al*., 1997). The enzyme was considered as a prototype for a novel family of oxidoreductases that contains a covalently bound flavin.

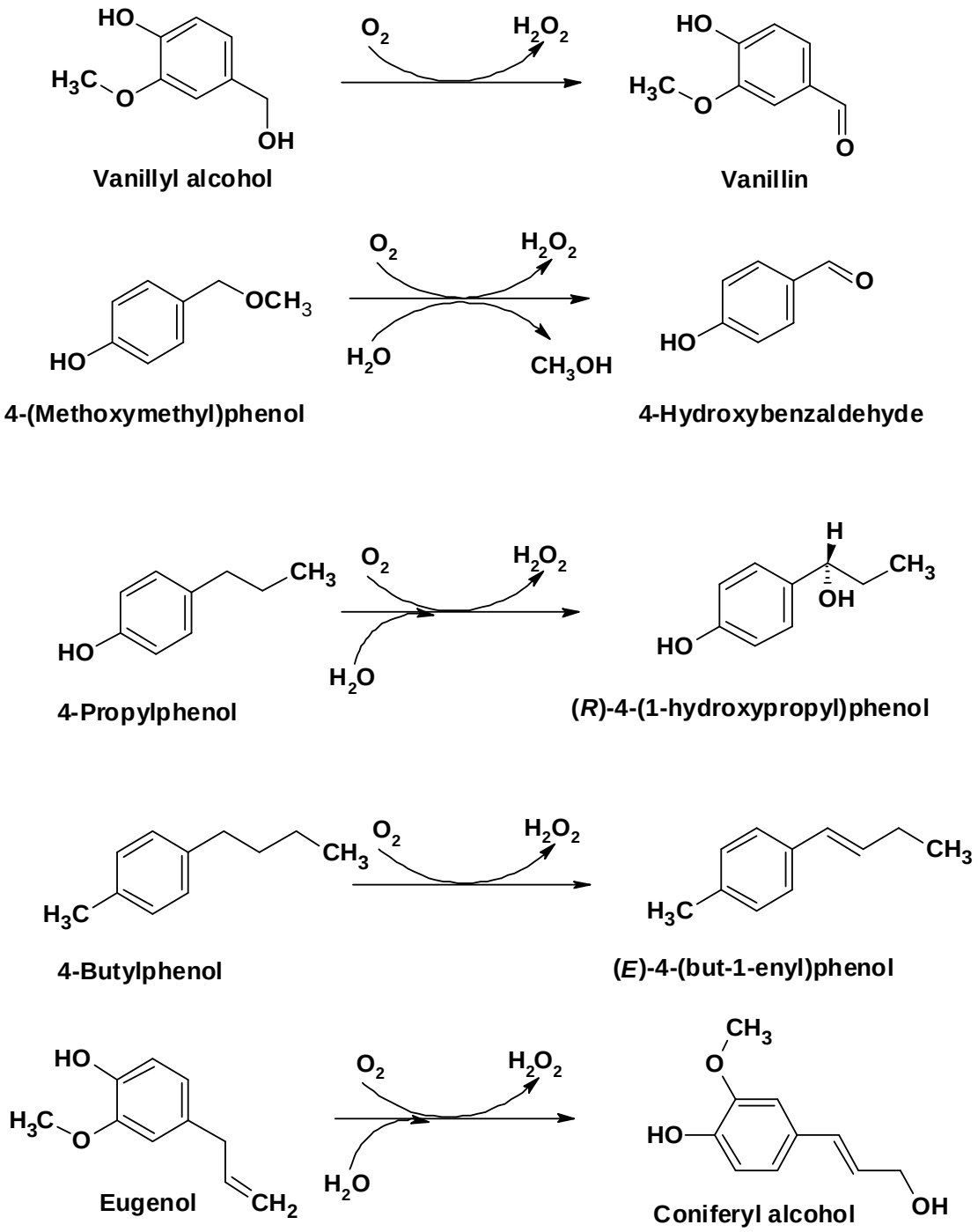

Fig 1.4: Different reactions catalyzed by VAO: oxidation of vanillyl alcohol; demethylation of 4-(methoxymethyl)phenol; hydroxylation of 4-propylphenol dehydrogenation of 4-butylphenol and hydroxylation of eugenol.

VAO is capable of oxidizing alcohols, performing amine oxidations, enantioselective hydroxylations, stereospecific dehydrogenations and oxidative ether cleavage reactions, forming flavouring compounds such as vanillin as well as 4-vinylphenols, which are present in beer and wine (van den Heuvel *et al*., 2001a; Jin *et al*., 2007) (Fig 1.4). The enzyme is capable of oxidizing a wide variety of phenolic compounds and is specifically induced when the fungus is grown on veratryl alcohol, anisyl alcohol or 4-(methoxymethyl)phenol (Fraaije *et al*., 1998). 4-(Methoxymethyl)phenol is efficiently demethylated into 4-hydroxybenzaldehyde, which is an important constituent of vanilla. Vanillin is also formed *via* the vanillyl-alcohol oxidase-mediated conversion of vanillyl amine, vanillyl alcohol and creosol.

Table 1.1: Kinetic data for the two isolated VAOs with different substrates.

Organism	Substrate	K_m (µM)	kcat (s^{-1})	Reference
P. simplicissimum	**Eugenol**	**2**	**14**	van den Heuvel *et al*., 2000c
	Vanillyl alcohol	75	1.6	van den Heuvel *et al*., 2004
	4-Ethylphenol	9	2.5	van den Heuvel *et al*., 2000c
	4- *n*-Propylphenol	4	4.2	van den Heuvel *et al*., 2000c
B. fulva V107	**Eugenol**	**6**	**Not given**	Furukawa *et al*., 1999
	Vanillyl alcohol	213	Not given	Furukawa *et al*., 1999
	4-Ethylphenol	78	Not given	Furukawa *et al*., 1999
	4- *n*-Propylphenol	77	Not given	Furukawa *et al*., 1999

Vanillyl-alcohol oxidase also stoichiometrically converts eugenol, which is the main component of clove, to coniferyl alcohol (van den Heuvel *et al.*, 2001a). Both the VAO enzymes that have been isolated in fact display the highest activity towards eugenol as substrate (Table 1.1). VAO also displays remarkable activity towards 4-alkylphenols with aliphatic side-chains of up to seven carbon atoms (van den Heuvel *et al*., 1998; van den Heuvel *et al*., 2000c; van den Heuvel *et al*., 2001a). Optimal catalytic efficiency occurs with 4-ethylphenol and 4-*n*-propylphenols. The shorter-chain 4-alkylphenols are

hydroxylated at the Cα-position to 1-(4'-hydroxyphenyl)alcohols. Medium-chain 4-alkylphenols are dehydrogenated to 1-(4'-hydroxy-phenyl)alkenes. The dehydrogenation of 4-alkylphenols can be promoted by the presence of monovalent anions and by low water content. The hydroxylation of 4-alkylphenols is also highly stereospecific to yield the *R*-isomer with an *e.e* of 94%. VAO also more efficiently oxidizes *S*-isomers of 1-(4'hydroxyphenyl) alcohols to the corresponding alkanones than the *R*-isomers, yielding highly pure (*R*)-1-(4'hydroxyphenyl)alcohols from a racemic mixture. In addition to stereospecific hydroxylation of 4-alkylphenols, VAO can also dehydrogenate medium-chain 4-alkylphenols stereospecifically into *cis*- or *trans*-1-(4'hydroxyphenyl)alkenes (van den Heuvel *et al.*, 2001a). This regiospecificity suggests that the site of the water attack depends on the delocalization of charge in the bound *p*-quinone methide intermediate (van den Heuvel *et al*., 1998).

Cis-trans stereospecificty is not unique for VAO. Examples of other flavoenzymes that exhibit this specificity include acyl-coenzyme A dehydrogenases that introduce a *trans* double bond between C-2 and C-3 of their coenzyme A substrates. Glyoxalate oxidases show specificity for abstraction of the *re* hydrogen when prochiral glycolate is used as a substrate (van den Heuvel *et al*., 1998). In contrast to these enzymes, the *cis-trans* specificity of VAO is dependent on the bulkiness of the alkyl side-chain of the substrate (van den Heuvel *et al*., 1998). Van den Heuvel and co-workers (1998) concluded that the regio- and stereospecificity of VAO was mainly due to three factors: (i) the intrinsic reactivity of the enzyme-bound *p*-quinone methide intermediate, (ii) the accessibility of water to the enzyme active site and (iii) the orientation of the hydrophobic alkyl side-chain of the substrate.

Vanillyl-alcohol oxidase was shown to have a bimodal distribution and is located in peroxisomes as well as the cytosol. VAO represents the first example of a covalent flavoprotein that is not strictly compartmentalized. The presence of active octameric VAO in the cytosol and peroxisomes shows that no specific organelle-bound assembly factors are required for flavinylation and oligomerization (Fraaije *et al*., 1998).

The enzyme forms stable homo-octamers of about 510 kDa, with each 64-kDa subunit containing two domains: a cap domain that covers the active site and a larger domain

that creates a binding site for the ADP-ribityl part of the FAD cofactor (van den Heuvel *et al*., 2000a; Fraaije *et al*., 2003). Mutagenesis studies have shown that the covalent flavin protein bond is crucial for efficient catalysis, and that the covalent flavinylation of the apoprotein proceeds *via* an autocatalytic event (Jin *et al.*, 2007).

A VAO was also isolated from *Byssochlamys fulva* V107, an anamorph of *Paecilomyces fulvus*. The homogeneity of the enzyme was confimed by HPLC elution. It showed a single symmetrical peak with a native molecular mass of 110 kDa, suggesting a homodimeric enzyme structure. The enzyme showed highest activity with eugenol, however, unlike vanillyl-alcohol oxidase, was not capable of oxidizing 4-hydroxybenzylamines and 4-(methoxymethyl)phenol (Furukawa *et al*., 1999).

1.3.1.1.1 Reaction mechanism

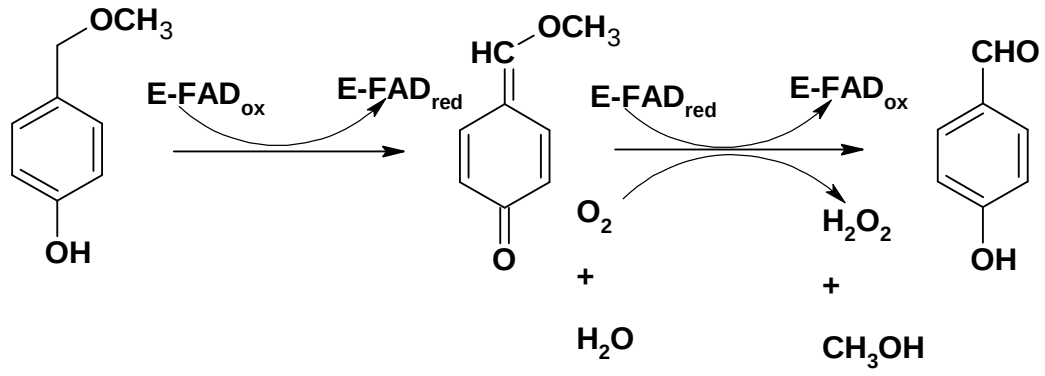

Fig 1.5: Catalytic mechanism of VAO with 4-(methoxymethyl)phenol as substrate, leading to the formation of 4-hydroxy benzaldehyde and methanol.

The expression of PsVAO (vanillyl-alcohol oidase from *P. simplicissimum*) is strongly induced by the presence of 4-(methoxymethyl)phenol in the growth medium and it has initially been proposed that 4-(methoxymethyl)phenol represents the physiological substrate for PsVAO (Mattevi *et al*., 1997), although activity towards eugenol has been shown to be much higher (Table 1). The catalytic cycle involves two half-reactions as

shown in Fig 1.5 for 4-(methoxymethyl)phenol. During the reductive half-reaction, a hydride is transferred from the Cα-atom of the substrate to the flavin N5-atom, forming a *p*-quinone methide intermediate. The enzyme achieves hydride transfer by positioning the ligand Cα atom 3.5 Å from the flavin N5-atom (Mattevi *et al*., 1997). This then reacts with molecular oxygen to regenerate the oxidized flavin during the oxidative half-reaction. Subsequently, the *p*-quinone methide product reacts with water in the enzyme active site, resulting in the formation of the final products, 4-hydroxy benzaldehyde and methanol via an unstable hemiacetal (van den Heuvel *et al.*, 2001b). Three residues, Arg504, Tyr503 and Tyr108 are ideally located for the stabilization of the phenolate negative charge. The propensity of these side-chains for binding anionic molecules is further underlined by the presence of aco- crystallized acetate ion directly interacting with the phenolate-binding cluster (Mattevi *et al*., 1997). The three-dimensional structure also suggests that charge balancing between the flavin, the quinone intermediate and Arg504 may determine the sequence of the catalytic steps. Arg504 is also well positioned to stabilize a negative charge on the N1-C2=O2 locus of the anionic cofactor. The C2 of the flavin is however located ~4 Å from the expected position of the oxygen atom of the *p*-quinone methide molecule, that is bound to the reduced enzyme. This should mean that in the reduced enzyme, electrostatic repulsion by the negative charge of the flavin C2 locus should prevent formation of the phenolate ion. This should stabilize the quinine form of the intermediate. On the contrary, upon flavin reoxidation, Arg504 is deprived of an anionic partner, triggering the development of a negative charge on the quinone oxygen atom. This increases the electrophilicity of the methide carbon, facilitating hydroxylation as in the case of 4-(methoxymethyl)phenol as substrate; or deprotonation (vanillyl alcohol as substrate) of the intermediate, producing the final product.

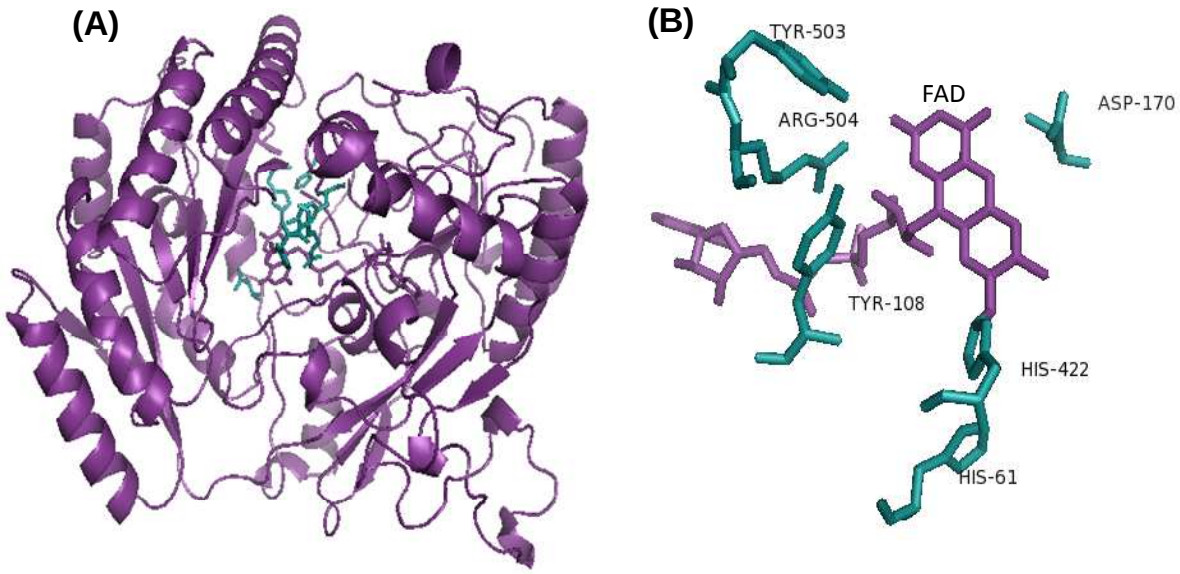

Fig 1.6: (A) The VAO isolated from *P. simplicissimum*. (B) Active-site residues involved in the covalent binding of the FAD cofactor, as well as in catalysis. The FAD molecule is visualized as a stick model in purple. The His61, His422, Asp170, Tyr503, Arg504 and Tyr108 residues are visualized as stick models in the colour teal. The protein model was generated using PYMOL (http://www.pymol.org/).

The X-ray structure of vanillyl-alcohol oxidase has revealed that the active site is located in the interior of the protein and contains an anionic binding pocket that facilitates substrate deprotonation (van den Heuvel *et al*., 1998) (Fig 1.6). The catalytic center of PsVAO is located on the *si* side of the flavin ring. It is delimited by hydrophobic and aromatic residues. The active site is occupied by a number of ordered solvent molecules. One of the surprising features of the active site is that it is completely inaccessible to solvent. The active site cavity is elongated with a volume of approximately 200 $Å^3$. The cavity has a rigid architecture, limiting the size and structure of the active-ligands. This solvent-protected environment is suited for binding the poorly soluble and hydrophobic VAO substrates. The low dielectric constant of the catalytic medium also strengthens the electrostatic and polar interactions, which activates the

substrate through phenolate formation. The solvent inaccessible catalytic site is also thought to affect the hydride transfer step leading to substrate oxidation. In other flavin dependent oxidases that have evolved the same strategy, a loop changes conformation during the catalytic cycle, thus controlling the accessibility of the catalytic site (Mattevi *et al*., 1997).

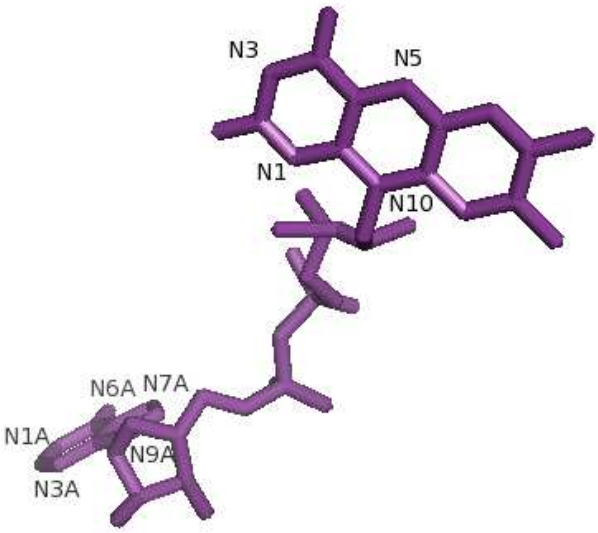

Fig 1.7: The FAD molecule displaying the relative positions of the all the N numbered atoms. The model was generated using PYMOL (http://www.pymol.org/).

In most flavin-dependent oxidoreductases with a known structure, the N5 atom of the flavin (Fig 1.7) contacts a hydrogen bond donor. However, in the case of VAO, Asp-170, an acidic residue is found in the vicinity of the N5-atom. The side-chain of this residue is positioned in a manner suggesting that during catalysis it might interact with the protonated N5-atom of the reduced cofactor (van den Heuvel *et al*., 2000a; van den Heuvel, 2002). Studies done using mutants in which the Asp170 residue was replaced with other amino acids *via* site-directed mutagenesis have shown that in some cases the FAD is not covalently bound in these variants and that Asp170 is critical for catalysis, since activity of the variants was markedly reduced when compared to the wild type, indicating slow flavin reduction. The mutant proteins could also not form stable complexes between the reduced enzyme and the *p*-quinone intermediate (van

den Heuvel *et al*., 2000a). The Asp170 residue has also been implicated in the stereospecific conversion of alkylphenols by PsVAO. Wild type PsVAO preferentially converts 4-ethylphenol to the (*R*)-enantiomer of 1-(4'-hydroxyphenyl)ethanol. Studies using double mutants that had been created through site-directed mutagenesis where the Thr457 and Asp170 residues had been relocated to the opposite face of the active site cavity, showed a reversal of enantioselectivity. Two possible reasons suggested for the (*S*)-selectivity was that the water molecule was attacking from the other side of the substrate or that the substrate was bound in a different orientation (van den Heuvel *et al*., 2000b). The crystal structures of these mutants revealed that the latter possibility was unlikely. It was revealed that in double mutants Glu457 directs the stereospecific attack to the planar quinine methide intermediate, presumably by acting as an active site base. In single mutants Asp170 favourably competes with Glu457 for the site of water attack resulting in the formation of the (*R*)-enantiomer (van den Heuvel *et al*., 2000b).

The His422 residue of the cap domain was identified as the residue responsible for the covalent binding of the flavin cofactor through the C8α-atom of the isoalloxazine ring of the FAD (van den Heuvel *et al*., 1998; van den Heuvel *et al.*, 2000b). Fraaije *et al*. (2003) studied the functional role of the covalent histidyl-FAD bond in VAO by creating mutants through site-directed mutagenesis. These mutants all contained tightly, but non-covalently-bound FAD. Steady-state kinetics with 4-(methoxymethyl)phenol indicated that the mutant enzymes were one order of magnitude slower than the wild-type VAO (Fraaije *et al*., 2003). The deletion of the histidyl-FAD bond decreases the midpoint redox potential from +55mV (for wild-type VAO) to -65mV, suggesting that the covalent bond may increase oxidative power (Fraaije *et al*., 2003).

Another residue at the catalytic site, His61 also located in the FAD domain, was also identified to be involved in the covalent binding of the FAD. It is however not directly involved, but rather indirectly. Mutant enzymes not containing the His61 residue only weakly bind FAD and were shown to be 10-fold less active with 4-(methoxymethyl)phenol than the wild type enzyme (Fraaije *et al*., 2003). His61 plays a crucial role in the autocatalytic flavinylation of VAO by activating the His422 residue.

1.3.1.2 Eugenol oxidase

Eugenol oxidase was isolated from a *Rhodococcus* sp. strain RHA1. It has a 45% sequence identity with VAO. Sequence alignments revealed that it also contains a histidine residue (His390) at the position equivalent to the FAD-binding histidine in VAO (Jin *et al*., 2008).

The enzyme was shown to exhibit a wide substrate spectrum. Eugenol was shown to be the best substrate. Eugenol was converted to coniferyl alcohol upon aerobic incubation with the enzyme (Jin *et al*., 2008). The reaction mechanism is similar to the one catalysed by vanillyl-alcohol oxidase, which includes attack by water to form the hydroxylated product, coniferyl alcohol, and the formation of hydrogen peroxide. Eugenol oxidase was also shown to exhibit high catalytic efficiencies (k_{cat}/K_m) for vanillyl alcohol (3.0×10^5 $s^{-1}M^{-1}$) and 5-indanol (1.0×10^5 $s^{-1}M^{-1}$). The k_{cat} value for vanillyl alcohol as substrate was in fact higher (12 s^{-1}) than for eugenol as substrate (3.1 s^{-1}) (Jin *et al*., 2008). The reverse is true for the catalytic efficiency with eugenol displaying a higher efficiency (3.1×10^6 $s^{-1}M^{-1}$) by a factor of 10. Vanillylamine and alkylphenols were shown to be poor substrates for this enzyme. 4-Methoxyphenol was hardly accepted by eugenol oxidase. The enzyme also has a wide pH range but works optimally between pH 9.0-10.0 (Jin *et al*., 2008).

Eugenol oxidase was shown to be a dimer, in contrast to VAO which is octameric. Comparison of the modeled structure of eugenol oxidase by Jin *et al*. (2008) to that of VAO revealed that the active sites are conserved. All residues of VAO that were previously shown to be involved in binding the phenolic moiety are conserved in eugenol oxidase. Residues that form the cavity that accommodates the *p*-alkyl side-chain are less conserved in eugenol oxidase, which may explain the differences in substrate specificity.

1.3.2. Flavoenzymes that use cytochrome *c* as electron acceptor

1.3.2.1 *p*-Cresol methyl hydroxylase

p-Cresol metabolism was first studied in *Pseudomonas putida*. It is the first enzyme in the protocatechuate pathway that is responsible for the degradation of *p*-cresol and other related phenols in *Pseudomonas* species (Cunane *et al*., 2000). *p*-Cresol is hydroxylated by a periplasmic *p*-cresol methylhydroxylase (PCMH) to *p*-hydroxybenzaldehyde, with the transient formation of *p*-hydroxybenzyl alcohol (Peters *et al*., 2007).

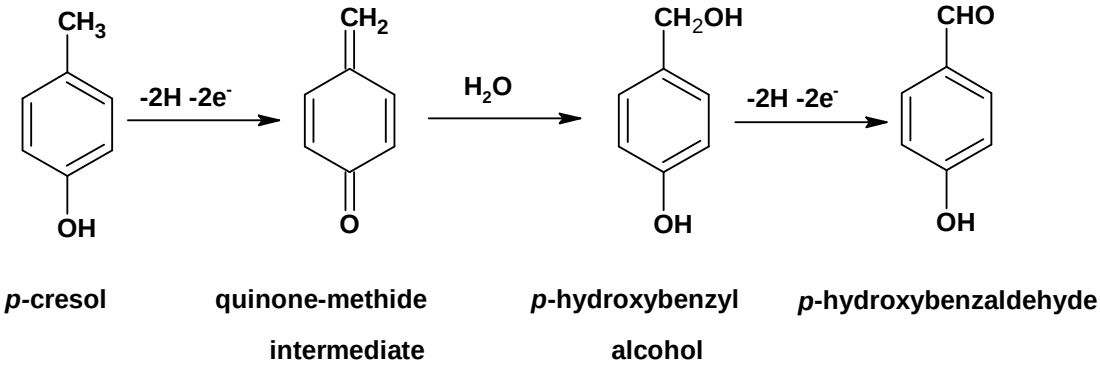

Fig 1.8: The reaction catalysed by PCMH. *p*-Hydroxybenzyl alcohol can serve as a substrate in a second reaction to form *p*-hydroxybenzaldehyde.

p-Cresol hydroxylation is achieved *via* the formation of a quinone methide intermediary by the removal of two electrons and two protons from *p*-cresol. Instead of passing the two electrons to oxygen, the enzyme passes the two electrons sequentially to an acceptor protein (Fig 1.8). The two protons are lost to the solvent. The quinone methide intermediate is then also hydrated through nucleophillic attack by water at the methide carbon atom. PCMH then also oxidizes *p*-hydroxybenzyl alcohol to *p*-hydroxybenzyl

aldehyde (Cunane *et al*., 2005). Electron equivalents generated by *p*-cresol oxidation are transferred to the blue copper protein azurin. In *P. putida* the product of PCMH, *p*-hydroxybenzaldehyde, becomes oxidized by a specific NAD^+- or $NADP^+$-dependent dehydrogenase to *p*-hydroxybenzoate (Peters *et al.*, 2007).

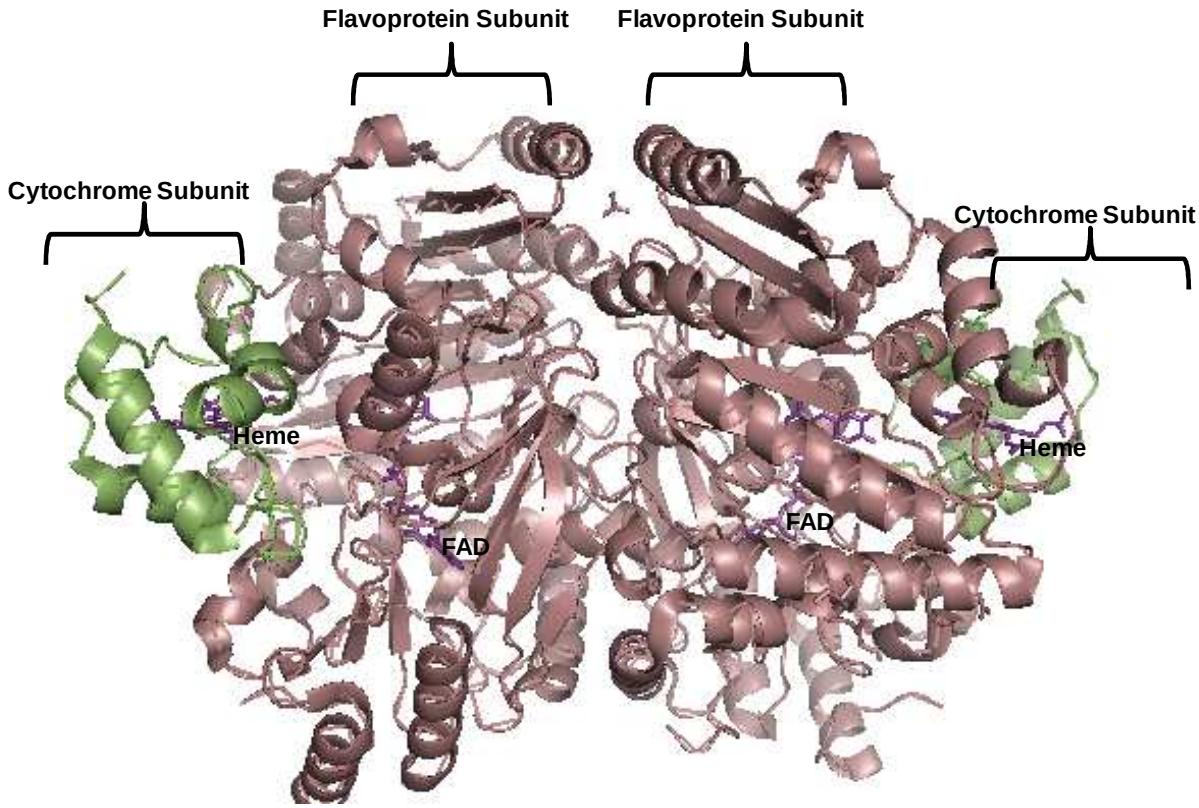

Fig 1.9: The PCMH heterotetramer showing the flavoprotein dimer and cytochrome subunits flank it on both sides. The location of the FAD and HEME cofactors are also indicated. The protein model was manipulated using PYMOL (http://www.pymol.org/).

Several X-ray structures are available for PCMH. PCMH consists of two subunits in a $\alpha_2\beta_2$-composition (Fig 1.9): an active-site α-subunit containing a flavin adenine dinucleotide (FAD) covalently linked to tyrosine and a *c*-type cytochrome β-subunit

(Cunane *et al*., 2000; Peters *et al*., 2007). The FAD cofactor is bound covalently through the 8-α methyl position to a tyrosine side-chain (Tyr384) (Cunane *et al*., 2000). This link has, as with VAO and EUGO, been proposed to form self-catalytically (Heuts *et al*., 2009). When separated, the components differ markedly in their biochemical properties from the holoenzyme. The isolated flavoprotein dimer has only about 2% of the catalytic activity towards *p*-cresol, while the redox potential of the isolated cytochrome subunit is lower by approximately 60 mV when compared to the native enzyme complex (250 mV). The holoenzyme $(\alpha_2\beta_2)^2$ can be reconstituted from the separated components and the enzymatic function and redox properties can be fully restored (Cunane *et al*., 2000).

It has been shown that the covalent link between the Tyr384 and the 8-methyl position of its isoalloxazine ring will not form when FAD is incubated with only the apo α-subunit (Heuts *et al*., 2009). Covalent binding will only occur when FAD is incubated with both the subunits; PchF and PchC together. FAD will first bind noncovalently to the α-subunit and when PchC binds to the holo α-subunit, a conformational change that leads to covalent flavinylation and further structural changes is induced in the latter (Heuts *et al*., 2009). When the covalent bond forms, the isoalloxazine moiety of FAD becomes reduced, which in turn reduces the β-subunits, as occurs during normal catalytic oxidation of the substrate (Heuts *et al*., 2009). Anaerobic titration of PCMH with either *p*-cresol or dithionite showed that reduction can be resolved into three distinct phases. The heme is reduced first, followed by the formation of the anionic flavin radical and finally the flavin becomes fully reduced (Cunane *et al*., 2000). It has also been shown that 5-deaza-FAD is capable of binding covalently to PCMH (Heuts *et al*., 2009).

The enzyme has also been found in *Geobacter metallireducens*. This organism is able to grow on aromatic compounds such as benzoate, toluene, phenol, *p*-cresol and *p*-hydroxybenzoate using Fe(III) as the terminal electron acceptor. (Peters *et al*., 2007). The PCMH activity was found in the membrane fraction of cell extracts. This is in contrast with the PCMH from *Pseudomonas* which is soluble. No PCMH activity was observed with cells grown on benzoate or acetate, suggesting strong regulation of the enzyme activity by *p*-cresol (Peters *et al*., 2007). The α subunit was present in two isoforms, suggesting an $\alpha\alpha'\beta_2$ composition. This is in contrast with the PCMH from *P.*

putida where the enzyme consists of a $\alpha_2\beta_2$-configuration. The unusual asymmetric architecture of PCMH in *G. metallireducens* with two different active-site α subunits might play a role in channeling electrons to different electron acceptors, to either cytochrome c during *p*-cresol metabolism or menaquinone during the oxidation of *p*-hydroxybenxyl alcohol (Peters *et al*., 2007). This could theoretically lead to a higher energy yield when compared to conventional soluble PCMH which uses cytochrome *c* or azurin as electron acceptor.

1.3.2.2 4-Ethylphenol Methylenehydroxylase

Another bacterial flavoenzyme, 4-ethylphenol methylenehydroxylase (4EPMH) was isolated from the bacterial strain *Pseudomonas putida* JD1. It is similar in structure and its mode of action to PCMH. It has however been shown to be a different enzyme (Reeve *et al*., 1989). It catalyses the first step in the degradation of 4-ethylphenol by dehydrogenation of the substrate to give a quinone methide intermediate. The quinone methide intermediate is then hydrated to give the hydroxylated product (Hopper and Cottrell, 2003). The product is the chiral alcohol 1-(4'-hydroxyphenyl)ethanol when 4-ethylphenol is used as substrate. Enantioselectivity is the same as with PsVAO with the *R*-enantiomer produced in excess of 98% (Hopper and Cottrell, 2003).

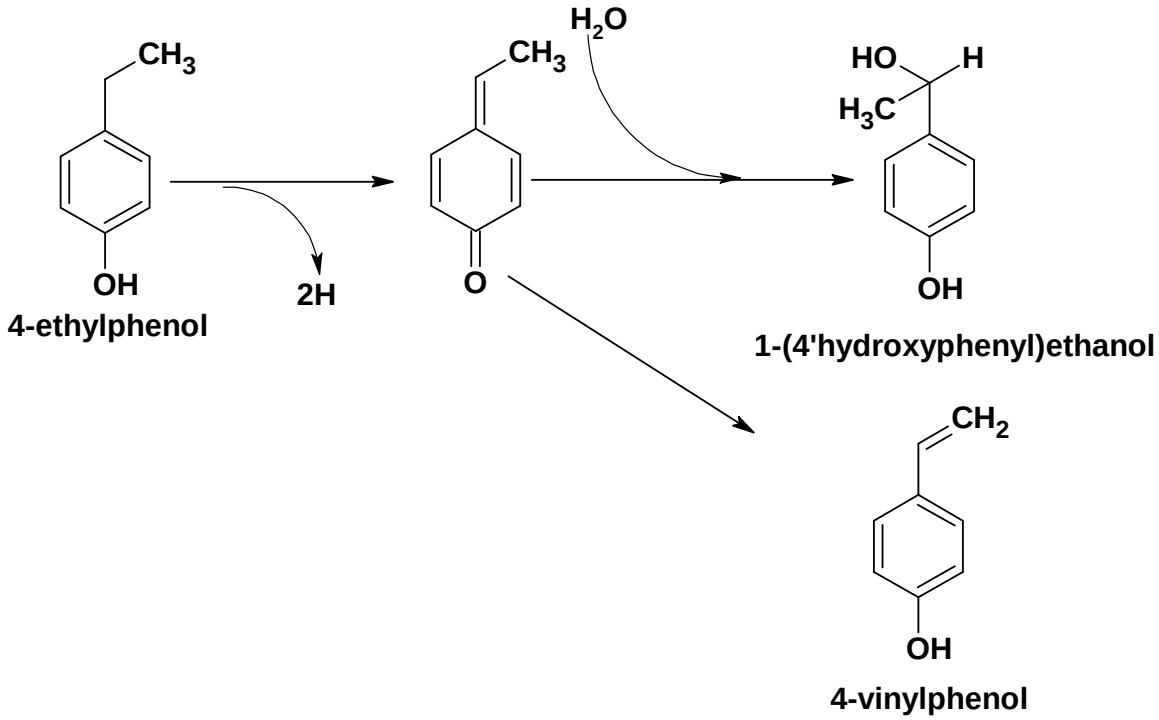

Fig 1.10: The formation of 1-(4'hydroxyphenyl)ethanol from 4-ethylphenol by 4-ethylphenol methylenehydroxylase.

4EPMH can accept a number of compounds including chavicol, 4-*n*-butylphenol, 4-*n*-propylphenol, 5-indanol, 4-isopropylphenol, 4-cyclohexylphenol, 6-hydroxytetralin and 4-hydroxydiphenylmethane (Hopper and Cottrell, 2003). 1-(4'-Hydroxyphenyl)propanol is formed from 4-*n*-propylphenol, while in the case of chavicol that has an unsaturated alkyl group, 1-(4' hydroxyphenyl)-2-propen-1-ol is formed. Branching or constraint of the alkylgroup of the substrate leads to the formation of vinyl compounds as the major products. This is usually the secondary product when the physiological substrate, 4-ethylphenol is used (Fig 1.10). Hopper and Cottrell (2003) also showed that these products are formed in high enantiomeric excess of >90%, favouring the *R*-enantiomer in all cases. The enzyme's activity decreases as the length of the side-chain of the substrate increases and in the case of a substrate like 4-hydroxydiphenylmethane, activity is greatly reduced.

The enzyme has been purified and characterised from *P. putida* growing on ethylphenol as carbon source by Reeve and co workers in 1989. They purified an enzyme with a M_r of 120 000, with a cytochrome subunit of M_r 10 000 and a flavin-containing subunit of M_r 50 000. The enzyme was also shown to be located in the periplasm. The major difference between PCMH and 4-ethylphenol methylenehydroxylase is specificity. 4-Ethylphenol methylenehydroxylase is better adapted to the hydroxylation of 4-alkylphenols with longer-chain alkyl groups. Longer-chain 4-alkylphenols also serve as substrates for 4-ethylphenol methylenehydroxylase, where in the case of PCMH they give little or no activity at all. 4-Ethylphenol methylenehydroxylase does not oxidize 3,4-xylenol, in contrast to PCMH (Reeve *et al*., 1989).

1.3.2.3 Eugenol hydroxylase

Eugenol hydroxylase is also part of the flavocytochrome *c* class of enzymes. It catalyses the initial reaction during eugenol catabolism in some *Pseudmonas* sp. It has been isolated from *Pseudomonas* sp. HR 199 and OPS 1 (Brandt *et al*., 2001; Priefert *et al*., 1999). Two genes (*ehyA* and *ehyB*) coding for the alpha and the beta subunit of the protein had been identified. The mass recorded for the β subunit (~57 kDa) corresponds with the mass of the α subunits of other flavocytchrome *c* proteins such as *p*-cresol methylhydroxylase from *P. putida* (57.9 kDa) and eugenol dehydrogenase (58 kDa) from *P. fluorescense* (Cronin and McIntyre, 2000; Furukawa *et al*., 1998; Priefert *et al*., 1999). The α subunit amino acid sequence showed a 29% homology to the corresponding subunit of PCMH from *P. putida*. Similarly, the β subunit showed a 55% homology to the corresponding subunit of PCMH from *P. putida*. A signal sequence had also been found for the *ehyA* gene product. This could indicate that eugenol hydroxylase is located in the periplasmic space.

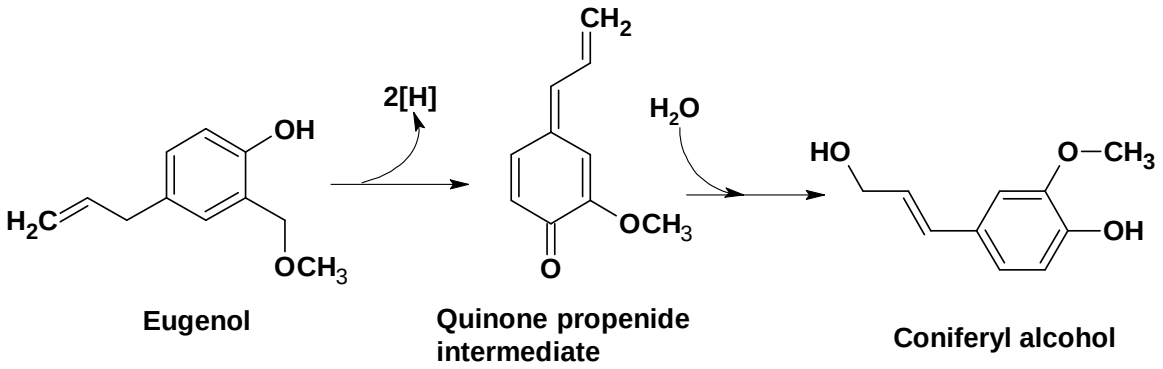

Fig 1.11: Proposed mechanism for eugenol hydroxylase (Taken from Priefert *et al*., 1999).

The strong sequence similarity with PCMH suggests that the enzyme will have a similar reaction mechanism (Fig 1.11) with the formation of a quinone propenide intermediate (Priefert *et al*., 1999). Another gene product known as *azu* had been identified downstream of the ehyA/B gene cluster in *Pseudomonas* sp. OPS1. It had been suggested that azurine, which is proposed to pass electrons from the cytochrome c subunit of the enzyme to cytochrome c oxidase of the respiratory pathway, represents the natural electron acceptor for eugenol hydroxylase (Brandt *et al*., 2001)

1.4 Covalent binding of flavin to flavoproteins

Most flavoproteins isolated thus far contain a dissociable flavin cofactor (Fraaije *et al*., 1998). However, some of these enzymes share a number of conserved FAD-binding domains with the flavin covalently linked to the protein (Leferink *et al.*, 2008). VAO was the first histidyl-FAD-containing flavoprotein for which the crystal structure was determined and serves as a prototype for this flavoprotein family (Jin *et al.*, 2007). Members of this family are involved in a wide variety of processes. One of the most distinct features of this family of enzymes is that it favours the covalent attachment of the flavin cofactor with the FAD in almost 25% of these proteins covalently linked (Leferink *et al.*, 2008). Members of this family have been shown to accommodate four

types of covalent attachment (Fig 1.12): 8α-N^3-histidyl-FAD, 8α-*O*-tyrosyl-FAD, 8α-N^1-histidyl-FAD and 8α-N^1-histidyl- 6-*S*-cysteinyl-FAD (Heuts *et al*., 2009).

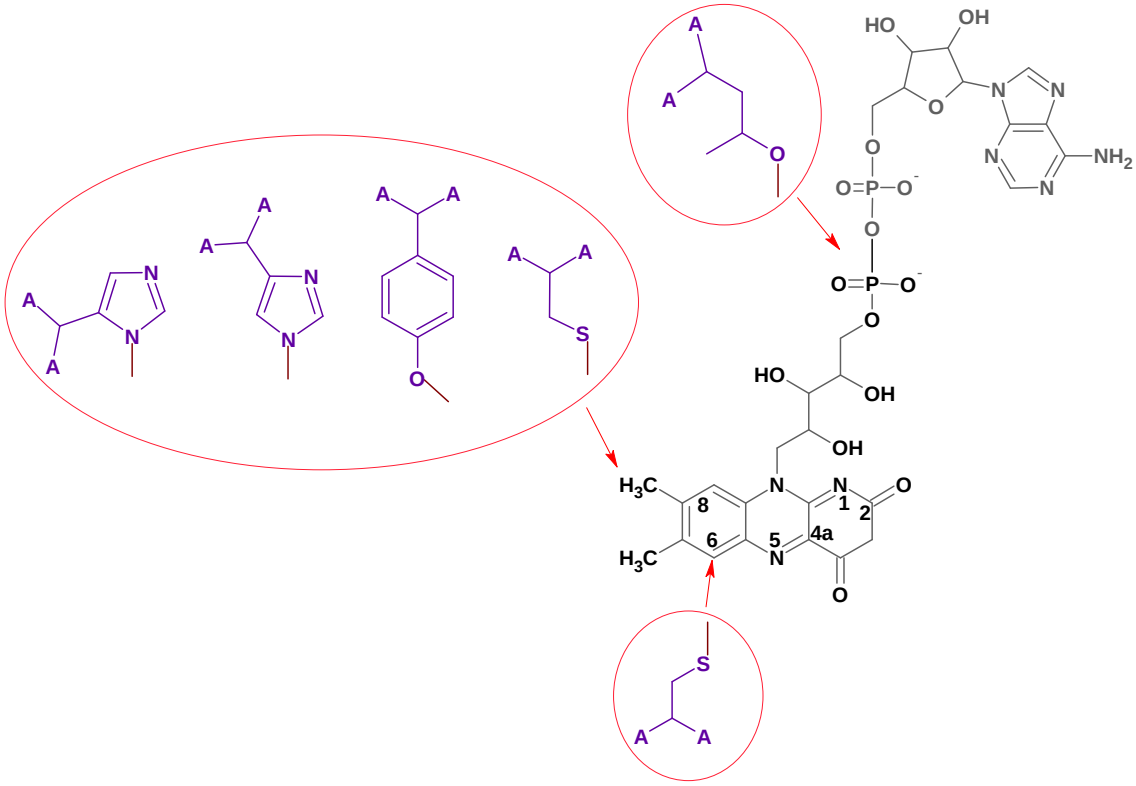

Fig 1.12: All known covalent flavin-protein linkages. FMN is shown in black. FAD is depicted in black and grey. Known linking amino acids are depicted in purple. The sites of covalent attachment are indicated by the arrows. The numbering of some of the isoalloxazine atoms is also indicated. (Taken from Heuts *et al*., 2009).

The function of covalent attachment of the flavin is still poorly understood, but some possibilities include the prevention of the inactivation of the cofactor or the facilitation of intramolecular electron transfer (Fraaije *et al*., 2003). The protein-flavin interaction might also improve protein stability or be favourable for flavoenzymes that are localized in a flavin-deficient environment (Fraaije *et al*., 2003). Several distinct types of covalent flavin binding have been recognized, but tethering of the 8α-methyl group of the flavin

isoalloxazine ring to a histidine residue is most frequently observed (Leferink *et al*., 2008). The isoalloxazine ring of the flavin group can also be covalently linked to a cysteine or tyrosine residue in the polypeptide structure (Fraaije *et al.*, 1998). Recently, enzymes have been discovered containing not one but two covalent FAD linkages. Each enzyme molecule of gluco-oligosaccharide oxidase (GOOX) isolated from the fungus *Acremonium strictum*, contains one FAD molecule that is covalently tethered *via* two bonds: an 8α-*N*^{1}-histidyl-FAD linkage, and a 6-*S*-cysteinyl-FAD linkage (Huang *et al*., 2005; Heuts *et al*., 2009). Other covalent flavoenzymes that also contain a similar flavin bond include aclacinomycin oxidoreductase, berberine bridge enzyme (BBE), hexose oxidase dbv29, Δ-tetrahydrocannabinolic acid synthase, cannabidiolic acid synthase and chito-oligosaccharide oxidase (ChitO) (Heuts *et al.,* 2009).

The enzymes belonging to the VAO family are diverse and found in diverse organisms (Table 1.2) where they perform a variety of different functions. Although enzymes belonging to this family share a similar structural fold, the residue that covalently tethers the FAD cofactor *via* the 8-methyl moiety is not conserved. The 8α-*N*^{1}-histidyl-FAD-containing homologs form an FAD linkage *via* a histidine close to the N-terminus, which is located in the FAD-binding domain. In contrast, the residues that form the 8α-*N*^{3}-histidyl-FAD and 8α-*O*-tyrosyl-FAD linkages are located at two different positions in the cap domain (Heuts *et al*., 2009). *p*-Cresol methylhydroxylase is the only enzyme isolated thus far with a tyrosyl linkage (Leferink *et al*., 2008).

Table 1.2: Enzymes of the VAO family with a covalently-bound FAD cofactor.

Covalent FAD cofactor	Enzyme	Organism	Reference
8α-Histidyl-6-S-cysteinyl	Glucooligosaccharide Oxidase	*Acremonium strictum*	Huang *et al*., 2005
	Chito Oligosaccharide Oxidase	*Fusarium graminarium*	Heuts *et al*., 2008

	Berberin Bridge Enzyme	*Eschcholzia californica*	Winckler *et al.*, 2006
	Hexose oxidase	*Chondrus Crispus*	Rand *et al*., 2006
	Aclacinomycin oxidoreductase	*Streptomyces*	Alexeev *et al*., 2007
	Δ-Tetrahydrocannabinolic acid synthase	*Cannabis sativa*	Taura *et al*., 2007
	Cannabidiolic acid synthase	*Cannabis sativa*	Taura *et al*., 2007
8α-Histidyl	Vanillyl-alcohol oxidase	*Penicillium simplissisimum*	Fraaije *et al*., 1999
	Cholesterol oxidase	Brevibacterium sterolicum	Coulumbe *et al*., 2001
	Alditol oxidase	*Streptomyces coelicolor*	Forneris *et al*., 2008
	6-Hydroxy-D-nicotine oxidase	*Arthrobacter nicotinovorans*	Koetter and Schulz, 2005
	Cytokinin dehydrogenase	*Arabidopsis thaliana*	Malito *et al*., 2004
	Eugenol oxidase	*Rhodococcus* sp.	Jin *et al*., 2007
	L-Glucono-$_{y}$-lactone oxidase	Mammals	Kenny *et al.*, 1976
	L-Gluconolactone oxidase	*Penicillium cyano-fulvum*	Shimizu *et al*., 1977
	L-Galactonolactone oxidase	*Saccharomyces cereviseae*	Kenny *et al*., 1979
	D-Arabino-1,4-lactone oxidase	*Candida albicans*	Huh *et a*l., 1994

	Sorbitol oxidase	*Streptomyces* sp.	Hiraga *et al*., 1997
	Xylitol oxidase	*Streptomyces* sp.	Yamashita *et al*., 2000
	Nectarin V	*Nicotiana langsdorffii X N. sanderae*	Carter and Thornburg, 2004
8α-*O*-Tyrosyl	PCMH	*Pseudomonas putida*	Mathews *et al*., 1991

*adapted from Heuts *et al*., 2009

1.5 Roles of covalent flavinylation

The role of covalent flavinylation has remained unclear for many years. Recently, studies on individual enzymes have provided some insights as to what the possible roles of covalent flavinylation may be. Possible roles that have been cited in literature include:

1.5.1 Increasing redox potential

It is known that the redox potential of flavins can be influenced by their environment. It has been shown that the covalent coupling of the flavin increases the midpoint potential significantly. This effect has also been observed when chemically-modified flavins such as 8α-*N*-imidazolylriboflavin are used. Using both the spectrocoulometric method and alternatively dithionite as reductant, the redox potential midpoint was determined as -154 mV at pH 7.0, compared to -200mV for free riboflavin (Williamson and Edmondson, 1985; Heuts *et al*., 2009).

The increase in redox potential allows an enzyme to oxidise the substrate more efficiently. This can however not be used as an accurate measurement for relative activity. As an example in a study where the redox potentials of the different subunits of PCMH was compared to PCMH, PCMH (redox potential of +93 mV) is fifty times more

active according to relative k_{cat} values (121 s^{-1} for PCMH) than one of its subunits $PchF^{c}$ (redox potential of +62mV and k_{cat} of 2.4 s^{-1}) containing covalently bound FAD (Effimov *et al*., 2004; Heuts *et al*., 2009).

An increase in redox potential also restricts the selection of electron acceptors that can be used. Molecular oxygen is often the only molecule that can be used as electron acceptor. This reason can serve as a possible explanation as to why most covalent flavoproteins are oxidases. PCMH is an exception to the rule and uses a high potential *c*-type heme (+230 mV) as the electron acceptor (Effimov *et al*., 2004; Heuts *et al*., 2009).

1.5.2 Structural integrity

Covalent flavinylation could also serve to enhance the stability of the protein. Removal of the covalent bonds leads to the production of incorrectly folded apoenzymes in several flavoenzymes (Heuts *et al*., 2009).

This is the case with the bicovalent flavoenzyme ChitO. Substanial effects were observed when the amino acids involved in covalent flavinylation were mutated. Not only does the removal of one of the covalent bonds affect the redox potential, but based on changes in the K_m value, it also appears to prevent the formation of a functional stable Michealis complex. The mutation also resulted in decreased structural stability. Protein aggregation was observed during redox potential measurements for the H94A mutant (Heuts *et al*., 2008).

The exception to this is PCMH and VAO. In both cases, the structural integrity of the apo forms seems to not be severely affected by removal of the covalent bond. The flavoprotein subunit can be expressed in *E. coli*, and when the cytochrome subunit of PCMH is not present, FAD is bound noncovalently to the isolated protein. The flavin is easily removed from the “holo” enzyme, and the stable apo protein can noncovalently rebind FAD. Exposure of this “holo” subunit to its partner cytochrome subunit results in

a fully formed and fully active native flavocytochrome that has covalently bound FAD (Cunane *et al*., 2005; Heuts *et al*., 2009).

Studies with the crystal structures of H61T apo-VAO, ADP-complexed H61T VAO, H61T holo-VAO and H422A holo-VAO, containing noncovalently bound FAD, revealed that binding of FAD and formation of the covalent FAD-protein bond do not cause any structural changes (Fraaije *et al*., 1999; Fraaije *et al*., 2000). It has also been shown that wild-type VAO can in the absence of FAD be produced and folded into a competent form able to bind FAD (Jin *et al*., 2008).

1.5.3 Flavin Reactivity

Another possible reason for covalent flavinylation has been suggested for TMADH, which oxidises trimethylamine to form dimethylamine and formaldehyde (Steenkamp and Mallison, 1976). This enzyme contains FMN that is covalently linked to a cysteine *via* the C-6 position of the flavin isoalloxizine moiety. Mutating the Cys30 to an alanine caused the removal of the covalent bond and results in the formation of a 6-hydroxy-FMN upon incubation with substrate. The 6-hydroxy moiety that is formed after oxidation of the substrate-reduced mutant, results from the reaction of reduced FMN with molecular oxygen. It was suggested that the covalent 6-*S*-cysteinyl-FMN has evolved to prevent wild type TMADH from forming the 6-hydroxy-FMN species, which renders the enzyme inactive (Huang *et al*., 1996).

1.5.4 Possible enhanced lifetime of the holo-enzyme

It has been suggested by Heuts *et al*. (2009) that some enzymes may have evolved with covalently bound flavins to increase the *in vivo* lifetime of the protein. If a flavin is noncovalently bound, the bond may weaken as the protein ages and may then dissociate (Heuts *et al*., 2009). Apo-flavoproteins are, in general, less stable than the holo forms. In cases where flavin re-association is impossible, the enzyme may be

rendered incompetent. This may be of particular importance for membrane-bound and extracellular flavoenzymes, which once inserted into the lipid bilayer or excreted, would have limited access to free flavin.

1.6 Heterologous expression of flavoenzymes

1.6.1 Vanillyl-alcohol oxidase

Vanillyl-alcohol oxidase has been heterologously expressed in bacteria including *E. coli* and the gram-positive strain *Amycolatopsis* sp. HR 167, as well as in the fungus *Aspergillus niger*. (Overhage *et al*., 2006; Benen *et al*., 1998).

The mRNA from *P. simplicissimum* was isolated and a cDNA library constructed. The gene was cloned and expressed in *E. coli*. The expression levels were however very low (less than 5% of total protein based on specific activity of vanillyl-alcohol oxidase), but could be picked up using specific antibodies. One reason for the low expression might be differences in codon usage. Codons that are considered modulator codons in *E. coli*, suppressing high expression, occur with high frequency in the cDNA of VAO from *P. simplicissimum*. Another reason may be the apparent absence of a ribosome-binding site. Introduction of a consensus *E. coli* ribosome-binding site at the correct distance from the start codon increased the expression level only 7-fold. This indicates that the low expression is probably related to codon usage (Benen *et al*., 1998). The problem can possibly be alleviated by gene optimization (Jin *et al*., 2007).

The *VAO* gene has also been transformed into *Aspergillus niger* under its own promoter. This means that it was under the control of carbon catabolite repression and induced by a limited number of aromatic compounds such as anisyl alcohol and veratryl alcohol (Benen *et al*., 1998). The highest level of induction was observed when *A. niger* was transferred to media containing veratryl alcohol and anisyl alcohol. Strong expression was observed with methoxybenzyl alcohols. Vanillyl-alcohol oxidase activity was also observed in the presence of vanillyl alcohol, vanillic acid and 4-hydroxybenzoic acid, indicating that the gene was under the control of at least one regulator involved in

the metabolism of aromatic compounds. The *VAO* gene was expressed from its own promoter, which means that the regulation mechanism present in *A. niger* was similar to that in *P. simplicissimum*. Induction was also observed with ferulic acid, vanillyl alcohol and 4-hydroxybenzoic acid. This is in contrast to what is seen in *P*. *simplicissimum*. The gene was introduced into *A. niger* in a high copy number (25 - 30 copies) which may explain the high level of induction that was observed (Benen *et al*., 1998).

The vanillyl-alcohol oxidase gene from *P. simplicissimum* was also heterlogously expressed in the vanillin-tolerant gram-positive strain *Amycolatopsis* sp. HR 167. This enabled the strain to use eugenol as sole carbon source. Coniferyl alcohol, coniferyl aldehyde, ferulic acid, guaiacol, vanillin and vanillic acid were detected by Overhage and co-workers, 2006. A high conversion rate of 2.3 mM. h^{-1} was achieved when using resting cells to transform eugenol to coniferyl alcohol, leading to a maximum concentration of conferyl alcohol of 4.7 $g.L^{-1}$. The biotransformation process is more effective than processes described using *Pseudomonas sp*. HR 99, which yielded 3.2 $g.L^{-1}$ coniferyl alcohol from 6.7 $g.L^{-1}$ eugenol. It was however not as effective as the fed-batch process using the fungus *Byssochlymus fulva* V107. The fungus produced a maximum concentration of 21.9 $g.L^{-1}$ coniferyl alcohol after 36 h of incubation. The molar yield was 94.6% (Furukawa *et al*., 1999; Overhage *et al*., 2006).

1.6.2 Eugenol oxidase

Eugenol oxidase from *Rhodococcus* sp. RHA1 has been heterologously expressed at high levels in *E. coli* TOP10 cells. Jin and co-workers were able to purify 160 mg of yellow-coloured recombinant eugenol oxidase from recombinant *E. coli*. No whole-cell biotransformation studies were done with the recombinant strain (Jin *et al*., 2007).

1.6.3 *p*-Cresol methyl hydroxylase

The genes encoding the subunits of PCMH have been cloned and sequenced. The subunits have been expressed either separately or together in *E. coli*. Expression of the flavoprotein occurs at levels of up to 15% of the soluble protein, with levels of cytochrome expression being much lower. Covalent flavinylation does not occur when the flavoprotein is expressed alone and leads to some bound FAD being lost during purification. The co-expression of the flavoprotein and the cytochrome subunits leads to the covalent flavinylation occurring at levels equivalent to the amount of cytochrome produced. Mixing the purified apo-protein with stoichiometric amounts of cytochrome and FAD *in vitro* leads to covalent flavin incorporation. This indicates that the covalent attachment of FAD to the enzyme is a self-catalytic process (Cunane *et al*., 2000; Engst *et al*., 1999; Kim *et al*., 1994). Whole-cell biotransformations were not attempted with the transformed strains (Kim *et al*., 1994).

1.6.4 Eugenol hydroxylase

The genes for eugenol hydroxylase from *Pseudomonas sp.* strain HR199 had been expressed in a variety of different species. It was first functionally expressed in *E. coli* XL1-Blue on pBluescript SK^- under the inducible *lacZ* promoter. The highest eugenol hydroxylase activity recorded was 0.16 $U.mg^{-1}$. The *ehyA* and *ehyB* genes had also been heterologously expressed in other *Pseudomonas* strains that were previously unable to grow on eugenol as sole carbon source or to convert eugenol to coniferyl alcohol. After transformation, these strains were streaked out on plates containing eugenol. It was reported that a bright yellow substance was excreted into the agar due to the formation of coniferyl alcohol (Priefert *et al*., 1999). The eugenol hydroxylase genes from *Pseudomonas* strain OPS1 had also been heterologously expressed using the same *E. coli* system. The production of coniferyl alcohol using whole cells were monitored but not reported. The activity in crude cell extract was reported to be 0.014 $U.mg^{-1}$ which is significantly lower than for the *ehy* from *Pseudomonas sp.* strain HR199 (Brandt *et al*., 2001).

The *ehy* genes had also been co-expressed along with various other genes in *Ralstonia eutropha* (Overhage *et al*., 2002). Co-expressing these genes along with other genes in the eugenol catabolic pathway enables the production of vanillin from an inexpensive feedstock. Co-expression along with genes coding for coniferyl alcohol dehydrogenase and coniferyl aldehyde dehydrogenase in the bacterium *Ralstonia eutropha* H16 resulted in a biocatalyst capable of producing ferulic acid from eugenol. During whole-cell biotransformations where 975 µmol eugenol was added as substrate, 915 µmol ferulic acid was produced after 20 h incubation. This equates to a maximum yield of 93.8 % without any further optimization (Overhage *et al*., 2002).

1.8 Practical Applications

Flavoprotein oxidases are seen as valuable biocatalysts for a variety of synthetic applications (Jin *et al*., 2007). They have broad substrate specificity and find application in the production of aromatic compounds such as benzaldehyde, cinnamic aldehyde and vanillin (Overhage *et al*., 2002; Jin *et al*., 2007). Enzymes such as vanillyl-alcohol oxidase and eugenol oxidase in particular are attractive as biocatalysts because of their ability to utilize molecular oxygen as a mild oxidant (Jin *et al*., 2007). This is in contrast to enzymes like *p*-cresol methyl hydroxylase and eugenol hydroxylase that need a proteinous electron acceptor (Jin *et al*., 2007).

Flavours and fragrances are widely used in the food, beverage and cosmetic industries. Most flavours in the world market are obtained by chemical synthesis. The prices of flavours produced *via* different means can vary greatly. On average, the price of vanilla extracted from vanilla beans can range between US\$1200 kg^{-1} and US\$4000 kg^{-1} (Xu *et al*., 2007). Chemically synthesized vanillin costs less than US\$15 kg^{-1}. According to FDA and European legislation, products obtained from biotechnological methods can also be considered as natural. Many studies have focused on the biotechnological production of flavours and fragrances, with focus on the microbial transformation of aromatic compounds (Xu *et al*., 2007). The relatively mild reaction conditions, high

substrate/product specificity to a single isomer and the reduced environmental risks also make the biotechnological approach more attractive.

Vanillin is widely used in food products as a flavour compound and in high concentrations has been shown to have antioxidant properties in mammalian cells (van den Heuvel *et al.,* 2001a). Natural vanillin supplies meet less than 1% of the total demand for vanillin and is produced from glucovanillin when the beans of the *Vanilla plantifolia* orchid are submitted to a multistep curing process (van den Heuvel *et al*., 2001a). The term “natural” can be applied when a product has been derived from a natural raw material via biological (enzymes or whole cells) and/or mild processing tools in the European Union and in the United States. Large numbers of studies have been done in recent years on microorganisms or isolated enzymes for the production of natural vanillin by biosynthesis (van den Heuvel *et al*., 2001a). The biotechnological production of vanillin is typically low yield and it has been reported that this may be due to the toxicity of vanillin and the low tolerance of organisms towards this compound in whole-cell biocatalysis (Overhage *et al*., 2002). Vanillyl-alcohol oxidase has been researched as a biocatalyst for the production of vanillin. Van den Heuvel and co-workers (2001a) investigated the enzymatic production of vanillin using isolated vanillyl-alcohol oxidase, purified from *E. coli* expressing the vanillyl-alcohol oxidase from *P. simplissisimum*. They used different substrates as starting materials including vanillyl alcohol, eugenol, vanillyl amine, capsaicin and creosol.

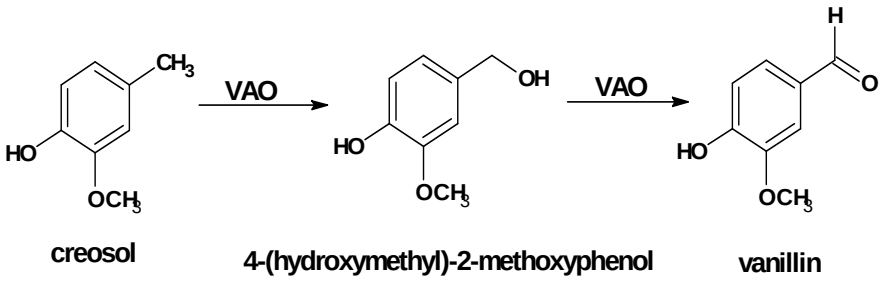

Fig 1.13: The formation of vanillin from creosol by vanillyl-alcohol oxidase.

Creosol is seen as an attractive feedstock and is obtained from creosote. It was found that the conversion of creosol to vanillin by vanillyl-alcohol oxidase reached 100%, but the catalytic efficiency is low (0.07 s^{-1}) when compared to vanillyl alcohol as substrate (3.3 s^{-1}). This is due to the formation of a nonreactive covalent adduct between creosol and the flavin prosthetic group of vanillyl-alcohol oxidase (Fig 1.13). This adduct is more stable at basic pH values. The competitive binding of creosol also inhibits the conversion of the intermediate product, vanillyl alcohol to vanillin. They reported the optimal conditions for production of vanillin by vanillyl-alcohol oxidase with creosol as feedstock to be at a pH of 7.5 and a substrate concentration of 150 µM. Vanillylamine, in contrast, was converted more efficiently at basic pH values between 9.0 and 10.5. The reason for this might possibly be that the binding of vanillylamine does not stimulate phenol deprotonation. Another alternative might be related to the preferred binding phenolate form of vanillylamine (van den Heuvel *et al*., 2001a).

1.9 Conclusion

One of the major differences between VAO and bacterial *p*-alkylphenol and *p*-allylphenol hydroxylases is the ability of VAO to use molecular oxygen as electron acceptor. The bacterial equivalents employ cytochrome domains to relay electrons towards azurin as electron acceptor (Jin *et al.*, 2007). The 8α-histidyl-FAD bond is the most abundant type of mono-covalent binding of FAD and can be found in all kingdoms of life. The covalent 8α-*O*-tyrosyl-FAD linkage present in the *p*-alkylphenol and *p*-allylphenol hydroxylases is on the other hand quite rare and has only been found in bacteria (Heuts *et al*., 2009). The ability of these enzymes to act on a wide range of phenolic substrates and the fact that they do not need cofactor recycling makes them attractive for use in various biocatalytic processes. Despite these advantages, relatively few of these enzymes have been studied. This is specifically the case with the fungal VAOs where only two have been isolated thus far. One of the main reasons for the lack of research into these enzymes might be the difficulty experienced with heterologous expression due to differences in codon usage.

With the above in mind, we set out to find a biocatalyst analogous to VAO in fungi capable of catalysing hydroxylation of alkylphenols and other aromatic compounds in order to produce value-added products.

The initial goals of the study included:

- To search the fungal database of the Broad Institute using BLAST searches in order to identify organisms carrying genes that are homologous to *VAO*.
- To screen for enzymes that would be able to perform these oxidative reactions with aromatic compounds, preferably with alkylphenols as substrates.
- To successfully isolate the gene and extract the mRNA coding for the putative *VAO*.
- To clone and investigate the expression of the putative VAO in different *E. coli* strains.
- To clone and investigate the expression of the putative VAO in the yeasts *Kluyvermyces marxianus* and *Arxula adeninivorans*.
- To compare the levels of activity of the putative VAO with that of the VAO from *P. simplicissimum*

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CHAPTER 2

A vanillyl-alcohol oxidase from *Fusarium moniliforme* – identification, cloning and heterologous expression in *E. coli*

2.1 Introduction

There are currently only two confirmed fungal vanillyl-alcohol oxidases (VAOs), the VAO isolated from *Penicillium simplicissimum* and the VAO from *Byssochlamys fulva*. The genomic sequence of only one of these is available, the VAO from *P. simplicissimum*. Traditionally the discovery of new biocatalysts such as the above mentioned VAOs had been problematic. New enzymes were initially discovered through enrichment of microorganisms under selective conditions. The process was often tedious and lengthy. The advent of recombinant DNA techniques has substantially simplified the process and allows for the production of high quality biocatalysts (Behrens *et al*., 2011).

Techniques such as the metagenome approach circumvents the cultivation of microorganisms completely, instead whole communities in an environment are screened for potential biocatalysts by extracting the genomic DNA from the environment. The DNA is fragmented and cloned yielding metagenome libraries. These libraries can then be screened for the desired activity or alternatively a sequence-based approach can be used in which genes are amplified based on homology to already described enzymes or all the DNA is sequenced (Behrens *et al*., 2011) and an *in silico* approach is followed.

In silico screening offers yet another approach for the discovery of new biocatalysts. Protein and DNA sequences of enzymes and entire organisms are deposited into public databases. If several enzymes with a distinct activity have already been described in literature then an alignment of their protein sequences together with experimentally confirmed activities or specificities can reveal conserved regions within the sequences

and a simple BLAST search can yield new and useful biocatalysts (Behrens *et al*., 2011).

An overwhelming amount of genetic data is currently available in these databases contributing to and simplifying the search for new biocatalysts. The explosion in genetic data really took off with the sequencing of the first eukaryotic genome, that of the yeast *Saccharomyces cerevisiae*, in 1996. It relied upon an international effort consisting of 600 scientists from Europe, North America and Japan. At the time it was the largest genome ever sequenced and was the first complete genome sequence of a eukaryote and only the sixth of any organism to be published (Foster *et al*., 2006; Goffeau *et al*., 1995). In 2002, six years after the publication of the *S. cerevisiae* genome, 111 new genome sequences had been published, of these only two were fungal, the fission yeast *Schizosaccharomyces pombe* and the microsporidian *Encephalitozoon cuniculi* (Foster *et al*., 2006). In other words, fungal sequences at that time represented only 16.5 Mb of 5900 Mb sequenced, a meager 0.3 % of all genomic data available at that stage (Foster *et al*., 2006). Shortly after release of the *S. cerevisiae* sequencing data, workshops were started to discuss genome projects for the filamentous fungi, *Aspergillus nidulans* and *Neurospora crassa.* It was not until the Fungal Genome Initiative (FGI) was formally established following meetings and workshops (initiated in November 2000 by Gerry Fink of the Whitehead Institute, now the Broad Institute), that the momentum in sequencing fungal genomes began to build.

One of the key points discussed in the FGI meetings was that a balanced selection of fungi would maximise the overall value for comparative genomics, evolutionary studies, eukaryotic biology and medical studies. After consultation of the wider fungal community this was the strategy that was presented in the 2002 FGI White Paper (Birren *et al*. 2002) and the three additional FGI proposals that followed (Birren 2003; Birren *et al*. 2003; Birren 2004). The FGI initially succeeded in obtaining funding for 24 fungal genome projects (Fig 2.1). Among the fungal genomes that were initially proposed to be sequenced was *Fusarium graminearum*. It was chosen because of its importance to the

agricultural industry. Since the initial proposal 3 more *Fusarium* species: *F. verticilliodes*, *F. solani* and *F. oxysporum* were also added to the project.

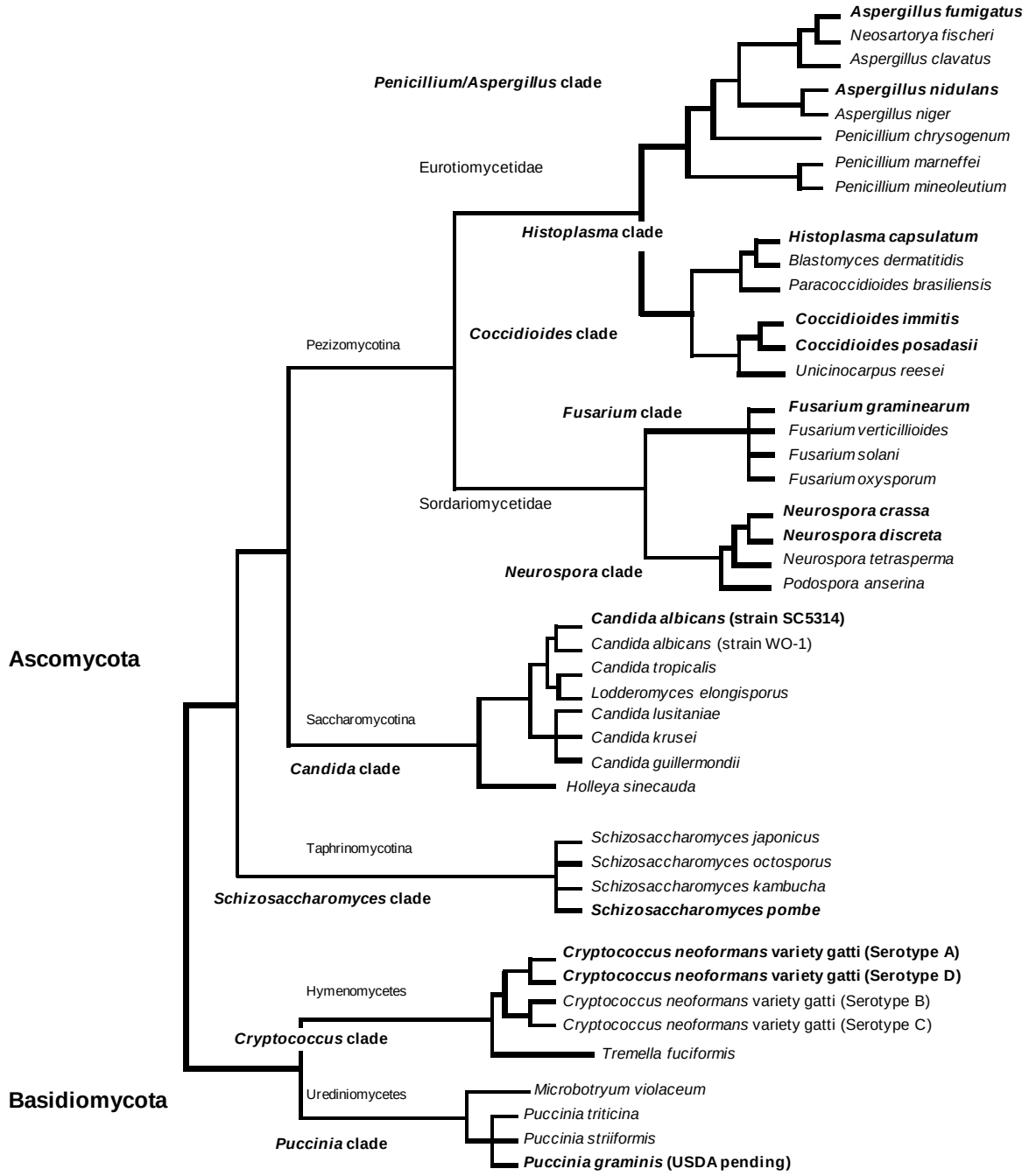

Fig 2.1: Clusters of fungal species proposed by the FGI as candidates for genome sequencing. The tree topology only represents the classification relationships among the selected taxa. The tree branches do not reflect the evolutionary divergence. (Taken from Fink and Lander, 2003).

The genus *Fusarium* contains a number of soil borne species with worldwide distribution, which have been known for a long time as important plant pathogens. Fungi in this genus cause a wide variety of blights, seedling diseases, root rots or wilts on nearly every species of cultivated plant (Fink and Lander, 2003). The most common species, *F. oxysporum* causes vascular wilt disease in a wide variety of economically important crops (Roncero *et al.*, 2003). *Fusarium graminearum* is increasingly becoming a threat to the world's food supply due to recent outbreaks of head blight in cereal crops in Asia, Canada, Europe, and South America. The fungus also causes disease on corn and rice. For many *Fusarium* diseases, effective fungicides and highly resistant plant cultivars are not available. The pathogen poses a two-fold threat: first, infested cereals show significant reduction in seed quality and yield; second, scabby grain is contaminated with mycotoxins, making it unsuitable for food or feed (Roncero *et al.*, 2003).

One group of mycotoxins known as fumonisins pose a very dangerous health threat. *F. verticilloides* and *F. proliferatum* are the main source of fumonisins. Fumonisins are mycotoxins structurally similar to the sphingolipid intermediates sphinganine and sphingosine, which affect sphingolipid metabolism by inhibiting the enzyme ceramide synthase. They cause severe mycotoxicosis in humans and animals, in particular fumonisin B1 (FB1), resulting in chronic and acute diseases and are catalogued as 2B group carcinogens by the IARC (International Agency for Research on Cancer) (Jurado *et al*., 2010).

A number of teleomorph genera have been associated with species of *Fusarium* (Summerell *et al*., 2010). Most of the teleomorphs are members of the Hypocreales in the Ascomycetes although some belong to other fungal orders (Summerell *et al*., 2010).

Over a number of years, most of the more divergent species have been removed from *Fusarium*, leaving clear associations with three teleomorph genera, *Gibberella*, *Haematonectria* and *Albonectria* (Summerell *et al*., 2010). Of the teleomorph genera, *Gibberella* is the most common, being linked with the majority of *Fusarium* species (Table 2.1).

Table 2.1: The anamorphic and the teleopmorphic stages of some of the agriculturally and medically important *Fusarium* species.

Anamorphic stage	Teleomorphic stage
Fusarium oxysporum	Only known by its anamorphic stage
Fusarium verticilloides (synonym *Fusarium moniliforme*)	*Giberella moniliformis*
Fusarium graminearum	*Giberella zeae*
Fusarium solani	*Nectria haematococca*

Although the *Fusarium* genus is mostly known as plant pathogens there have also been studies on its ability to produce lignolytic enzymes and its capability to utilize various aromatic compounds. Different strains of *Fusarium* have been known to have the ability to degrade aromatic compounds. *Fusarium flocciferum* has been shown to be capable of degrading phenolic compounds in a mixture from an initial concentration of 350 $mg.L^{-1}$ to below detection limits within 8 h of incubation (Mendonça *et al.*, 2004). A strain of *Fusarium proliferatum* capable of degrading lignin has been isolated. Evidence has been found that it produces the extracellular enzymes laccase and aryl alcohol oxidase, both of which are involved in lignin degradation processes (Regalado *et al*., 1999). *Fusarium oxysporum* was used in the transformation of aromatic components of olive-mill dry residue which also reduced its phytotoxicity. A laccase-like phenol oxidase has also been purified and partially characterised from *Fusarium oxysporum f. sp. dianthi* (Curir *et al.*, 1997; Sampedro *et al*., 2007). Resting cells of *Fusarium moniliforme* strain MS31 have been reported to convert various alkylbenzenes at the

benzylic position to produce optically active alcohols. The *R* absolute configuration of the products was more abundant. Aromatic compounds with linear side chains were converted to their corresponding alcohols with an enantiomeric excess of 94% to 100%. Very little further oxidation of these alcoholic products was detected (Uzura *et al.*, 2001a; Uzura *et al*., 2001b).

It is clear from the experimental data available in literature that *Fusarium* is capable of utilizing various aromatic compounds. Due to its agricultural importance and the health risks it poses, a wealth of genetic data is also available to us. The aim of the study was to mine the databases and to identify possible vanillyl-alcohol oxidases in *Fusarium* strains, to investigate different culture conditions for optimal enzyme production, to isolate a gene coding for a putative VAO and to successfully express the *VAO* gene in *E. coli*.

2.2 Materials and methods

2.2.1 Microorganisms

Fusarium strains (Table 2.2) were originally received from the Forestry and Agricultural Biotechnology Institute of the University of Pretoria, South Africa. All strains were stored in LN broth (see below) containing glycerol (7% v/v) under liquid N_2 in the MIRCEN yeast culture collection of the University of the Free State, South Africa. Strains were revived by streaking out on YM plates (see below) supplemented with a vitamin solution (1% v/v).

LN broth contained (per liter distilled water): 40 g glucose, 10 g tryptone and 10 g yeast nitrogen base (YNB) containing amino acids and ammonium sulfate. YM solid media contained (per liter water): 10 g peptone (Biolab), 10 g glucose (Biolab), 20 g malt extract (Biolab), 3 g yeast extract (Biolab) and 20 g agar (Biolab).

Table 2.2: Strains received and used in this study:

Strain	Number
Fusarium moniliforme [1]	MRC 6437
Fusarium moniliforme[1]	MRC 6155
Fusarium graminearum	MRC 4712
Fusarium graminearum	MRC 4927
Fusarium oxysporum	MRC 8437
Fusarium oxysporum	MRC 3239
Fusarium verticillioides	Freshly isolated uncatalogued strain

[1]*Fusarium moniliforme* is the older less commonly used synonym for *Fusarium verticillioides,* but these strains were catalogued under this name and will be referred to as *F. moniliforme* throughout this document.

2.2.2 Growth conditions for the cultivation of *Fusarium* spp.

Separate glycerol stocks were made from the freshly revived strains on YM plates and stored at -80 ºC. Strains revived from these stocks for experimental use were streaked out on Bennets agar plates. The freshly streaked out plates were incubated at 25 ºC for 48 h, and then transferred to a liquid medium.

Cultivation in liquid media was, unless stated otherwise, performed in 25 ml Bennets broth (see below) in 250 ml Erlenmeyer flasks for pre cultures and 50 ml Bennets broth in 500 ml Erlenmeyer flasks in the case of the main cultures on a rotary shaker at 180 rpm and 25 ºC (Uzura *et al*., 2001b). Shake-flasks were inoculated with 24 h old Bennets cultures.

Bennets broth contained (per liter water): 10 g glucose (Biolab), 5 g peptone (Biolab), 2 g yeast extract (Biolab), 2 g beef extract (Biolab). For solid medium 7.5 g agar (Biolab) was added.

2.2.3 Induction of VAO activity in *Fusarium* spp.

To study induction of VAO activity, loopfuls of each *Fusarium* strain were transferred from Bennets agar plates to 12.5 ml Bennets broth in 25 ml Erlenmyer flasks. The flasks were incubated at 25 ºC for 24 h, after which 500 µl of culture was transferred (Uzura *et al*., 2001b) to 50 ml of a chemically defined medium (see below) in 500 ml Erlenmeyer flasks. It was decided on using this specific volume to volume ratio in order to achieve maximum aeration within the shake flasks. The medium was supplemented with either vanillyl alcohol (0.1% w/v) (Sigma-Aldrich), anisyl alcohol (0.1% v/v) (Sigma-Aldrich) or veratryl alcohol (Sigma-Aldrich) (0.1% v/v) to serve as possible inducers. The strains were cultivated for 48 h after addition of possible inducers at 25 ºC on a rotary shaker (180 rpm). 500 µl Samples were taken and analyzed for possible product formation and substrate utilization with thin layer chromatography.

The chemically defined medium contained (per liter water): 5 g $NaNO_3$ (Merck), 2 g KH_2PO_4 (Merck), 3 g K_2HPO_4 (Merck), 2 g NaCl (Merck), 0.2 g $MgSO_4.7H_2O$ (Merck), 0.5 g yeast extract (Biolab), 2 ml glycerol (Merck) and 2 ml trace element solution (Uzura *et al*., 2001). The pH of the medium was adjusted to 8.

Trace element solution contained per 100 ml distilled H_2O: 400 mg $MnCl_2.2H_2O$ (Merck), 350 mg $FeCl_2.4H_2O$ (Merck), 200 mg $ZnCl_2$ (Merck), 20 mg $CoCl_2$ (Merck), 20 mg $CuCl_2.2H_2O$ (Merck), 10 mg $Na_2MoO_4.2H_2O$ (Merck) 10 mg $Na_2B_4O_7.10H_2O$ (Merck) and 2 ml concentrated HCl (Merck).

2.2.4 Extraction and thin layer chromatography analysis

Samples (500 µl) were taken at regular intervals and extracted twice with 300 µl ethyl acetate in a microcentrifuge tube. The samples were vortexed for 5 min and the phases were separated by centrifugation (10 000 x *g* for 10 min) and the extracts combined.

For TLC (Thin Layer Chromatography) analysis samples were spotted onto Alugram Sil G/UV_{245} TLC plates (Merck) containing a fluorescent indicator. Plates were developed using a mobile phase consisting of hexane (Merck), diethylether (Merck) and formic acid (Merck) in a 60:35:5 ratio. Plates were visualized under short wavelength UV-light.

2.2.5 Preparation of fungal crude protein extracts for use in activity assays

Strains were harvested after 48 h growth using centrifugation (3000 x *g* for 10 min) and re-suspended in 50 mM potassium phosphate buffer, pH 8. The fungal biomass was disrupted using glass beads and vortexing on ice. Separation of cell debris was achieved by further centrifugation at 10 000 x *g* for 30 min. Protein concentration was determined spectrophotometrically using Bradford reagent (Sigma) and a Beckman Coulter spectrophotometer at 595 nm according to manufacturer's protocol.

2.2.6 Activity assays with crude cell-free extracts from fungi

Crude cell-free extracts were assayed for vanillyl-alcohol oxidase activity using a Beckman Coulter spectrophotometer. The reaction mixtures were made up to a final volume of 1 ml in a cuvette. The reaction was carried out in 50 mM potassium phosphate buffer, pH 8. The final concentration of crude protein in the reaction mixtures was 100 µg/ml. Each reaction mixture contained either vanillyl alcohol (Sigma-Aldrich), eugenol (0.1% v/v) (Sigma-Aldrich), ethylphenol (0.1% w/v) (Sigma-Aldrich) or propylphenol (Sigma-Aldrich) (0.1% v/v) as substrate. Product formation was followed at

340 nm (vanillin formation), 320 nm (coniferyl alcohol formation) and at 270 nm for formation of 1-(4'-hydroxyphenyl) alcohols from ethylphenol and propylphenol. Activities were determined by measuring the increase in absorbance at the relevant wavelengths every 30 s for 5 min. Reaction rates for formation of the following products were calculated using the extinction coefficients given in parentheses: vanillin (ε_{340} = 15.0 mM^{-1} cm^{-1}) from vanillyl alcohol; 4-vinylphenol (ε_{255} = 14.3 mM^{-1} cm^{-1}) and 1-(4-hydroxyphenyl) ethanol (ε_{270} = 1.2 mM^{-1} cm^{-1}) from 4-ethylphenol; 1-(4-hydroxyphenyl)propanol (ε_{270} = 1.3 mM^{-1} cm^{-1}) from propylphenol; coniferyl alcohol (ε_{290} = 3 mM^{-1} cm^{-1}) from eugenol (van den Heuvel *et al*., 1998; van den Heuvel *et al*., 2004).

2.2.7 Nucleic acid isolation

All restriction and modifying enzymes were obtained from Fermentas. Oligonucleotides were obtained from Inqaba Biotechnological Industries and Bioneer Incorporated. PCR amplification was performed using Expand High Fidelity Plus (Roche Applied Sciences) Taq polymerase. PCR and gel-band purification was performed using the Biospin gel extraction kit (Lasec). Subcloning of PCR products was performed using the pGEM-T® Easy Vector (Promega). Nucleic acids were isolated from *Fusarium moniliforme* (MRC 6155 and MRC 6437) following cultivation in 50 ml Erlenmeyer flasks containing a chemically defined medium (see section 2.2.3). The medium was supplemented with veratryl alcohol (0.1% v/v) as inducer and cultivated for 72 h at 30 ℃. Cells were harvested by centrifugation. The pellet was frozen at -70 ℃ for RNA isolation or suspended to a final concentration of 20% (w/v) in 50 mM phosphate buffer, pH 7.5 containing 20% (v/v) glycerol and frozen at -70 ℃ for DNA isolation. Genomic DNA isolation was carried out according to the method described by Labuschagné and Albertyn (2007).

Total RNA isolation entailed grinding 10 g wet cells under liquid nitrogen to a fine powder. The liquid powder (0.5 ml) was transferred to a 1.5 ml pre-cooled microcentrifuge tube. The powder was thawed by the addition of TRIzol®solution

(Invitrogen). Further isolation of total RNA using TRIzol® was carried out according to the manufacturer's instructions (Invitrogen). The isolated total RNA was resuspended in 50 µl formamide and frozen at -70 ℃ for further use.

2.2.8 Cloning of the putative *VAO* gene from *Fusarium moniliforme*

Gene specific primers, VAO-GEN-F and VAO-GEN-R (Table 2.3) were designed based on the sequence of the putative VAO gene from *F. verticillioides* (*FvVAO*) to PCR amplify the putative VAO gene from *Fusarium moniliforme* (*FmVAO*) from genomic DNA (initial denaturation for 2 min at 94 ℃; followed by 30 cycles of 94 ℃ for 30 s, 54 ℃ for 30 s, 72 ℃ for 1.5 min, and a final elongation of 72 ℃ for 10 min).

Table 2.3: Primers used for cloning of the VAO gene from *F. moniliforme*

Primer name	Sequence in 5' to 3' orientation	Restriction sites introduced/comments
VAO-GEN-F	GATGACTACTGTTAATCCTCTTGTTCTGCC	gDNA isolation primer
VAO-GEN-R	GCTACAGTTTTGTAGAGCGCTGTGG	gDNA isolation primer
PVAO-F	GCTAGCATGTCCAAGACACAGGAATTCAGG	*Nhe*I
PVAO-R	GGAAGCTTTTACAGTTTCCAAGTAACATGAC	*Hin*dIII
FVAO-His-F	GCTAGCATGACTACTGTTAATCCTCTTGTTCTGCC	*Nhe*I
FVAO-F1	CCATGGCTACTGTTAATGCTCTTGTTTGCC	*Nco*I
FVAO-F	TCATGACTACTGTTAATGCTCTTGTTTGCC	*Bsp*HI
FVAO-R	GAAGCTT CTACAGTTTTGTAGAGCGCTGTGG	*Hin*dIII

Underlined characters in the primer sequence indicate the introduced restriction sites.

A two-step RT-PCR reaction was used to amplify the cDNA. First-strand cDNA synthesis was performed on total RNA, using Expand Reverse Transcriptase (Roche Applied Science) in combination with primer VAO-GEN-R at 42 ℃ for 1 h, followed by heat inactivation for 2 min at 95 ℃. The confirmed newly-synthesised cDNA was further amplified using a combination of the following primers listed in table 2.3: FVAO-His-F and FVAO-R to create a His-tagged version after cloning into pET28b(+) or FVAO-F1 and FVAO-R to clone without the His-tag (initial denaturation for 2 min at 94 ℃; followed by 30 cycles of 94℃ for 30 s, 54 ℃ for 3 0 s, 72 ℃ for 1.5 min, and a final elongation of 72 ℃ for 10 min). cDNA synthesis was confirmed *via* gel electrophoresis.

The *VAO* gene from *P. simplicissimum (PsVAO)* was received from Prof. M Fraaije to be used as a positive control. It was amplified using the specific primers PVAO-F and PVAO-R under the following PCR conditions: initial denaturation for 2 min at 94℃; followed by 30 cycles of 94 ℃ for 30 s, 54 ℃ for 30 s, 72 ℃ for 1.5 min, and a final elongation of 72 ℃ for 10 min.

PCR products were cloned into pGEM®-T Easy vector (Table 2.4) and transformed into *E. coli* Top 10 or XL10-Gold (Table 2.5), followed by DNA sequencing of two independent clones of both gDNA and cDNA amplicons. Sequencing was initially done by Inqaba Biotech and later in the Molecular Biology Laboratory at the University of the Free State. Sequencing of the samples were done on a Hitachi 3130xl genetic analyser (Applied Biosystems) and samples were prepared using the BigDye terminator cycle sequencing v.3.1 kit (Applied Biosystems).

Table 2.4: Description of vectors used in study.

Plasmid	Description	Source
pGEM®-T Easy	*E. coli* vector used for subcloning. It contains T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the α-peptide region of β-galactosidase. Insertional inactivation of the α-peptide allows for recombinant clone selection *via* colour screening on indicator plates. It also carries an ampicillin resistance marker for selection.	Promega
pSMART® HCKan	*E. coli* vector used for subcloning. It contains SL1 and SR2 RNA polymerase promoters flanking a multiple cloning region. The vector is supplied with blunt dephosphorylated ends and also contains a kanamycin resistance marker for positive selection on plates.	Lucigen
pET-28b(+)	*E. coli* vector for cloning and expression. It carries an N-terminal His•Tag®/thrombin/T7•Tag® configuration plus an optional C-terminal His•Tag sequence. It also carries a kanamycin resistance marker for positive selection on plates and a *lacI* coding sequence for IPTG induction.	Novagen

Sequencing of the cDNA amplicon indicated that mutations had been introduced in the first 99 bp on the 5' end (before the *Bbv*CI restriction site) and in the last 211 bp on the

3' end (after the *Cla*I restriction site). This mutated gene was subsequently referred to as *FmutVAO*. In order to correct these mutations introduced during cDNA synthesis, *FmutVAO* cDNA and *FmVAO* gDNA was digested with *Bbv*CI and *Cla*I. The internal part of *FmutVAO* between the *Bbv*CI and *Cla*I restriction sites was ligated into the *FmVAO* gDNA in pGEM®-T Easy. In order to ligate into pET28b(+) (Table 2.4) a second round of PCR was carried out using the following primer sets: FVAO-His-F and FVAO-R to create a His-tagged version or FVAO-F and FVAO-R to clone without the His-tag. The fragments were initially subcloned into the pSMART vector and transformed into *E. coli* XL-10Gold in order to obtain high yields of vector for sequencing purposes before finally being cloned into pET28b(+). Two pSMART constructs of each type with and without His-tag were submitted for sequencing.

Table 2.5: Table of the different competent *E. coli* strains used in study.

Strain	Genotype	Description	Supplier
Top10	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G.	Strain used for subcloning	Invitrogen
XL10-Gold	*end*A1 *gln*V44 *rec*A1 *thi*-1 *gyr*A96 *rel*A1 *lac* Hte Δ(*mcr*A)183 Δ(*mcr*CB-*hsd*SMR-*mrr*)173 tet^R F'[*pro*AB $lacI^qZ$ΔM15 Tn10(Tet^R Amy Cam^R)]	Strain used for subcloning	Stratagene
BL21(DE3)	F^- ompT gal dcm lon *hsd*S_B(r_B^- m_B^-) λ(DE3 [*lac*I *lac*UV5-T7 gene 1 ind1 *sam*7 *nin*5])	Strain used for expression	Invitrogen
Rosetta-gami2(DE3)pLysS	Δ(*ara-leu*)7697 ΔlacX74 Δ*pho*A *Pvu*II *pho*R *ara*D139 *ahp*C *gal*E *gal*K *rps*L (DE3) F'[lac^+ $lacI^q$ pro] *gor*522::Tn10 trxB pLysSRARE2 (Cam^R, Str^R, Tet^R)	Strain used for expression	Novagen
BL21(DE3) pLYsS-RARE2	F^- ompT gal dcm lon *hsd*S_B(r_B^- m_B^-) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])(Cam^R)	Strain used for expression	Constructed by Dr. Van Marwijk

Plasmids were purified from *E. coli* Top10 using the Biospin plasmid isolation kit according to manufacturers protocol (GE Health) and was treated with *Nhe*I/*Hin*dIII to add the N-terminal His-tag sequence and *Nco*I/*Hin*dIII (*FmutVAO*) or *Bsp*HI/*Hin*dIII (*FmVAO*) to negate the N-terminal His-tag sequence in pET28b(+). The cDNA fragments were ligated into the pET28b(+) vector using T4 DNA ligase to join the compatible ends (*Bsp*HI and *Nco*I give compatible ends).

2.2.9 Transformation of *E. coli* strains

E. coli strains (Table 2.5) were transformed according to the method described by Inoue *et al*. (1990). The transformants were plated out on LB plates containing ampicillin (30 µg/ml) and X-gal (40 µg/ml) for blue/white colony selection in the case of *E. coli* Top10 and XL10-Gold transformed with pGEM®-T Easy vector.

pET-28b(+) constructs were transformed into *E. coli* BL21(DE3) and plated onto LB plates containing kanamycin (30 µg/µl). These constructs were also transformed into *E. coli* Rosetta-gami2(DE3)pLysS and BL21(DE3)pLYsS-RARE2 (Table 2.5) and plated out onto LB plates containing kanamycin (30 µg/ml) and chloramphenicol (34 µg/ml).

All plates were incubated at 37℃ for 24 h before b eing checked for colony formation. LB broth contained per liter water: 5 g Yeast Extract (Biolab), 10 g NaCl (Biolab), 10 g Tryptone (Biolab), pH adjusted to 7.5 with 5 M NaOH (Biolab)(50 µl).

2.2.10 Growth conditions and induction of the recombinant *E. coli* strains expressing *PsVAO* and *FmVAO*

The three *E. coli* strains BL21(DE3), Rosetta-gami 2(DE3)pLysS and BL21(DE3)pLYsS-RARE2 were inoculated into LB broth supplemented with kanamycin (30 µg/ml) or kanamycin (30 µg/ml) and chloramphenicol (30 µg/ml) in the case of the Rosetta-gami2(DE3)pLysS and BL21(DE3)pLYsS-RARE2 strains and cultured overnight at

37℃. Flasks (500 ml) containing 50 ml of the main culture media for optimal aeration (LB broth supplemented with kanamycin (30 µg/ml) or kanamycin (30 µg/ml) and chloramphenicol (30 µg/ml)) were inoculated with 1 % of the pre-culture broth and incubated at 37℃ until an optical density (OD_{600}) of ~ 0.6 was reached. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM to induce the *VAO* genes and the cultures were incubated for 24 h at 30℃.

2.2.11 VAO activity in crude cell-free extracts of the recombinant *E. coli* strains

Recombinant *E. coli* cells were harvested by centrifugation (3000 x *g* for 10 min) and lysed by one pass through a One Shot Cell Disrupter System (Constant Cell Disruption Systems) at 32 kpsi. The resultant lysate was centrifuged at 4000 x *g* for 10 min at 4 ^{o}C. Crude protein extract was obtained through further centrifugation (10 000 x *g* for 30 min). SDS-PAGE analysis was carried out using the Biorad system. Protein concentration was determined using Bradford solution (Sigma-Aldrich) and determined spectrophotometrically at 595 nm according to manufacturer's protocol. Different protein amounts (40 or 80 µg) were used in assays. Stock solutions in ethanol (0.1 M) were prepared for eugenol and vanillyl alcohol.

The assay mixtures (final volume 200 µl) were made up as set out in table 2.6. All assays were done in triplicate.

Table 2.6: Composition of the reaction mixtures used for the crude assay of VAO activity in recombinant *E. coli* strains.

Strain	Protein 40 µg (µl)	Protein 80 µg (µl)	Substrate 1mM (µl)	Buffer (µl) 40 µg protein	Buffer (µl) 80 µg protein
Control	22	44	2	178	156
FmVAO	13	26	2	187	174
PsVAO	12.9	25.8	2	187.1	174.2

All reactions were carried out at 30 ºC. Solutions of 50 mM Tris-HCl pH 8 containing 1 mM of the different substrates were used as blanks. Spectrophotometric analysis was done using a microtiterplate reader (M2 Spectramax). Coniferyl alcohol formation (ε_{300} = 7.4 mM/cm) and vanillin formation (ε_{340} = 8.74 mM/cm) was read every 0.5 min for 15 min. The path length of a 200 µl sample in the microtiter plate was 0.596 cm.

2.2.12 Whole cell analysis of the recombinant *E. coli* strains

Substrates, eugenol (1% v/v), vanillyl alcohol (1% w/v) or ethylphenol (1% w/v), was added 8 h after IPTG addition and samples (500 µl) were taken at regular intervals and extracted twice with 300 µl ethyl acetate (Sigma-Aldrich). The phases were separated by centrifugation (10 000 x *g* for 10 min) and the extracts combined.

Samples were analysed as discussed in section 2.2.4. Spectrophotometric analysis was done using a microtiterplate reader. Vanillin formation was followed at 340 nm and coniferyl alcohol formation at 300 or 320 nm. The concentrations of the products formed were calculated using the following extinction coefficients obtained from standard curves of vanillin and coniferyl alcohol dissolved in ethyl acetate: vanillin ε_{340} = 0.33 mM^{-}

[1] cm^{-1} and coniferyl alcohol ε_{300} = 3.2 mM^{-1} cm^{-1} or at ε_{320} = 0.39 mM^{-1} cm^{-1}). The path length was 0.596 cm.

2.2.13 Phylogenetic Analysis

VAO-like sequences were retrieved from public databases including the Fungal Genome Institute (FGI) of the Broad Institute (BI) and National Center for Biotechnology Information (NCBI) using the BLAST algorithm. Multiple sequence alignments of the amino acid sequences were performed using the MUSCLE EBI web tool (http://www.ebi.ac.uk/Tools/msa/muscle) with the default parameters (Edgar, 2004). Alignments were manually inspected and partial sequences or sequences with obvious frame-shifts removed. The best amino acid substitution model was estimated for tree building using the MEGA 5 software (Kumar *et al*., 2004). The Whelan and Goldman (WAG) model was selected with a discrete Gamma distribution with 5 rate categories and by assuming that a certain fraction of sites are evolutionary invariable. An un-rooted maximum likelihood tree was constructed using MEGA 5. The phylogenetic tree was inferred using Nearest-Neighbor-Interchange (NNI) with bootstrap support for individual nodes calculated on 500 replicates.

2.3 Results and Discussion

2.3.1 Mining for a new biocatalyst: BLAST analysis

BLAST searches of the predicted protein sequences of the Fungal Genome Initiative (FGI) of the Broad Institute (BI) (**http://www.broadinstitute.org/science/projects/fungal-genome-initiative/fungal-genome-initiative),** carried out during the early stages of this project (2007 - 2008), using the *VAO* sequence from *P. simplisissimum* (*PsVAO*) as query, returned hits of possible VAOs from several ascomycetous fungi. A phylogenetic analysis with the highest scoring sequences according to % identity, from the BLAST searches and the confirmed *PsVAO,* revealed

the clustering of predicted proteins from *F. verticilliodes* and *F. oxysporum* with *PsVAO* along with the sequence from *Microsporum gypseum* (Fig: 2.2). The sequence from *F. verticillioides* shared 63% amino acid identity with PsVAO. These results gave us confidence that it should be possible to obtain new VAOs from *Fusarium* spp. and encouraged us to continue screening the seven *Fusarium* strains listed in Table 2.2 for possible VAO activity.

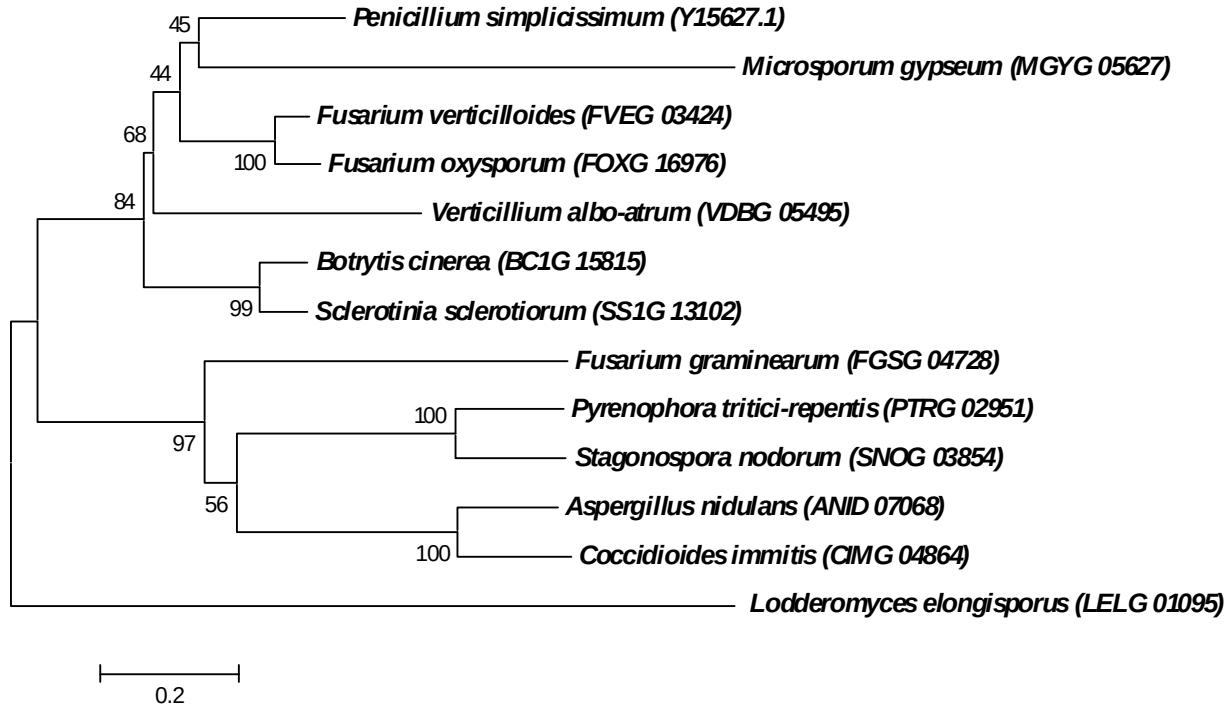

Fig 2.2: Phylogeny of the putative VAOs which gave the highest BLAST scores when the *VAO* from *P. simplicissimum* was used as query in a search of the database of the FGI of the BI done in 2008. FGI locus numbers are included. The Entrez protein accession number is indicated for PsVAO. A ClustalW alignment was done and phylogenetic and molecular evolutionary analysis were conducted using MEGA version 3.1 (Kumar *et al*., 2004), using the neighbor-joining method. Confidence values were estimated from bootstrap

analysis of 1000 replicates. The bar length corresponds to 20% amino acid dissimilarity.

2.3.2 Preliminary screening of *Fusarium* strains for potential VAO activity

PsVAO is capable of accepting a range of different 4-alkylphenols and 4-alkenylphenols as substrates, while it is induced by veratryl alcohol and anisyl alcohol (Fraaije *et al*., 1998a). To determine the ability of the different *Fusarium* strains to catalyze biotransformation of typical substrates of PsVAO, veratryl alcohol and anisyl alcohol, added at 0.1% v/v, were used as possible inducers. Mycelia were harvested after 48 h incubation with these inducers, and acquired biomass was resuspended in 50 mM phosphate buffer (pH 8). Vanillyl alcohol (0.1% w/v), eugenol (0.1% v/v), ethylphenol (0.1% w/v), and propylphenol (0.1% v/v) were added as substrates. Samples were taken routinely and analyzed using TLC.

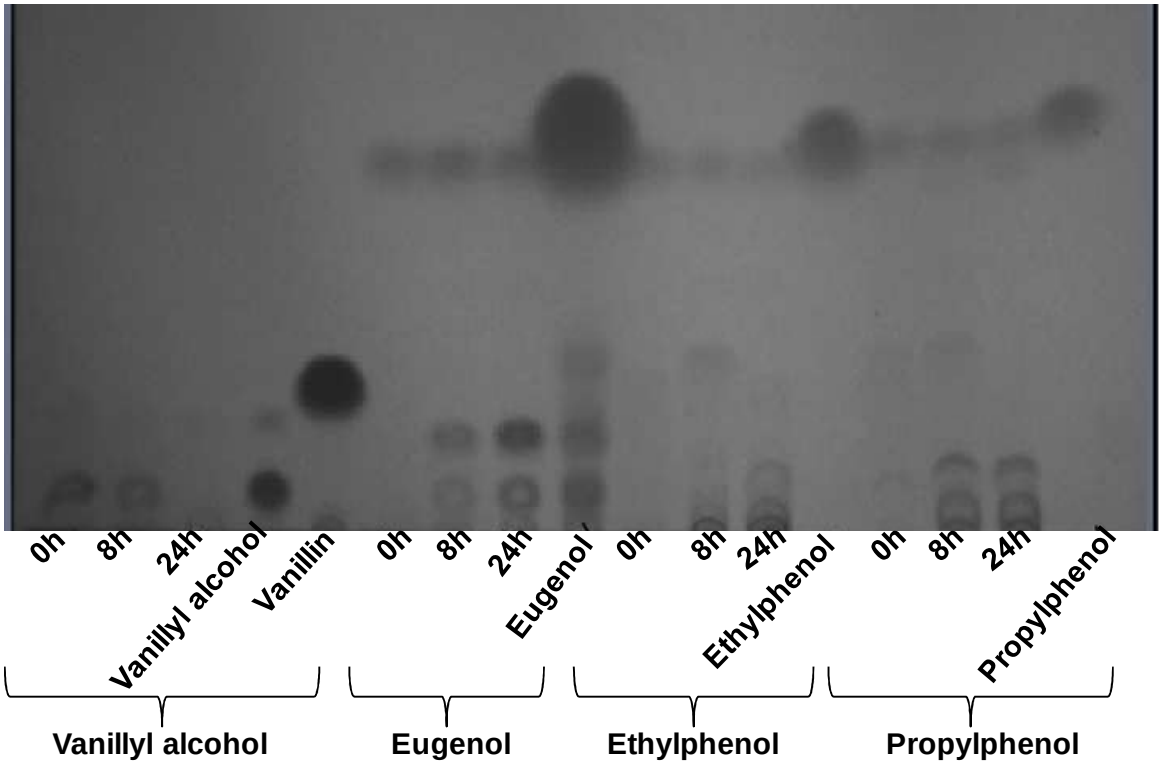

Fig 2.3: Typical results obtained with whole-cell biotransformations using different *Fusarium* strains. TLC of samples taken at 0, 8 and 24 h from *F. moniliforme* MRC 6437 with 0.1% w/v vanillyl alcohol, 0.1% v/v eugenol, 0.1% w/v ethylphenol and 0.1% v/v propylphenol added as substrates. Anisyl alcohol (1% v/v) had been used as inducer.

TLC analysis was used to observe possible product formation by the strains. Product formation from vanillyl alcohol as substrate could not be observed using TLC; however the utilization of substrate was observed. Vanillyl alcohol was completely utilized after 24 h of incubation with all the strains tested. Clear product formation could be observed when eugenol was added as susbtrate (Fig 2.3). The strongest product formation from eugenol was observed with *F. moniliforme* MRC 6437. In the case of ethylphenol as well as propylphenol, faint spots were also observed after 8 h of incubation. Results were similar for the other *Fusarium* strains that were screened.

2.3.3 VAO activity in cell free extracts of *Fusarium* strains

Since vanillyl alcohol was completely degraded by whole mycelia of all the *Fusarium* strains tested, assays with vanillyl alcohol were repeated with crude cell free extracts. Vanillyl alcohol (0.1% w/v), anisyl alcohol (0.1% v/v) and veratryl alcohol (0.1% v/v) were again added as possible inducers and the cultures incubated for 48 h before biomass was harvested. The crude extracts were assayed spectrophotometrically. Vanillyl alcohol (0.1% w/v) was used as substrate and vanillin formation was followed by measuring increase in absorbance at 340 nm every 30 s for 5 min. Protein concentrations were determined using the Bradford solution (Invitrogen). All assays were done in triplicate.

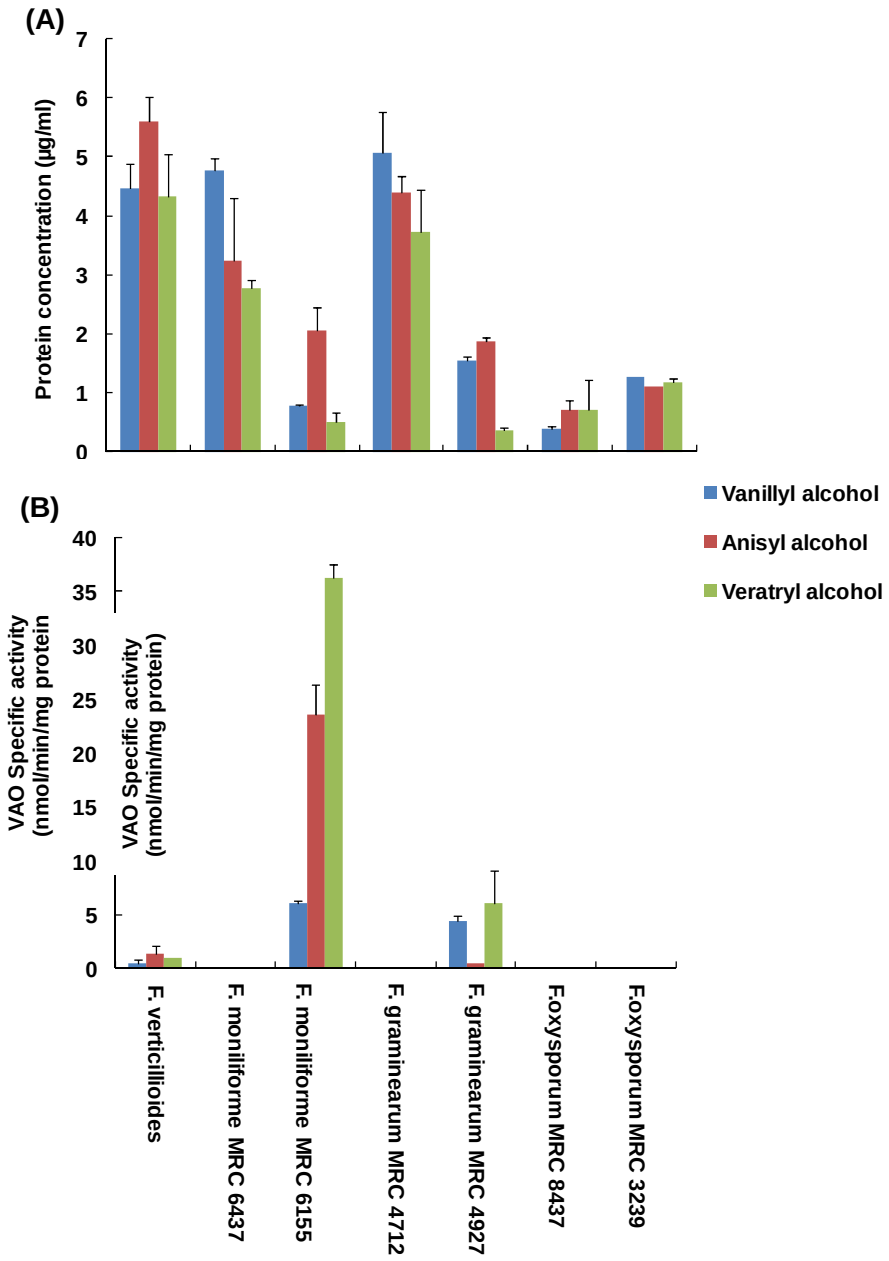

Fig 2.4: Protein concentration (A) and VAO specific activity (B) in cell-free extracts of the different *Fusarium* strains harvested after 48 h of incubation with different inducers added. Vanillyl alcohol (0.1% w/v) was used as the substrate.

From the *Fusarium* strains tested only three, namely *F. verticilliodes*, *F. moniliforme* MRC 6155 and *F. graminearum* MRC 4927 showed detectable activities towards vanillyl alcohol. The specific activities for *F. moniliforme* MRC 6155 was 6, 24 and 36 nmol/min/mg protein when vanillyl alcohol, anisyl alcohol and veratryl alcohol were used respectively. Veratryl alcohol was also the best inducer in the case of *F. graminearum* MRC 4927 giving a specific activity of 6 nmol/min/mg protein. Vanillyl alcohol was the second best inducer with a specific activity of 4 nmol/min/mg protein achieved. *F. verticilliodes* showed the weakest specific activity when compared to the other two strains, 1 nmol/min/mg protein with vanillyl alcohol and veratryl alcohol as inducers. The protein concentration of the two *Fusarium* sp. showing the best activity was low when compared to the other *Fusarium* strains <1 µg/ml when the inducer veratryl alcohol was added.

Experiments to measure VAO activity in cell-free extracts of the *Fusarium* strains were repeated 3 times and specific activities measured in different experiments were quite variable. Table 2.7 summarizes the highest activities observed with each strain. No activity was ever observed with the *Fusarium oxysporum* strains or with *F. graminearum* MRC 4712 despite the fact that he BLAST search with *PsVAO* had revealed a hit for a putative *VAO* in the sequenced genome of *F. oxysporum* and the phylogenetic analysis clustered this putative VAO with the putative VAO from *F. verticilloides.* Closer inspection showed that the protein obtained from the BLAST search was incomplete since it consists of only 187 amino acids. It was therefore not surprising that no activity was detected for this species.

Table 2.7: Comparison of the highest VAO activities obtained with cell-free extracts of the different *Fusarium* strains (measured in 50 mM potassium phosphate buffer pH 8) compared to the VAO activities in crude protein extracts of *P. simplicissimum* (measured in 42 mM glycine/NaOH buffer pH 10, (Fraaije *et al*., 1998b) and *B. fulva* (estimated from eugenol oxidase activity, probably measured at pH 7, no buffer given, Furukawa *et al*., 1999).

Strain	Inducer	Activity Crude extract (nmol/min/mg protein)
F. moniliforme MRC 6347	Vanillyl alcohol	1
F. moniliforme MRC 6347	Anisyl alcohol	0
F. moniliforme MRC 6347	Veratryl alcohol	0
F. moniliforme MRC 6155	Vanillyl alcohol	6
F. moniliforme MRC 6155	Anisyl alcohol	24
F. moniliforme MRC 6155	Veratryl alcohol	36
F. verticilliodes	Vanillyl alcohol	1
F. verticilliodes	Anisyl alcohol	1
F. verticilliodes	Veratryl alcohol	1
F. graminearum MRC 4927	Vanillyl alcohol	4
F. graminearum MRC 4927	Anisyl alcohol	1
F. graminearum MRC 4927	Veratryl alcohol	6
P. simplicissimum	Vanillyl alcohol	<1
P. simplicissimum	Anisyl alcohol	74.5
P. simplicissimum	Veratryl alcohol	69.8
B. fulva	Vanillyl alcohol	~60

It should however be noted that VAO activities of the *Fusarium* spp. were measured at pH 8, while the *P. simplicissimum* activities were measured at pH 10. It has been demonstrated that activity of PsVAO is optimal between pH 9.5 and 10. It has only 70% of its maximum activity at pH 8 (de Jong *et al*., 1992). The pH optimum for the *Fusarium* VAOs has not yet been established.

Activity assays with crude cell-free extracts were also done with eugenol, ethylphenol and propylphenol, but no activity could be detected with any of these substrates with any of the strains tested, even though possible products were observed on TLC during the initial assays with whole mycelia. We thought this might indicate the presence of a true VAO with little or no activity towards the other substrates.

2.3.4 Cloning and sequencing of gDNA and cDNA of the *VAO* gene from *Fusarium moniliforme* MRC 6155

To determine the optimal time of induction of the putative *VAO* genes in *F. moniliforme* strains MRC 6437 and MRC 6155, these strains were cultured for 48, 72 and 96 h, with veratryl alcohol (0.1% w/v) as inducer. The crude extracts were spectrophotometrically assayed in triplicate for VAO activity using vanillyl alcohol as substrate (0.1% w/v).

Surprisingly this time strain *F. moniliforme* MRC 6437, which previously had displayed very little VAO activity after 48 h growth, gave higher levels of specific VAO activity (13 nmol/min/mg protein) after 96 h growth. Activity of *F. moniliforme* MRC 6155 peaked earlier after 72 h growth (12 nmol/min/mg protein) after which it decreased to 9 nmol/min/mg protein (Fig 2.5). VAO production in strain MRC 6437 was apparently slower than in strain MRC 6155, explaining the very low activities observed with strain MRC 6437 in the initial experiments when biomass was harvested after 48 h growth.

It was decided, based on the above results, to harvest *F. moniliforme* MRC 6437 after 96 h growth in the presence of veratryl alcohol (0.1% v/v) and *F. moniliforme* MRC 6155 after 72 h for total RNA isolation.

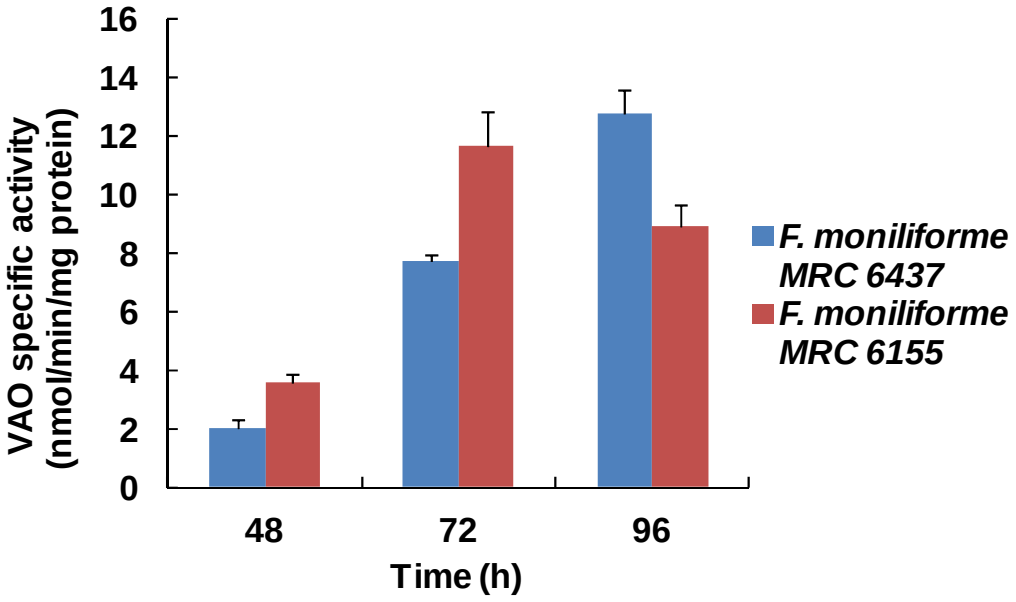

Fig 2.5: Specific activity after 48, 72 and 96 h of induction for *F. moniliforme* MRC 6437 and 6155 with veratryl alcohol as inducer (0.1% v/v) and vanillyl alcohol (0.1% w/v) as substrate.

Total RNA was successfully isolated from both strains of *F. moniliforme* (Fig 2.5), however the RT-PCR only proved successful for *F. moniliforme* MRC 6155, with the expected band of approximately 1.7 kilobases observed on the electrophoresis gel (Fig 2.6).

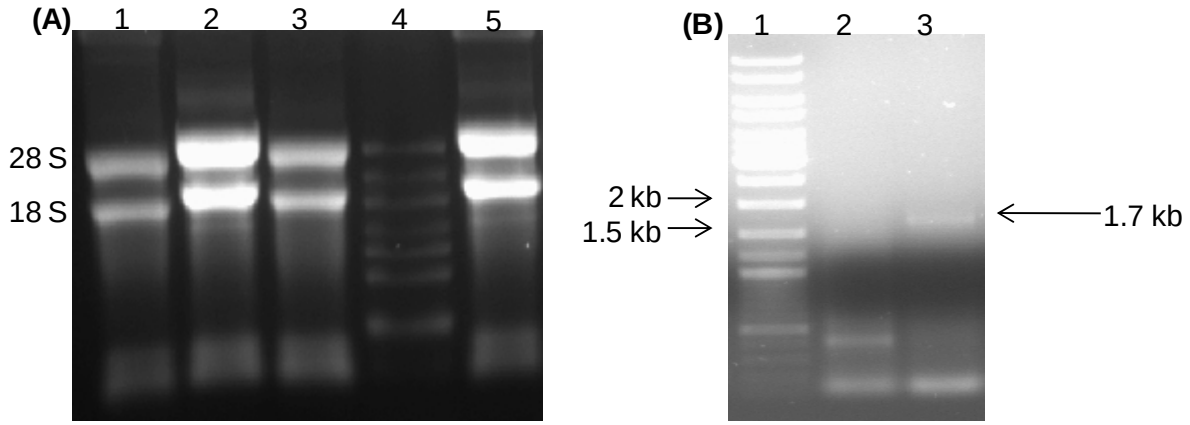

Fig 2.6: (A) Successful isolation of total RNA from *F. moniliforme* MRC 6437 and MRC 6155. Lane 1: *F. moniliforme* MRC 6437, lane 2: *F. moniliforme* MRC 6155, lane 3: *F. moniliforme* MRC 6437, lane 4: kilobase ladder (Fermentas RiboRuler high range RNA ladder for 200-6000 bases) and lane 4: *F. moniliforme* MRC 6155. (B) Amplification of cDNA for putative *VAO* gene using primers FVAO-F1 and FVAO-R. Lane 1: kilobase ladder (Fermentas 1 kb GeneRuler), lane 2: *F. moniliforme* MRC 6437 and lane 3: *F. moniliforme* MRC 6155.

A second PCR was done to introduce the necessary restriction sites for future cloning into pET28b(+) with or without an additional His-tag. The *VAO* gene received from Prof. M. Fraaije was also amplified to introduce the necessary restriction sites for future cloning into pET28b(+) with addition of a His-tag. The resultant products were successfully subcloned into pGEM®-T Easy vector and transformed into *E. coli* Top 10. The positive clones were isolated and plasmids were purified. A restriction digest was run to confirm the presence of the insert (Fig 2.7). Plasmids with correct inserts were sent for sequencing and the *VAO* genes were excised from pGEM®-T Easy and ligated into pET28b(+).

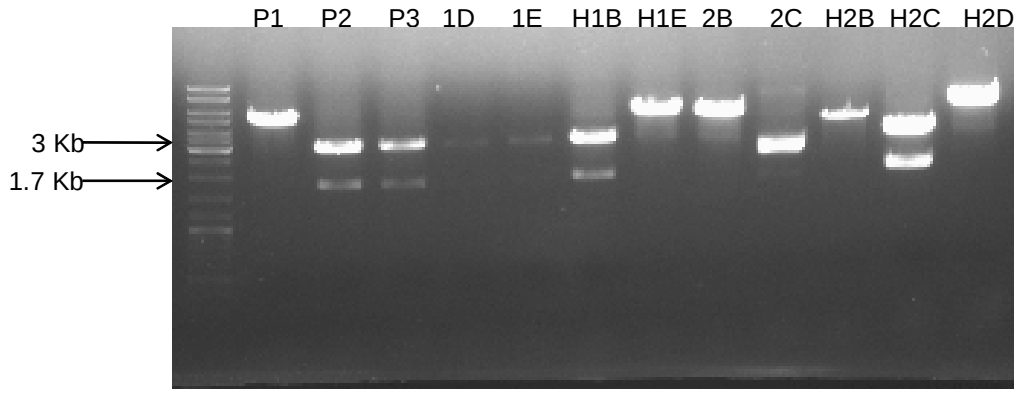

Fig 2.7: Restriction analysis of pGEM®-T Easy Vector containing putative *FmVAO* cDNA inserts and *PsVAO* cDNA insert. Lane 1: Kilobase ladder (Fermentas GeneRuler), lanes P1-P3: *P. simplicissimum PsVAO* constructs digested with *NheI* and *Hin*dIII, lanes 1D, 1E, 2B and 2C: *FmVAO* constructs digested with *NcoI* and *Hin*dIII, lanes H1B, H1E, H2B, H2C and H2D: *FmVAO* constructs digested with *Nhe*I and *Hin*dIII. Clones P2, P3, H1B, H2B and 2C displaying a 1.7 Kb band were selected for further sequencing and cloning.

Comparison of the *FmVAO* cDNA sequence with the gDNA sequence and the *FvVAO* cDNA sequence revealed that the *FmVAO* cDNA had in the first 99 bases on the 5' side one silent mutation and two mutations that would result in amino acid changes, while it had in the last 211 bases on the 3' side 12 silent mutations and four mutations that would result in amino acid changes (see Appendix A). The FVAO-F1 primer used to introduce the *Nco*I restriction site in the initial cDNA synthesis, had introduced a T/A mutation at the second amino acid. It was unfortunate that so many mutations had been introduced during cDNA synthesis. This gene was subsequently referred to as *FmutVAO*. The final sequencing results only became available after the *FmutVAO* cDNA had already been cloned into the expression vector and some expression experiments had already been done (section 2.3.5).

A corrected *FmVAO* cDNA was obtained by cloning the internal part of *FmutVAO* between the *Bbv*CI and *Cla*I restriction sites into the *FmVAO* gDNA in pGEM®-T Easy (Fig 2.8). The corrected cDNA was again PCR amplified to introduce the desired restriction sites for cloning into pET28b(+). To avoid introduction of the T/A mutation at the second amino acid, primer FVAO-F1 was replaced with primer FVAO-F, which introduced a *Bsp*H1 restriction site. Digestion with the *Bsp*H1 restriction enzyme gave a 5' overhang that is compatible with the 3' overhang formed by digestion of the pET28b(+) vector with *Nco*I enzyme. The fragments were initially subcloned into the pSMART vector and two pSMART constructs of each type, with and without His-tag, were submitted for sequencing. Sequencing confirmed that the FmVAO cDNA to be cloned into pET28b(+) was now correct. Unfortunately cloning of the *Bsp*H1/*Hin*dIII fragment into pET28b(+) digested with *Nco*I/*Hin*dIII was not successful. It was finally decided to only continue with the His-tagged construct to investigate expression in *E. coli.*

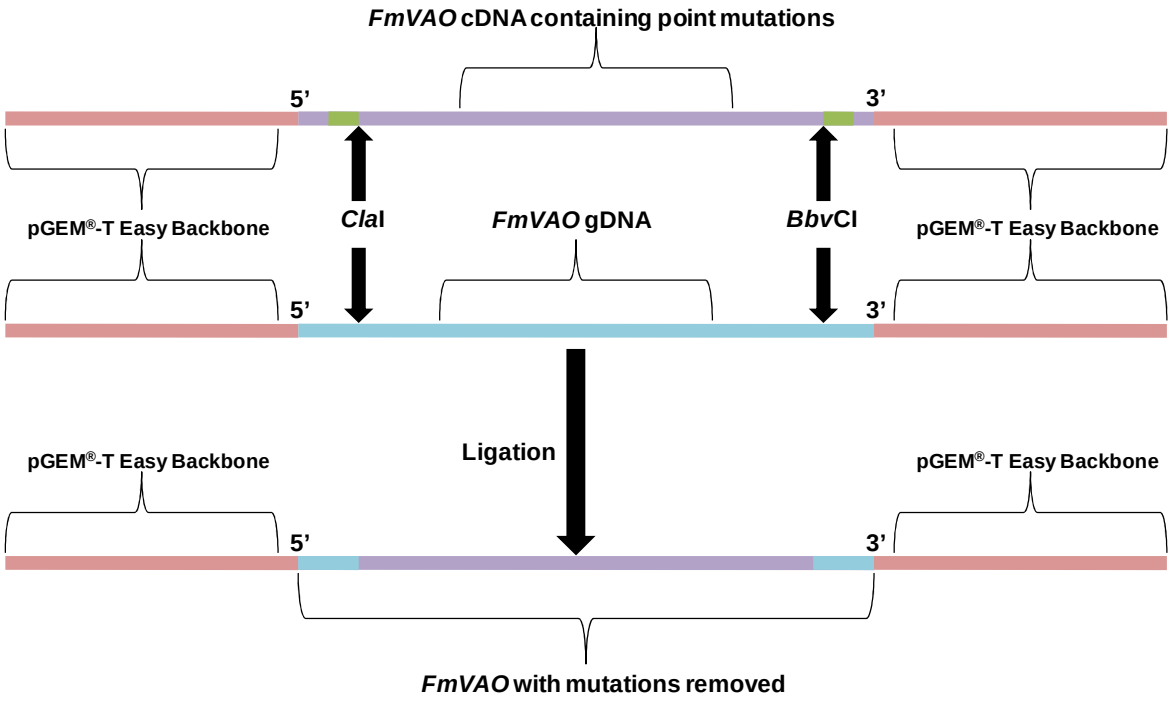

Fig 2.8: Schematic representation of the method used for removal of point mutations caused during cDNA synthesis by PCR.

Comparison of the final correct *FmVAO* cDNA sequence with the *FvVAO* cDNA sequence obtained from the initial BLAST search of the FGI database revealed that the *FmVAO* sequence still contained 31 nucleotide differences 23 are silent mutations and 7 give rise to amino acids changes in the protein sequence (Appendix A). The two gDNA sequences contain a total of 42 nucleotide differences (97.8% identity). The new *FmVAO* is therefore completely novel and the sequences have been submitted to GenBank (NCBI), accession number JQ410355.

A comparison between the gDNA and cDNA of the VAOs from *P. simplicissimum* and *F. moniliforme* (Fig 2.9) reveals that introns are situated in different positions. The *PsVAO* gene also contains 1 extra intron with a total of 5 introns present in the gene itself compared to the 4 introns present in the putative *FmVAO* gene. The position of introns in protein encoding-genes tend to be less conserved in homologous genes in organisms

that diverged 500-1500 million years ago. The relative positions of introns in homologous genes across organisms are usually conserved if the organism diverged less than a couple of hundred million years ago (Bon *et al*., 2003).

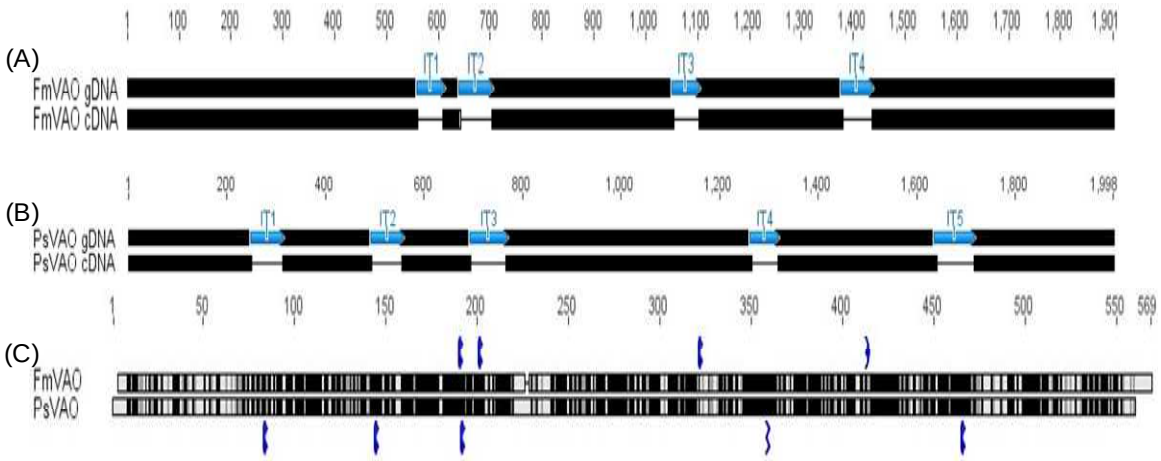

Fig 2.9: Alignment of the gDNA and cDNA sequences of *FmVAO* (A) and *PsVAO* (B) and the alignment of the amino acid sequences of PsVAO and FmVAO (C) showing the positions of introns.

The introns in both genes also adhere to the GT/AG rule. There are exceptions to this rule and in some cases the intron can begin with an AT and end with AC (Bon *et al*., 2003). Since both the *PsVAO* and *FmVAO* genes adhere to the GT/AG rule it serves as a good indication that both genes are excised by the same spliceosome namely the U2 spliceosome. This spliceosome is mostly prevalent in eukaryotes.

2.3.5 Heterologous expression of *PsVAO* and the mutated *FmVAO* in *E. coli* BL21(DE3), *E. coli* Rosetta-gami 2(DE3)pLysS and *E. coli* BL21(DE3) pLysS -RARE2

The use of commercial pET vectors for the expression of *PsVAO* has not been reported. Benen *et al*. (1998) cloned the *PsVAO* cDNA into the pEMBL19 plasmid. This vector did not contain an *E. coli* ribosome binding site, which is routinely supplied by pET vectors. Introduction of a ribosome binding site improved expression levels 7-fold and this vector transformed into *E. coli* TG2 was used in most of the subsequent publications where *E. coli* was used for expression of *PsVAO*. An additional explanation given by Benen *et al*. (1998) for the initial low heterologous expression of *PsVAO* by *E. coli* was the high number of so called "rare" codons in the *PsVAO* gene. These "rare" codons comprise a subset of codons for Arg, Ile, Gly, Leu, and Pro which are infrequently used by *E. coli.* They are considered modulator codons, capable of suppressing expression of the cDNA of a cloned gene.

To compare the occurrence of rare codons in the cDNAs of *PsVAO* and *FmVAO*, a codon calculator was used (http://nihserver.mbi.ucla.edu/RACC/). The results of these analyses are summarized in Table 2.8. It revealed that all the codons that are considered rare (AGG, AGA, CGA coding for arginine; CTA, coding for leucine; ATA coding for isoleucine; CCC, coding for proline) are present in the cDNA of the both genes. There are a total of 40 rare codons in the *PsVAO* cDNA while there are 29 in the *FmVAO* cDNA. There is one occurrence of a double rare codon in the *PsVAO* cDNA and none in the *FmVAO* cDNA, while neither of the cDNAs has triple rare codons.

Table 2.8: Analysis of the occurrence of rare codons in *PsVAO* and *FmVAO*. Results were obtained with the codon calculator available at http://nihserver.mbi.ucla.edu/RACC/. The double rare codons in *PsVAO* are in bold.

Rare codons	*PsVAO* Total Number	Positions (codon numbers)	*FmVAO* Total Number	Positions (codon numbers)
Arg (AGG, AGA, CGA)	17	8 30 104 129 161 183 211 325 350 **364 365** 446 463 482 489 504 526	8	101 180 292 307 319 393 458 478
Leu (CTA)	9	131 217 228 253 283 301 411 478 525	2	206 486
Ile (ATA)	2	250 479	6	22 37 60 248 463 543
Pro (CCC)	12	14 60 96 99 144 223 248 271 284 298 309 333	13	40 51 74 93 110 202 230 243 279 304 374 401 422
Total	40		29	

In the pET system and other *E. coli* expression systems giving high-level expression, the presence of a small number of rare codons often does not severely repress target protein synthesis. However, heterologous protein expression can be very low when a gene encodes clusters of and/or numerous rare *E. coli* codons. The most severe effects on expression have been observed when multiple consecutive rare codons occur near the N-terminus of a coding sequence (Novy *et al*., 2001). Several laboratories have shown that expression yields of proteins whose genes contain rare codons can be dramatically improved when the corresponding tRNA is increased within the host. tRNA levels can be elevated by increasing the copy numbers of the respective tRNA genes. This is typically accomplished by inserting the wild type tRNA gene on a multiple copy plasmid. The tRNA gene is either inserted into the expression vector itself or placed on a compatible plasmid (Novy *et al*., 2001). Various combinations of rare tRNA genes

have been assembled to optimize the expression of genes isolated from organisms with AT or GC rich genomes that have corresponding codon usage bias. One such assembly, the pRIG plasmid, encodes tRNA genes argU, ileX and glyT under their native promoters on a pACYC backbone, which carries the p15a origin of replication. The presence of pRIG in the host strain was shown to significantly enhance the expression of several genes derived from an AT-rich *Plasmodium* genome (Novy *et al*., 2001). To further extend the utility of pRIG for the expression of genes having rare *E. coli* codons, Novagen has added the leuW and proL tRNA genes to create the pRARE plasmid. pRARE encodes tRNA genes for six of the "problematic" rarely used codons encoding Arg, Ile, Gly, Leu and Pro, except for Arg CGA/CGG (Novy *et al*., 2001).

E. coli Rosetta-gami 2(DE3)pLysS (Novagen) is a strain that was specifically designed to enhance expression of eukaryotic proteins that contain codons rarely used in *E. coli*. Rosetta-gami™ host strains are K-12 derivatives. They are sensitive to kanamycin and carry the *trxB* and *gor* mutations for disulfide bond formation in the cytoplasm. The cells carry the chloramphenicol-resistant plasmid, pLYsS-RARE2, which supplies tRNAs for seven rare codons, AUA, AGG, AGA, CUA, CCC, GGA, and CGG under the control of their native promoters (Fig 2.10). The *gor* mutation is selectable on tetracycline. (Novagen Innovations 18; Novy *et al*., 2001). The pLYsS-RARE2 plasmid was isolated from *E. coli* Rosetta-gami 2(DE3)pLysS by Dr. J. van Marwijk in the Molecular Biology laboratory at the UFS and *E. coli* BL21(DE3) was transformed with it to give strain *E. coli* BL21(DE3) pLYsS-RARE2.

To investigate the codon usage problem the pET28b(+) vectors carrying the *PsVAO* and *FmutVAO* genes were transformed into *E. coli* BL21(DE3), *E. coli* Rosetta-gami 2(DE3)pLysS and *E. coli* BL21(DE3) pLYsS-RARE2. Expression experiments were carried out with cultures grown in LB broth and induced with IPTG.

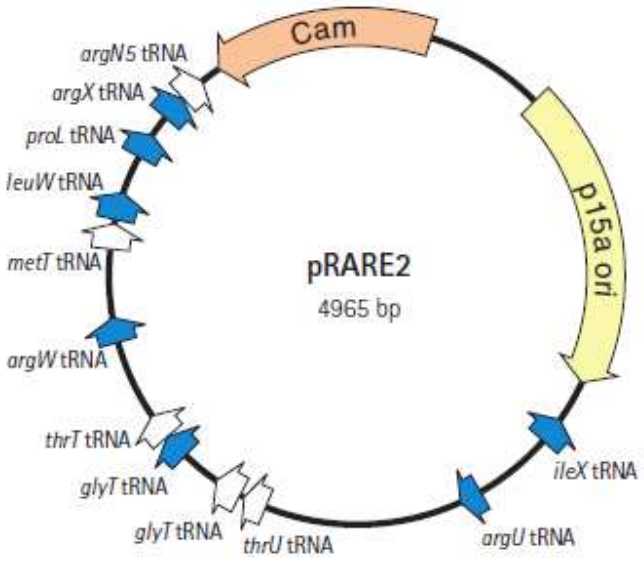

Fig 2.10: Map of the pLYsS-RARE2. Also indicated are the chloramphenicol resistance gene (Cam), origin of replication (p15a ori) and tRNA genes. tRNA genes corresponding to rare codons in *E. coli* are indicated in blue (Taken from Novagen Innovations 18).

Crude protein extracts were prepared from the *E. coli* BL21(DE3), *E. coli* Rossetta-gami 2(DE3)pLysS and *E. coli* BL21(DE3)pLYsS-RARE2 strains expressing *PsVAO* and *FmutVAO* to establish whether any differences in expression could be visualized on SDS-PAGE when compared to a empty vector control (Fig. 2.11 and Fig. 2.12). Analysis of the crude protein extracts of the three different strains with *PsVAO* and *FmutVAO* cloned did not reveal any clear sign of expression in the soluble fractions. The expected size of the protein is ~65 kDa and no clearly distinguishable bands could be observed in this range. A clear band of approximately 60 kDa could however be observed in the insoluble fractions of the *E. coli* Rossetta-gami 2(DE3)pLysS and *E. coli* pLYsS-RARE2 strains expressing *FmutVAO*.

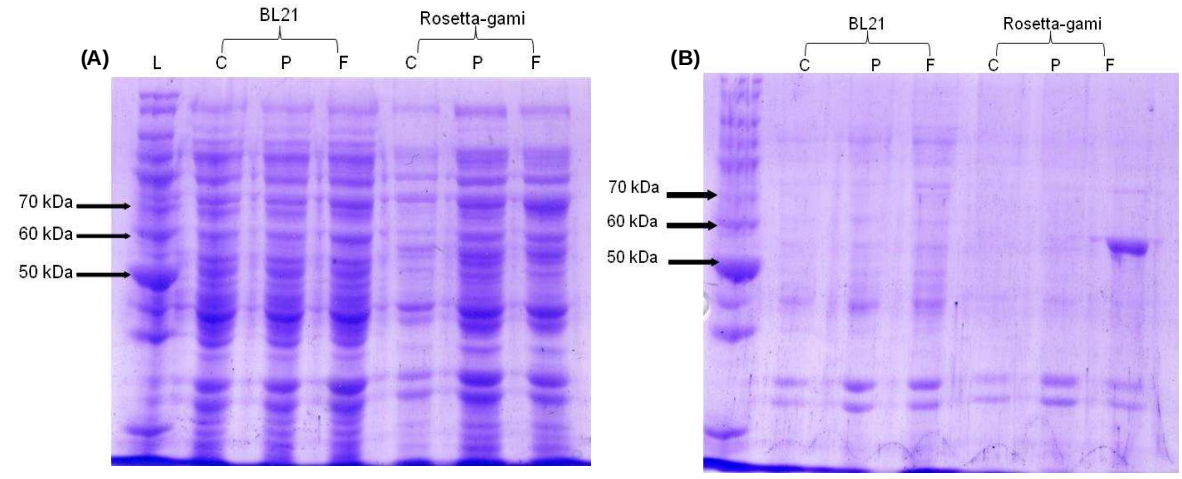

Fig 2.11: SDS-PAGE of the soluble (A) and insoluble (B) fraction of *E. coli* BL21(DE3) and Rosetta-gami 2(DE3)pLysS containing the different *VAO* inserts. SDS-PAGE gel (A) L: Protein ladder (Fermentas), lane C: Control, lane P: PsVAO, lane F: FmutVAO.

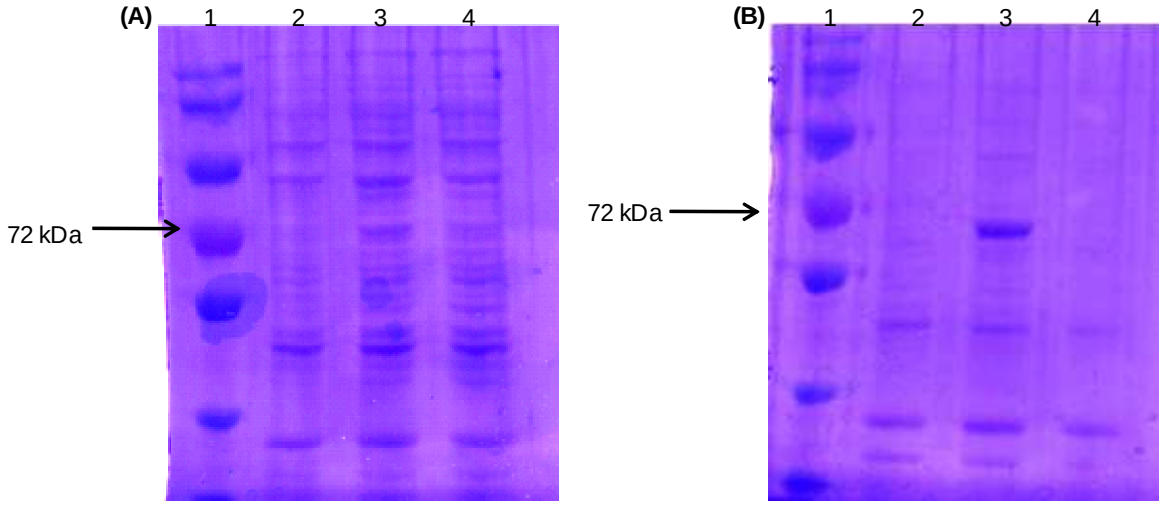

Fig 2.12: SDS-PAGE of the soluble (A) and insoluble (B) fraction of *E. coli* BL21(DE3) containing the different *VAO* inserts. SDS-PAGE gel (A) Lane 1: Protein ladder (Fermentas), lane 2: Control, lane 3: FmutVAO, lane 4: PsVAO (B) Lane 1: Protein ladder (Fermentas), lane 2: PsVAO, lane 3: FmutVAO, lane 4: Control

Whole cell biotransformations were carried out using the different *E.coli* strains expressing *PsVAO* and *FmutVAO* in order to compare VAO activity. Eugenol (1%, v/v) was used as the substrate and the coniferyl alcohol formed as product was monitored at 320 nm using UV spectroscopy. No activity was observed with transformants expressing *FmutVAO* and this result combined with the sequencing results (section 2.3.4) and the observation that the FmutVAO protein was expressed in the insoluble fractions, urged us to correct the gene as described above.

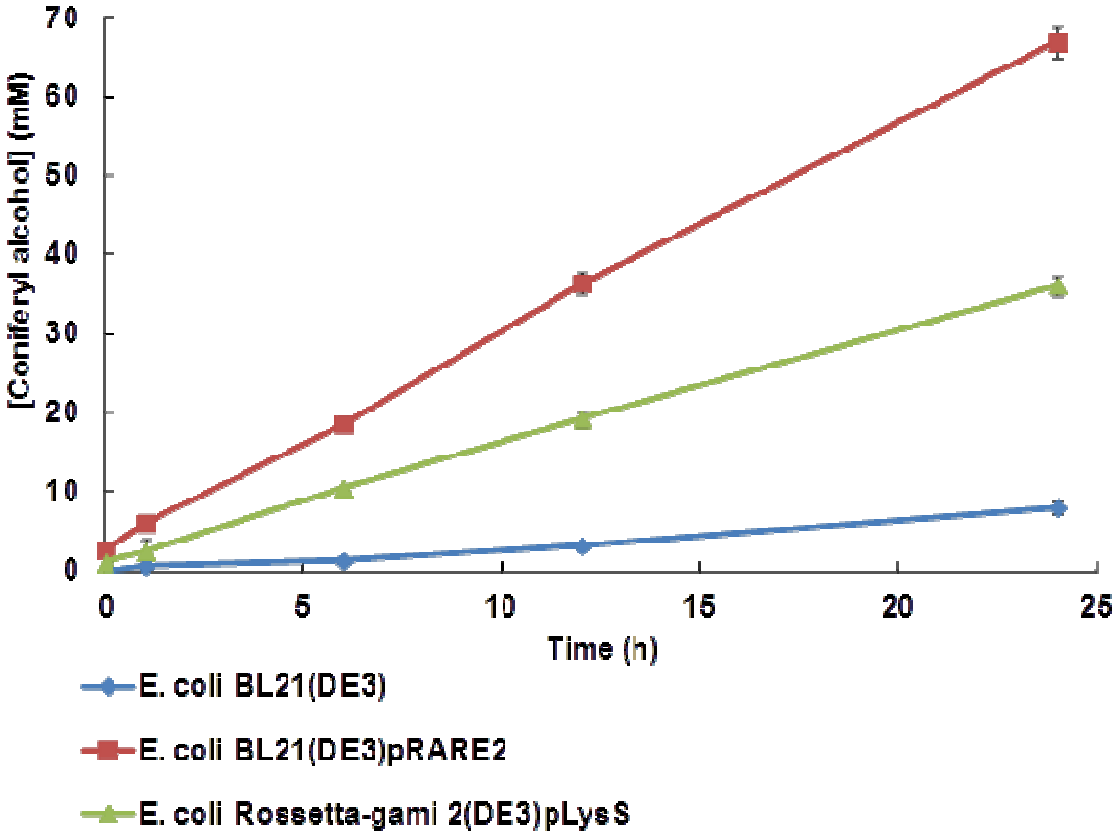

Fig 2.13: Whole cell biotransformations of eugenol (1% v/v) carried out with different *E.coli* strains producing PsVAO. Samples were taken on a regular basis and extracted with ethyl acetate. Coniferyl alcohol formation was monitored spectrophotometrically at 320nm.

Expression of *PsVAO* in *E. coli* BL21(DE3)pLYsS-RARE2 greatly improved the activity. Complete conversion of 65 mM eugenol (*i.e*. 66 mM coniferyl alcohol measured) was obtained after 24 h of incubation with substrate. *PsVAO* expressed in *E. coli* Rossetta-gami 2(DE3)pLysS gave a maximum coniferyl alcohol concentration of 36 mM (~ 55 % conversion) after 24 h while *PsVAO* expressed in *E.coli* BL21(DE3) performed the worst with a maximum coniferyl alcohol concentration of 7.9 mM obtained after 24 h (Fig 2.13). These results clearly revealed the positive impact that the pLYsS-RARE2 has on the expression of *PsVAO* in *E. coli*.

2.3.6 Heterologous expression of *PsVAO* and the corrected *FmVAO* in *E. coli* BL21(DE3) pLYsS-RARE2

Expression of the corrected *FmVAO* gene was eventually only tested in the *E. coli* BL21(DE3)pLYsS-RARE2 strain and compared with expression of *PsVAO* in this strain. Crude protein extracts were again prepared for all the *E. coli* BL21(DE3)pLYsS-RARE2 strains to determine whether expression of specifically the corrected *FmVAO* could be visualized on SDS-PAGE.

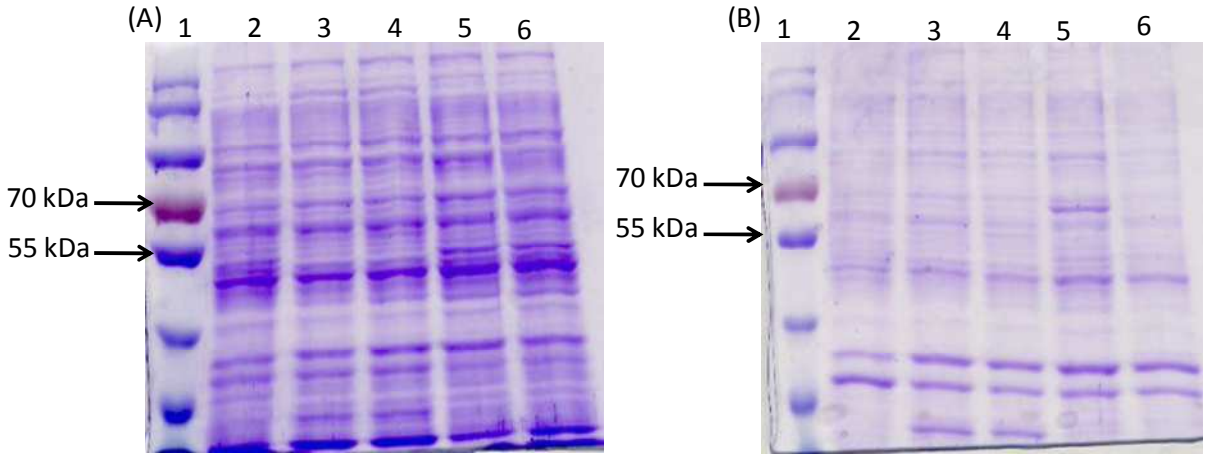

Fig 2.14: SDS-PAGE of the soluble (A) and insoluble (B) fraction of *E. coli* BL21(DE3)pLYsS-RARE2 containing the different *VAO* inserts. Lane 1: Protein ladder, lane 2: PsVAO, lane 3: FmVAO (no histag), lane 4: FmVAO (histagged), lane 5: FmutVAO, lane 6: Control containing empty vector.

The results were similar to previous results for SDS gels and no clear protein band corresponding to the VAO's could be identified in the soluble fractions. Also, as with the previous gels, a band at approximately 60 kDa was only visualised in the insoluble fractions from the strain expressing the *FmutVAO* gene (Fig 2.14).

A second round of biotransformations of eugenol and vanillyl alcohol were carried out to compare activity of whole cells of *E. coli* BL21(DE3)pLYsS-RARE2 producing PsVAO and FmVAO. Extractions were done with ethyl acetate and analysis was done with TLC and UV spectrophotometry. All samples were taken in triplicate.

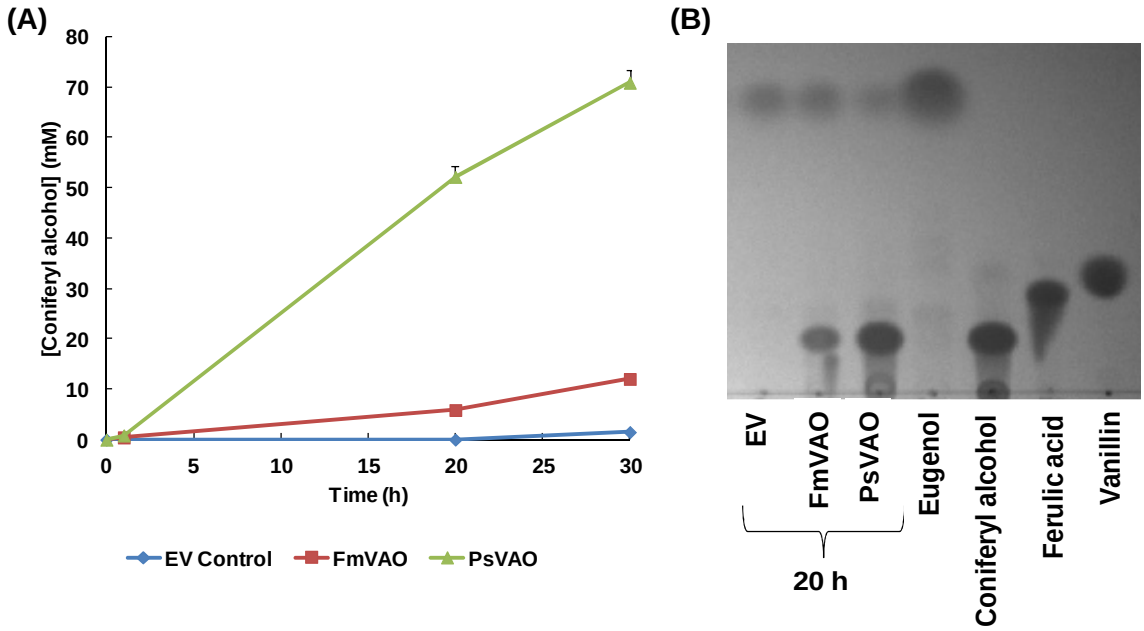

Fig 2.15: The formation of coniferyl alcohol by *E. coli* BL21(DE3)pLYsS-RARE2 containing empty pET28b(+) control (EV), *FmVAO* and *PsVAO*. Eugenol was added as substrate at a concentration of 1% (v/v). Formation of product (coniferyl alcohol) was followed using UV spectroscopy (A) and TLC (B).

Whole cell biotransformations revealed that after 30 h of incubation the strain producing PsVAO had produced 70 mM coniferyl alcohol, indicating complete conversion of the eugenol added, with the UV assay possibly giving a slight overestimation of coniferyl alcohol concentration (Fig 2.15). The specific eugenol oxidase activity of this PsVAO producing strain was 11.0 U/g dry weight, which was approximately the same as in the previous experiment. The maximum product concentration obtained with the strain producing FmVAO was 12 mM coniferyl alcohol. The specific eugenol oxidase activity of the strain producing FmVAO was 2.2 U/g dry weight (Table 2.9). TLC analysis of samples taken after 20 h confirmed the higher activity of the PsVAO producing strain.

Table 2.9: Specific activity of *PsVAO* and *FmVAO* expressed in *E. coli* BL21(DE3)pLYsS-RARE2 during a whole cell biotransformation using eugenol (1 v/v) as substrate.

***E. coli* strains**	**VAO gene expressed**	**Eugenol Specific activity (U/ g dry weight)**	**Vanillyl alcohol Specific activity (U/ g dry weight)**
BL21(DE3)	*PsVAO*	1.9	not done
Rossetta-gami 2(DE3)pLysS	*PsVAO*	8.7	not done
	PsVAO	13.9	not done
BL21(DE3)pRARE 2	*PsVAO*	11.0	6.1
	FmVAO	2.2	5.7

UV analysis of the whole cell biotransformations of vanillyl alcohol with PsVAO and FmVAO expressed in *E. coli* BL21(DE3) pLYsS-RARE2, showed that PsVAO produced 34 mM vanillin after 30 h while FmVAO produced only 14 mM. The specific activities over the first 6 h were however not so different with the PsVAO producing strain having a specific activity of 6.1 U/g dry weight and the FmVAO producing strain (5.7 U/g dry weight). TLC analysis again confirmed the UV assays showing that there was after 20 h around 50% conversion by PsVAO and less vanillin formed by FmVAO. Both TLC and UV analysis also showed a small amount of vanillin formed by the empty vector control strain.

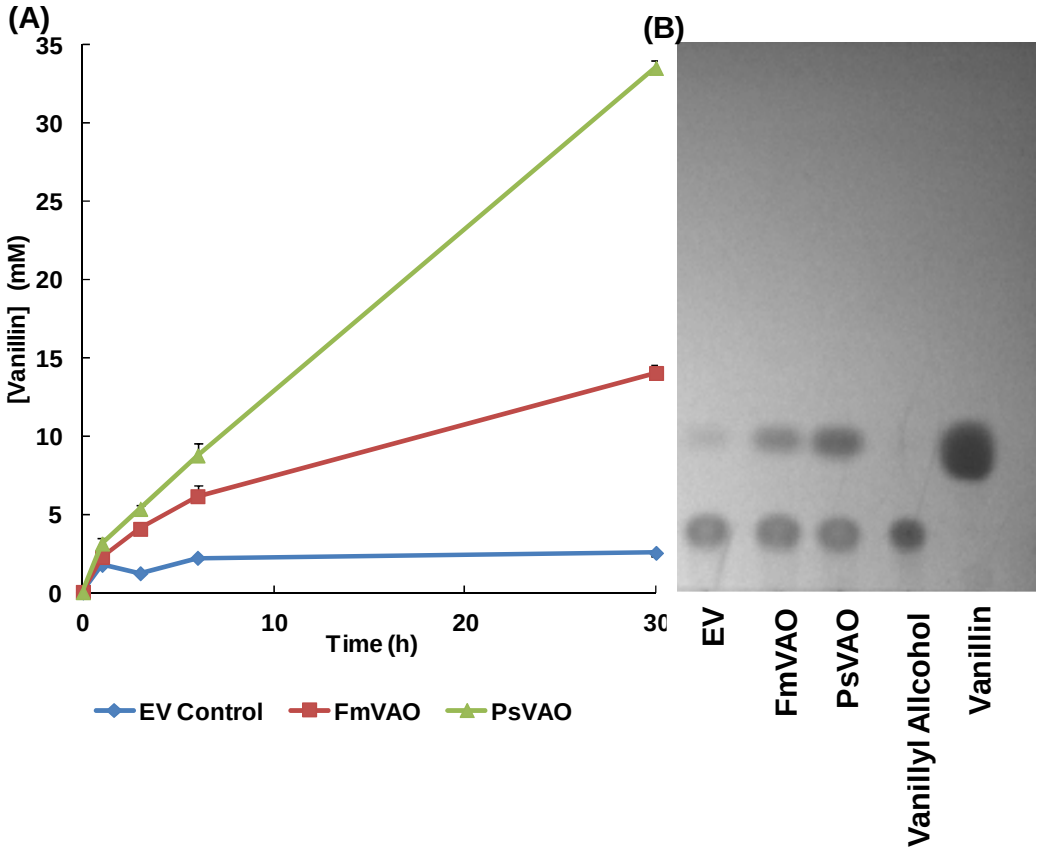

Fig 2.16: The formation of vanillin by *E. coli* BL21 pLYsS-RARE2 containing empty pET28b(+) control (EV), *FmVAO*, *PsVAO*. Vanillyl alcohol was added as substrate at a concentration of 1% (w/v). Formation of product (vanillin) was followed using UV spectroscopy (A) as well as visually with TLC (B).

Whole cell biotransformation of ethylphenol (1% w/v) was tested with the strains producing PsVAO and FmVAO. Samples taken at regular intervals were extracted with ethyl acetate and analyzed with TLC. The formation of an unidentified product could be observed for both *PsVAO* and *FmVAO* after 2 hours of incubation. This product is most likely 1-(4-hydroxyphenyl)alcohol, since it has been established that PsVAO produces mainly 1-(4-hydroxyphenyl)alcohol from ethylphenol (van den Heuvel *et al*., 1998). Formation of product appeared to be higher for the *FmVAO* strain. It is known that PsVAO is subject to inhibition by 4-vinylphenol (van den Heuvel *et al*., 1998). It

appeared however as if the reaction with both strains stopped within the first six hours, since product concentrations did not increase after the first 2 h (Fig 2.17).

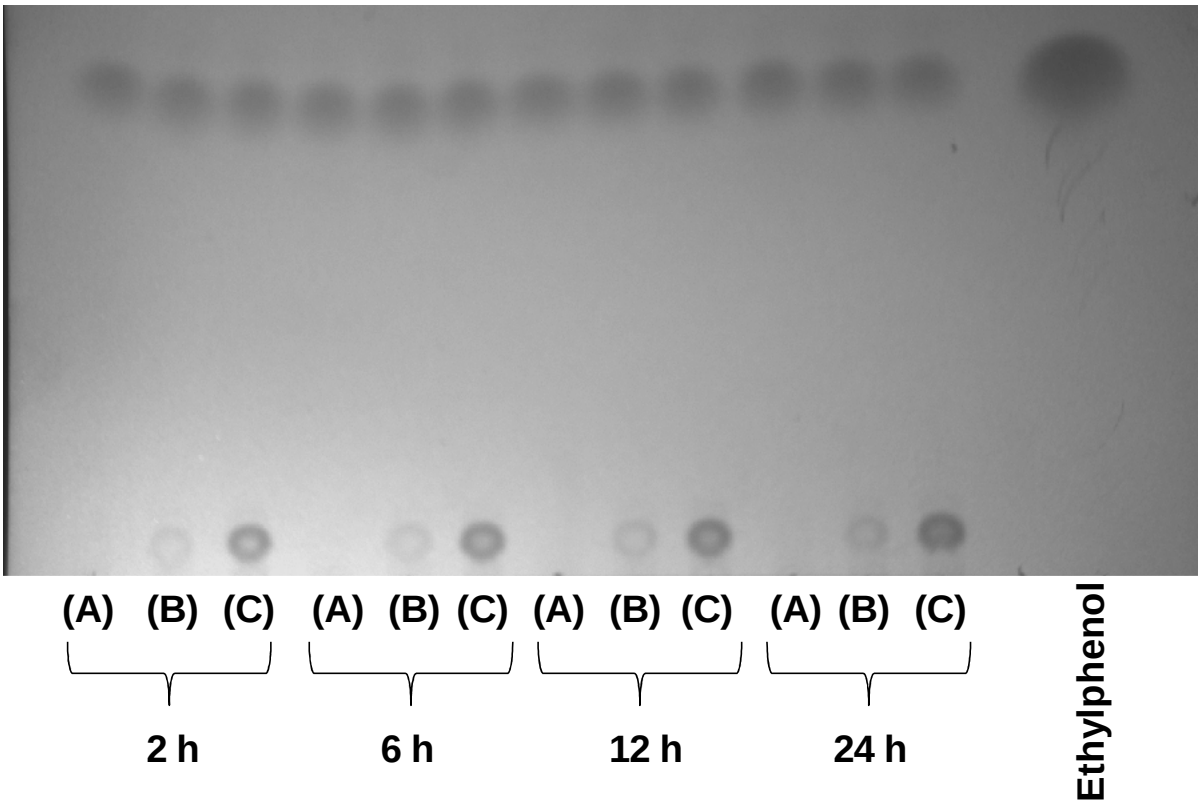

Fig 2.17: Biotransformation of ethylphenol (1 % w/v) by an (A) empty vector control strain of *E. coli* BL21(DE3)pLYsS-RARE2 as well as strains producing (B) PsVAOand (C) FmVAO.

2.3.7 Assays with crude cell-free extracts of *E. coli* BL21(DE3)pLYsS-RARE2 expressing *PsVAO* and *FmVAO*

E. coli BL21(DE3) pLYsS-RARE2 strains were used to evaluate the activity of PsVAO and FmVAO in crude cell-free extracts. The activity was evaluated using both vanillyl alcohol and eugenol as described in section 2.2.11. Results obtained from these experiments revealed that PsVAO had higher specific activity with both substrates that

were tested. The specific activity of PsVAO when eugenol was used as substrate was 0.030 U/mg protein as compared to 0.005 U/mg protein obtained with FmVAO (Table 2.10). With vanillyl alcohol as substrate PsVAO specific activity was 1.6 fold higher (0.028 U/mg prt) when compared to that of FmVAO (0.018 U/mg protein). Benen *et al*. (1998) obtained 0.017 U/mg protein vanillyl alcohol oxidase activity in cell free extracts of *E. coli* TG2 expressing *PsVAO* while Overhage *et al,*. (2003) obtained 0.02 U/mg protein with *E. coli* XL1-Blue(pSK*vao*mP*cal*Am*cal*B) with *PsVAO* cloned under a *lac* promoter using IPTG induction.

Table 2.10: Summary of the specific activity of *PsVAO* and *FmVAO* with eugenol and vanillyl alcohol as substrates.

Strains	Substrate	Protein concentration	Specific activity
		µg/ml	U/mg prt
PsVAO	Eugenol	200	0.030
	Eugenol	400	0.025
	Vanillyl alcohol	400	0.028
FmVAO	Eugenol	200	0.005
	Eugenol	400	0.005
	Vanillyl alcohol	400	0.018

FmVAO evidently prefers vanillyl alcohol as substrate with its activity towards vanillyl alcohol almost four times higher than activity towards eugenol. In our assays PsVAO activities towards eugenol and vanillyl alcohol were within experimental error the same (Table 2.10). Based on k_{cat} values reported in literature activity of wild-type PsVAO isolated from *P. simplicissimum* towards eugenol is approximately double the activity towards vanillyl alcohol (Fraaije *et al*., 1998a), while eugenol oxidase activity of recombinant PsVAO expressed in *E. coli* was almost nine time higher than vanillyl alcohol oxidase activity (van den Heuvel *et al*., 2004). The reason for these differences is not clear, except that assays were done in different buffers and at different pH values.

2.3.8 Comparison of the amino acid sequence of FmVAO with that of other enzymes with confirmed vanillyl alcohol and/or eugenol oxidase/dehydrogenase activity

We know now that FmVAO is an active VAO that prefers vanillyl alcohol to eugenol at substrate concentrations of 1 mM (v/v). The other two fungal VAOs described to date in fact prefer eugenol as substrate. The translated protein sequence of *FmVAO* was aligned to that of similar enzymes from the VAO family with confirmed activity towards vanillyl alcohol or eugenol (Fig. 2.18). Proteins included in the alignment are PsVAO, eugenol oxidase (EUGO) from *Rhodococcus* strain sp. RHA1, eugenol dehydrogenase (EUGH) from *P. putida* and para-cresol methyl hydroxylase (PCMH) from *Pseudomonas* sp. The FvVAO sequence obtained from the initial BLAST database search was also included for comparison. TBLASTN searches of the *F. verticilliodes* genome with PsVAO yielded a second protein with a lower BLAST score. This putative protein can be considered a paralogue of FvVAO and we named it FvVAOpara. This protein was also included in the alignment.

The alignment shows that these proteins all share a highly conserved PCMH-type FAD-binding domain in the N-terminal half. There are four residues in the active site of PsVAO that are critical for the reaction mechanism (Fig. 2.19) and deemed essential for activity (van den Heuvel *et al*., 2000). These are Asp170, Tyr108, Tyr503 and Arg504. Three of these residues (Tyr108, Tyr503, Arg504) are conserved in FmVAO and FvVAO as well as the other four aligned proteins. Asp170 is replaced by an Ala in FvVAOpara and PCMH and by Thr in EUGH. Asp170 is involved in the deprotonation of the substrate during catalysis and thus plays a crucial role in the functionality of these enzymes. It also plays a role in autocatalytic covalent flavinilation of PsVAO (see later). Its replacement by non-acidic residues in PCMH and EUGH, both dehydrogenases with a slightly different reaction mechanism, and also in FvVAOpara might indicate that FvVAOpara is not an oxidase, but a dehydrogenase. In PsVAO three residues were shown to be important in covalent binding of FAD, namely His422 that forms the covalent link to the C8α-atom of the isoalloxazine ring of the FAD as well as His61 and

Asp170 that have been proposed to be responsible for autocatalytic flavinylation. Independent mutations of His61 and Asp170 in PsVAO largely abolished covalent binding of FAD and yielded enzymes that were less active (Fraaije *et al*., 2003). His422 (PsVAO numbering) is conserved in all the aligned proteins except PCMH and EUGH. It is known that covalent linkage to FAD in PCMH is through Tyr384 (PCMH numbering), which is also present in EUGH. Interestingly His61 is only present in PsVAO. It has already been established that covalent flavinylation of EUGO is also an autocatalytic process (Jin *et al*., 2007). We have not established covalent flavinylation of FmVAO, but since the level of activity appears very similar to that of PsVAO we can assume that it is also covalently flavinilated. It thus appears that in these other enzymes without a His61 residue but with a conserved His422 (PsVAO numbering) autocatalytic flavinilation is catalyzed by different residues. PsVAO has been shown to have a dimer-dimer interacting loop. These dimer-dimer interacting loops are often involved in conformational changes within the enzyme and play a role in the accessibility of the substrate towards the enzyme (Mattevi *et al*., 1997). Based on the alignment FvVAO and FmVAO can also be expected to have such dimer-dimer interacting loops, but it is absent from the other aligned proteins.

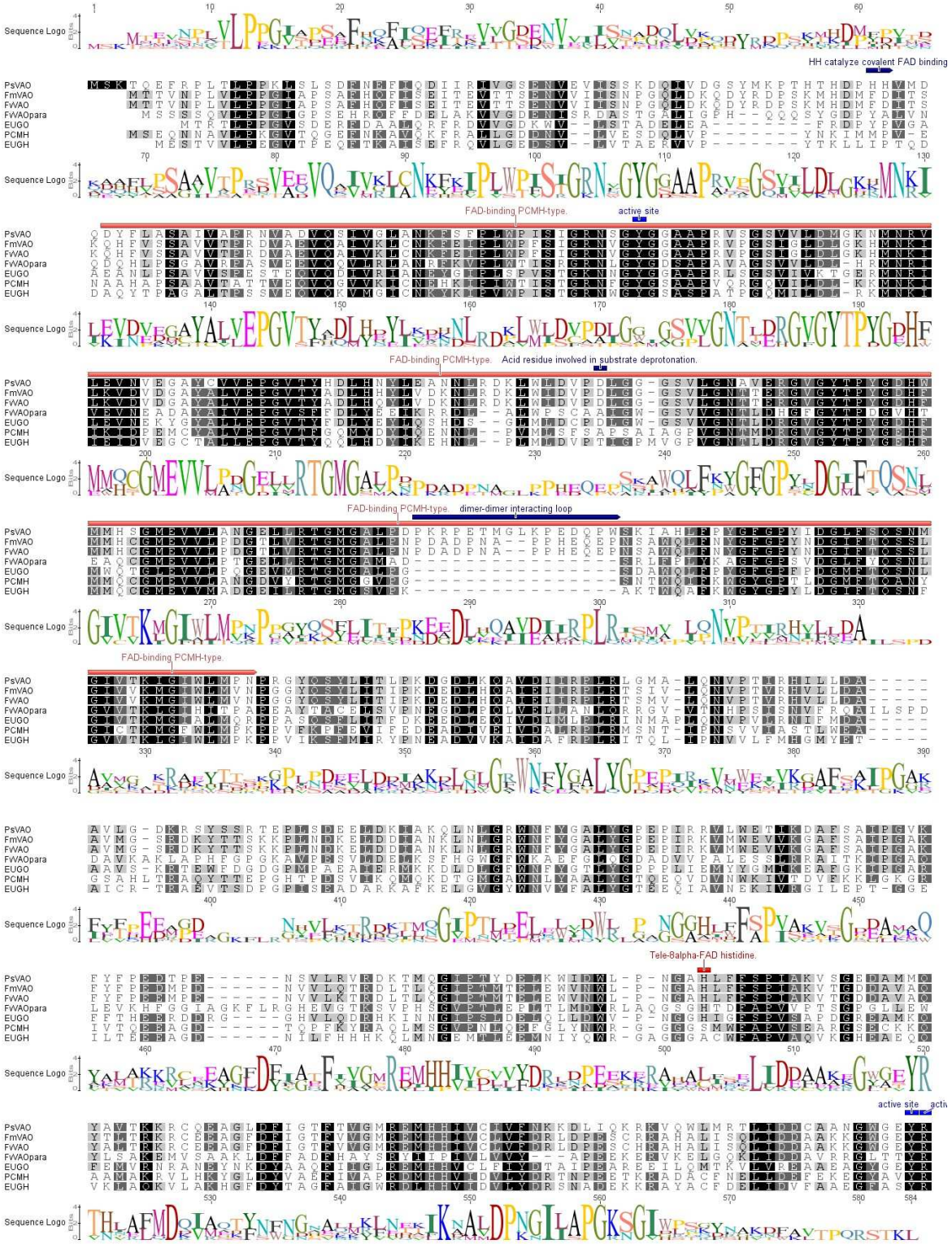
Sequence Logo
HH catalyze covalent FAD binding
PsVAO
FmVAO
FvAO
FvAOpara
EUGO
PCMH
EUGH
FAD-binding PCMH-type.
active site
Acid residue involved in substrate deprotonation.
dimer-dimer interacting loop
Tele-8alpha-FAD histidine.

Fig 2.18: Alignment of the FmVAO, FvVAO and FvVAOpara protein sequences with sequences of similar enzymes from the VAO family with confirmed activity towards vanillyl alcohol or eugenol namely PsVAO, eugenol oxidase (EUGO), eugenol dehydrogenase (EUGH) and *para*-cresol methyl hydroxylase (PCMH). A MUSCLE alignment was done in Geneious Pro 5.5.5 using default settings and it was also used to generate the annotated alignment with catalytically important residues labeled.

2.3.9 Putative VAOs from currently available genome sequences

Seventy two sequences of VAO-like proteins obtained through BLAST searches of several databases were submitted to a MUSCLE alignment (Appendix B) which was used to explore the phylogenetic relationship of FvVAO with closely related sequences using an unrooted maximum likelihood phylogenetic tree. The phylogenetic tree was inferred based on the full length amino acid sequences. Based on the topology of the tree, we subdivided the sequences into five clusters (I-V) (Fig. 2.20). The conservation in the different clusters of the catalytically essential residues identified in PsVAO (Asp170, Tyr108, Tyr503 and Arg504) as well as the histidine involved in the covalent 8α-*N3*-histidyl FAD linkage (His422) are logged in Table 2.11 (Fig. 2.19). Of the four catalytically essential residues only the two tyrosine residues and the arginine residue are conserved throughout most of the seventy two sequences. The multiple alignments further showed either the presence of the tyrosine or the histidine involved in the covalent bond with the flavin co-factor. The fourth catalytically-essential residue of PsVAO, Asp170, is the least conserved with alanine, threonine and serine more prevalent in the corresponding position (Appendix B).

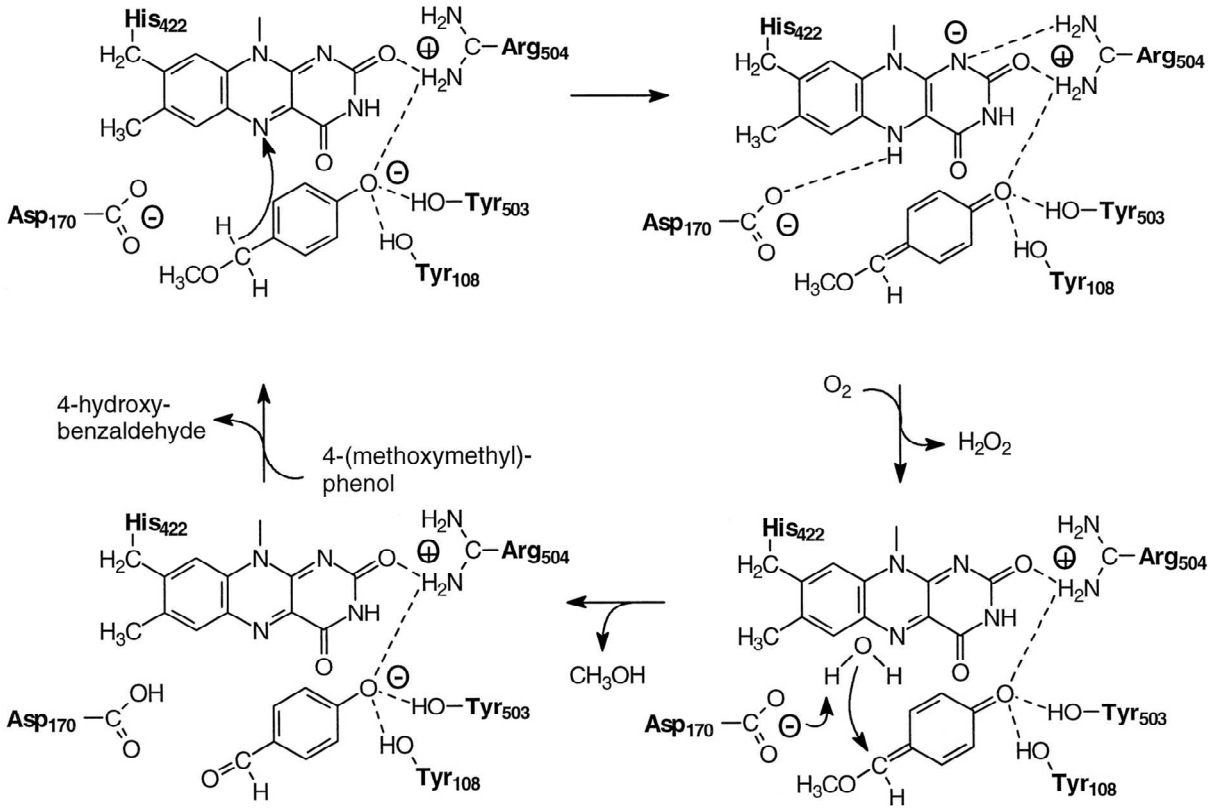

Fig 2.19: Proposed role of Asp170 in the VAO-mediated conversion of 4-(methoxymethyl)phenol (4, 7, 44). (Taken from van den Heuvel *et al*., 2000).

Cluster I contains both the eugenol hydroxylase (EUGH) and *p*-cresol methylhydroxylase (PCMH) from *Pseudomonas*. The multiple alignment shows the tyrosine residue involved in the 8α-*O*-tyrosyl FAD linkage to be conserved within this group. Members of this cluster are restricted to only bacterial proteins. Cluster II shows early divergence from Cluster I and contains the VAOs from *P. simplicissimum* and *F. verticillioides*. Except for two sequences, that of *Talaromyces stipitatus* and *Fusarium solani*, the histidine residue involved in the 8α-*N*3-histidyl-FAD linkage is conserved within this group, suggesting early divergence between the different modes of covalent linkages of the FAD cofactor.

Cluster II shows the relatedness of eugenol oxidase (EUGO) from *Rhodococcus jostii* to the prototypic fungal VAOs which populates most of this cluster. Cluster II contains mostly proteins from *Penicillium* and *Fusarium* species and shows further divergence and the evolution of the dimer-dimer interacting loop within cluster IIB. Both the *FvVAO* as well as the *PsVAO* clusters into this smaller subgroup of proteins containing the dimer-dimer interacting loop. It is known from other flavin dependant oxidases that such loops are responsible for changing conformation during the catalytic cycle and so controls the accessibility of the catalytic site to the substrate (Mattevi *et al*., 1997).

Clusters III and IV contain only VAO-like proteins from bacteria, notably *Burkholderia* species. A second group of fungal VAO-like proteins are found within Cluster V. This cluster includes the *F. verticillioides* paralogue (FvVAOpara) as well as VAO-like proteins from 9 other fungal genera including *Aspergillus* and *Fusarium*. Although the histidine residue implicated in the covalent histidyl-FAD linkage is conserved, members of this entire fungal sub-cluster, as was observed with the FvVAOpara, do not contain the aspartic acid essential for catalysis and covalent flavinylation. The activity of substrate specificity and the reaction mechanism is unkown and it will be worthwhile investigating, given that these enzymes are more prevalent in fungi than the confirmed VAOs of cluster IIB.

Table 2.11: Summary of the presence of catalytically- and structurally-conserved residues identified in PsVAO within the phylogenetically distinct clusters. Residues are numbered according to PsVAO. Grey shading indicates presence of a conserved residue.

	Cluster				
	I	II	III	IV	V
Tyr108					
Asp170					
His422*					
Tyr503					
Arg504					

* Members of Cluster I that lack the conserved His422 have a 8α-*O*-tyrosyl FAD linkage through a conserved tyrosine (Tyr384 in PCMH)

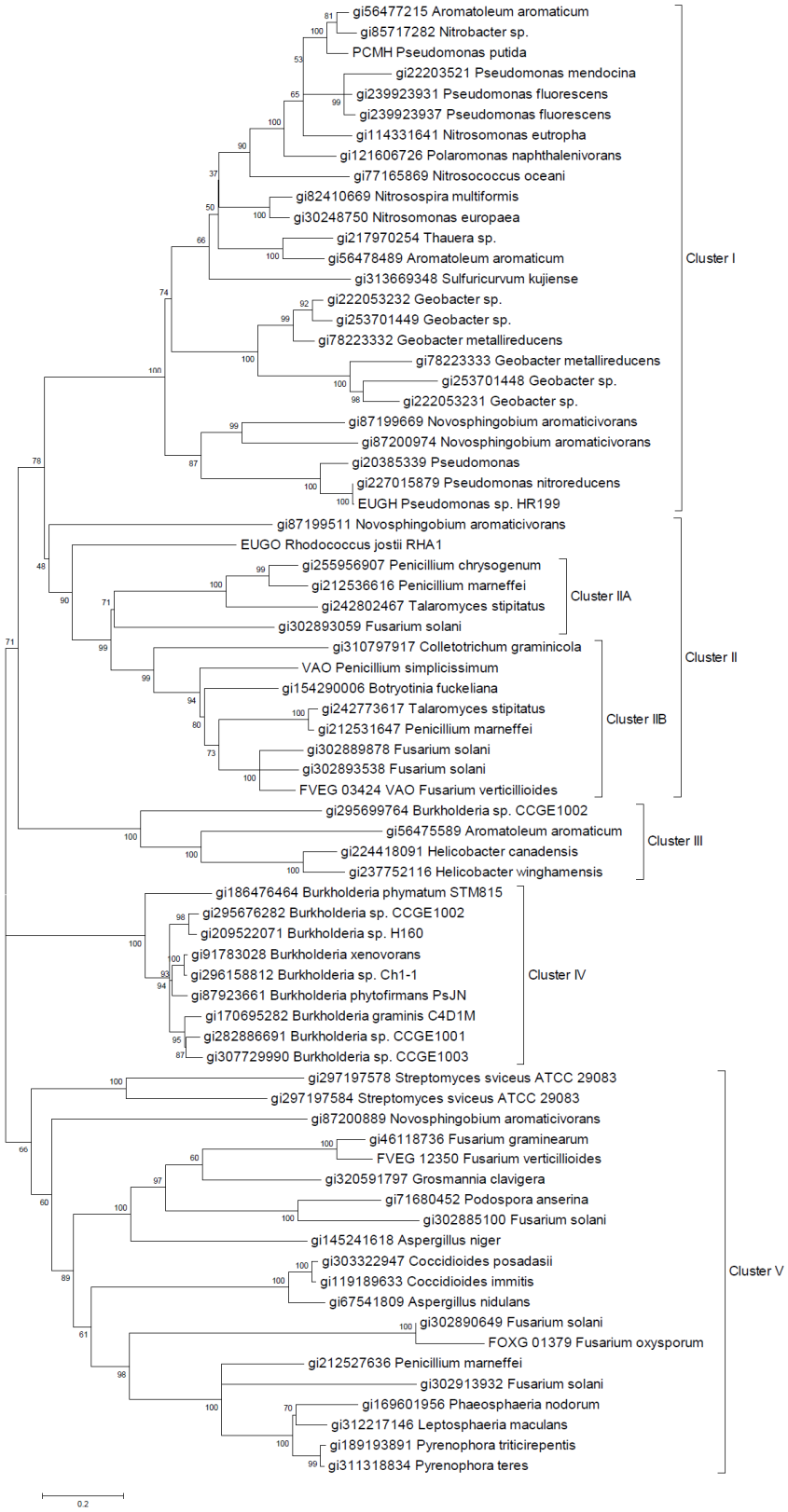
gi56477215 Aromatoleum aromaticum
gi85717282 Nitrobacter sp.
PCMH Pseudomonas putida
gi22203521 Pseudomonas mendocina
gi239923931 Pseudomonas fluorescens
gi239923937 Pseudomonas fluorescens
gi114331641 Nitrosomonas eutropha
gi121606726 Polaromonas naphthalenivorans
gi77165869 Nitrosococcus oceani
gi82410669 Nitrosospira multiformis
gi30248750 Nitrosomonas europaea
gi217970254 Thauera sp.
gi56478489 Aromatoleum aromaticum
gi313669348 Sulfuricurvum kujiense
gi222053232 Geobacter sp.
gi253701449 Geobacter sp.
gi78223332 Geobacter metallireducens
gi78223333 Geobacter metallireducens
gi253701448 Geobacter sp.
gi222053231 Geobacter sp.
gi87199669 Novosphingobium aromaticivorans
gi87200974 Novosphingobium aromaticivorans
gi20385339 Pseudomonas
gi227015879 Pseudomonas nitroreducens
EUGH Pseudomonas sp. HR199
Cluster I
gi87199511 Novosphingobium aromaticivorans
EUGO Rhodococcus jostii RHA1
gi255956907 Penicillium chrysogenum
gi212536616 Penicillium marneffei
gi242802467 Talaromyces stipitatus
gi302893059 Fusarium solani
Cluster IIA
gi310797917 Colletotrichum graminicola
VAO Penicillium simplicissimum
gi154290006 Botryotinia fuckeliana
gi242773617 Talaromyces stipitatus
gi212531647 Penicillium marneffei
gi302889878 Fusarium solani
gi302893538 Fusarium solani
FVEG 03424 VAO Fusarium verticillioides
Cluster IIB
Cluster II
gi295699764 Burkholderia sp. CCGE1002
gi56475589 Aromatoleum aromaticum
gi224418091 Helicobacter canadensis
gi237752116 Helicobacter winghamensis
Cluster III
gi186476464 Burkholderia phymatum STM815
gi295676282 Burkholderia sp. CCGE1002
gi209522071 Burkholderia sp. H160
gi91783028 Burkholderia xenovorans
gi296158812 Burkholderia sp. Ch1-1
gi87923661 Burkholderia phytofirmans PsJN
gi170695282 Burkholderia graminis C4D1M
gi282886691 Burkholderia sp. CCGE1001
gi307729990 Burkholderia sp. CCGE1003
Cluster IV
gi297197578 Streptomyces sviceus ATCC 29083
gi297197584 Streptomyces sviceus ATCC 29083
gi87200889 Novosphingobium aromaticivorans
gi46118736 Fusarium graminearum
FVEG 12350 Fusarium verticillioides
gi320591797 Grosmannia clavigera
gi71680452 Podospora anserina
gi302885100 Fusarium solani
gi145241618 Aspergillus niger
gi303322947 Coccidioides posadasii
gi119189633 Coccidioides immitis
gi67541809 Aspergillus nidulans
gi302890649 Fusarium solani
FOXG 01379 Fusarium oxysporum
gi212527636 Penicillium marneffei
gi302913932 Fusarium solani
gi169601956 Phaeosphaeria nodorum
gi312217146 Leptosphaeria maculans
gi189193891 Pyrenophora triticirepentis
gi311318834 Pyrenophora teres
Cluster V
0.2

Fig 2.20: Molecular Phylogenetic anaylsis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Whelan And Goldman model (Whelan and Goldman, 2001. The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.0560). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 1.6315% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 72 amino acid sequences. All ambiguous positions were removed for each sequence pair. There were a total of 874 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2007). Multiple sequence alignments of the amino acid sequences were performed using the MUSCLE EBI web tool (http://www.ebi.ac.uk/tool/msa/muscle) with the default paramaters (Edgar, 2004).

2.4. Conclusion

Despite the very interesting, unique and useful reactions catalyzed by vanillyl-alcohol oxidases, there were at the beginning of this project only two confirmed fungal VAOs, those from *Penicillium simplicissimum and Byssochlamys fulva*, and only the gDNA and cDNA sequences of the one from *P. simplicissimum,* which we call *PsVAO,* were available in public available databases. By combining activity information from assays carried out with whole cells and crude cell free extracts of seven *Fusarium* strains from three species (*Fusarium oxysporum, Fusarium verticilloides* (synonym *Fusarium moniliforme*) and *Fusarium graminearum*) with genome sequence information obtained from the Fungal Genome Initiative of the Broad Institute we were able to amplify and clone a new VAO, which we named FmVAO, from *F. moniliforme* MRC 6155. Sequencing revealed that the FmVAO is on gDNA and cDNA level 98% identical to the putative VAO from *Fusarium verticilliodes*, which we called FvVAO, identified in the sequenced genome of *Giberella moniliformis* (anamorph *Fusarium verticilliodes*). The deduced amino acid sequences of the two proteins are 99% identical. FvVAO and FmVAO both share 63% amino acid identity with PsVAO.

Both PsVAO and the new FmVAO were cloned into pET28b(+) with addition of N-terminal His-tags and expressed in *E. coli* BL21(DE3)pLYsS-RARE2. Although expression levels were too low to observe any discernable bands on SDS-PAGE gels, activity was observed with both PsVAO and FmVAO in whole cell biotransformation studies as well as assays with crude cell-free extracts using eugenol and vanillyl alcohol as substrates. FmVAO like PsVAO converts eugenol to coniferyl alcohol and vanillyl alcohol to vanillin. In whole cell biotransformations of ethylphenol both PsVAO and FmVAO produced an unidentified product which is most likely 1-(4-hydroxyphenyl)alcohol, since it is known that PsVAO produces mainly this alcohol from ethylphenol (van den Heuvel *et al.*, 1998). FmVAO has different substrate specificity when compared to PsVAO. In crude cell-free extracts PsVAO showed 1.6 fold higher activity towards vanillyl alcohol than FmVAO (0.028 vs 0.018 U/mg protein), while with

eugenol as substrate PsVAO activity was much higher than FmVAO activity (5.5 fold) (0.028 *vs* 0.005 U/mg protein). Compared to PsVAO that either prefers eugenol as substrate (according to the literature) or displays the same activity towards eugenol and vanillyl alcohol (our results), FmVAO clearly prefers vanillyl alcohol as substrate and might be regarded as the first true vanillyl alcohol oxidase. In whole cell biotransformations FmVAO also displayed higher activity towards ethylphenol than PsVAO. It thus might have more potential to develop into an alkylphenol hydroxylase.

The *FmVAO* sequences (cDNA and gDNA) have been submitted to Genbank making this only the second confirmed fungal VAO of which the genetic information has been deposited. Comparison of the FmVAO and FvVAO amino acid sequences with VAO-like proteins with confirmed activity towards vanillyl alcohol and/or eugenol (*i.e*. PsVAO, EUGO, EUGH and PCMH), confirmed the conservation of Tyr108, Asp170, His422, Tyr503 and Arg504 (PsVAO numbering) in all enzymes with confirmed oxidase activity *i.e*. PsVAO, FmVAO and EUGO. Asp170 and His422 are not conserved in the dehydrogenases, *i.e*. EUGH and PCMH. However, His61, which according to studies done with PsVAO is critical for autocatalytic covalent flavinilation of His422, is absent from EUGO and replaced by phenylalanine in FvVAO and FmVAO. Covalent flavinilation has been confirmed for EUGO and seems likely for FmVAO, given that activity levels of the recombinant FmVAO are similar to that of recombinant PsVAO. It thus appears that residues at other positions might also assist autocatalytic covalent flavinilation. FmVAO of course needs to be purified and rigorously characterized to confirm covalent flavinilation as well as differences in substrate specificity.

Alignment and phylogenetic analysis of 72 sequences of VAO-like proteins obtained through BLAST searches with FvVAO showed that PsVAO and FvVAO (and thus FmVAO) belong together with 10 other fungal sequences to a cluster of proteins that, with two exceptions, all have the conserved Tyr108, Asp170, His422, Tyr503 and Arg504 (PsVAO numbering). This cluster and the organisms these sequences originate from can be targeted for the cloning of more vanillyl alcohol and/or eugenol oxidases through gene synthesis or amplification of the genes of interest from mRNA.

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CHAPTER 3

Heterologous expression of fungal vanillyl-alcohol oxidases in yeasts

3.1 Introduction

Codon usage has been suggested as a reason for the low expression of *Penicillium simplicissimum VAO* in *E. coli*, since there is a relatively high number of so called "rare" codons present in the cDNA sequence of *P. simplicissimum VAO* (Benen *et al*., 1998). The cloning of both *PsVAO* and the newly isolated *FmVAO* into the pET28b(+) vector and expression in *E. coli* strains specifically designed to alleviate the codon usage problem, only resulted in a marginal improvement in specific activity. The amounts of PsVAO and FmVAO formed were still too little to detect on SDS gels (Chapter 2).

Another option to improve the expression of these genes would be to clone and express them in a similar eukaryotic fungal host. *PsVAO* has previously been expressed in the fungus, *Aspergillus niger* under the original promoter of the *VAO* gene. With anisyl alcohol and veratryl alcohol, established strong inducers of VAO in *P. simplicissimum,* very high levels of VAO was produced *i.e*. 0.48 and 0.34 U/mg protein. This VAO titer was almost 30 times higher than that obtained with *E. coli*. High expression was even observed when using compounds that are not naturally considered as good inducers of the enzymes such as ferulic acid, vanillyl alcohol and 4-hydroxybenzoic acid. It was postulated that this might have been due to the insertion of a high copy number of the VAO gene (25 - 30 copies) into *A. niger* (Benen *et al*., 1998).

The possibility also exists for the expression of these genes in yeasts. Yeasts as hosts for heterologous expression offer the same ease and simplicity of use as bacterial expression systems. Yeast-based production processes have been established with product yields in a multigram range (Böer *et al*., 2007). These organisms meet safety aspects in that they do not harbor pathogens, viral inclusions, or pyrogens. Easy fermentation regimens have been established that allow rapid growth to high cell densities on simple media. Being eukaryotic organisms, yeasts are able to secrete

recombinant proteins and to modify them according to a general eukaryotic scheme (Böer *et al*., 2007).

One of the first yeasts to be used as expression host was *Saccharomyces cerevisiae*, due to the knowledge that has been accumulated about this yeast. It does however have some limitations which sometimes make it not ideal for use as an expression host. These limitations include undesired hyperglycosylation, instability of the production strain, low yields and low secretory capacity (Zhang *et al*., 2003). Recently non-conventional yeast expression systems have been developed to overcome these problems. Most notable among these are the expression systems that have been developed for the methanotrophic yeast *Pichia pastoris* and the alkane utilization yeast *Yarrowia lipolytica* (Böer *et al*., 2007).

Despite the superior characteristics of yeast hosts in various developments, there is no single system that is optimal for production of all possible proteins. The use of a wide-range yeast vector system enables the assessment of several yeasts in parallel for their capability to produce a particular protein in desired amounts and quality. Two such wide-range yeast vector systems, CoMed and Xplor, are commercially available (Steinborn *et al*., 2006; Böer *et al*., 2009). These vectors mainly rely on rRNA targeting sequences based on the 25S or 18S rRNA for stable integration into the yeast chromosome (Wartmann *et al*., 2002; Wartmann and Kunze, 2000; Erik *et al*., 2009; Gellissen *et al*., 2005). Other elements that are commonly integrated into these vector systems include the dominant *hph* gene from *E. coli* conferring hygromycin resistance, while markers used for auxotrophic strains include, *AILV1, ALEU2 and ATRP1* (Wartmann *et al*., 2003). The strong promoter of the constitutive *TEF1* (translation elongation factor) gene is commonly used as promoter in conjunction with the *PHO5* terminator gene from *S. cerevisiae* or the *trpC* terminator gene from *Aspergillus nidulans* (Erik *et al*., 2009). These wide range vectors have been tested in *S. cerevisiae*, *P. pastoris*, *Hansenula polymorpha*, *Debaryomyces polymorphus*, *Debaryomyces hansenii* and *Y. lipolytica*.

A wide-range vector system was also developed at the University of the Free State by Dr. M. Labuschagné in the laboratory of Prof. J. Albertyn (Smit *et al*., 2011). The pKM118 vector (Fig 3.1) forms the basis of this wide range vector system and consists of (i) 18S rDNA fragments from *Kluyveromyces marxianus* flanking the "yeast casette" for genomic integration, (ii) the *Xho*I/*Avr*II cloning site flanked by the constitutive *TEF* promoter from *Y. lipolytica* and the *K. marxianus* inulinase terminator (iii) the hygromycin resistance marker under control of the *Saccharomyces cerevisiae TEF* promoter for selection of yeast transformants and (iv) the kanamycin resistance marker for subcloning into *E. coli*. PsVAO was used by Dr. Labuschagné as an intracellular reporter protein for testing this wide range vector system. pKM118 with *PsVAO* cloned between the *Xho*I and *Avr*II restriction sites was digested with *Not*I and the yeast cassette transformed into seven different yeasts namely *Arxula adeninivorans*, *Candida deformans*, *H. polymorpha*, *Kluyveromyces lactis*, *K. marxianus*, *S. cerevisiae* and *Y. lipolyitca.* VAO activity was evaluated using TLC and UV spectrophotometry (Fig 3.2). The best results were obtained when expressing in *K. marxianus* and *A. adeninivorans*. If one considers that the best performing transformant was probably an *A. adeninivorans* strain with two copies of the cassette integrated, while the *K. marxianus* transformants probably had only single copies integrated, then *K. marxianus* performed better than *A. adeninivorans*.

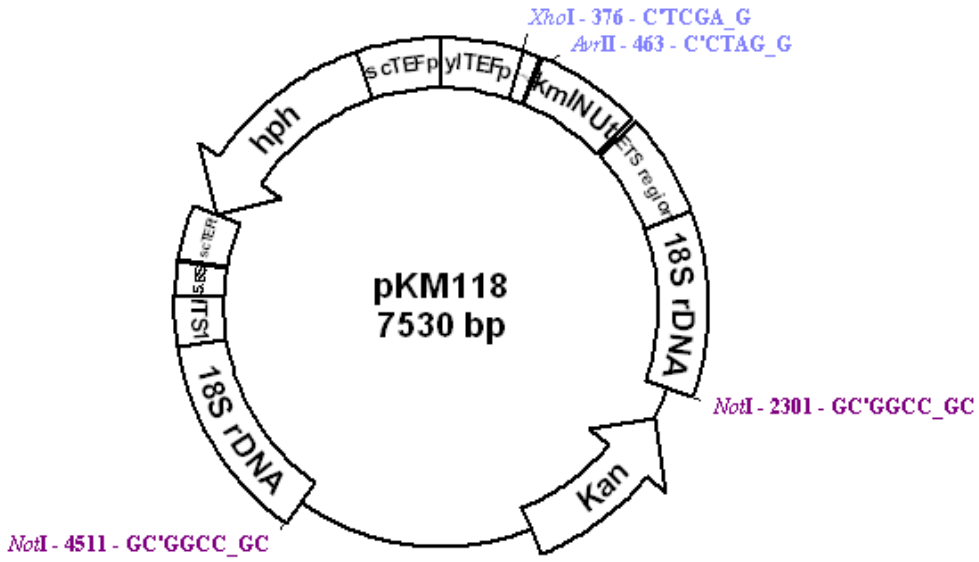

Fig 3.1: The pKM118 vector, containing the the *Xho*I/*Avr*II cloning site flanked by the *Y. lipolytica TEF* promoter (*ylTEFp*) and the *K. marxianus* inulinase terminator (*kmlNUt*) as well as the kanamycin (*Kan*) and hygromycin (*hph*) resistance markers was constructed by Dr. M. Labuschagné.

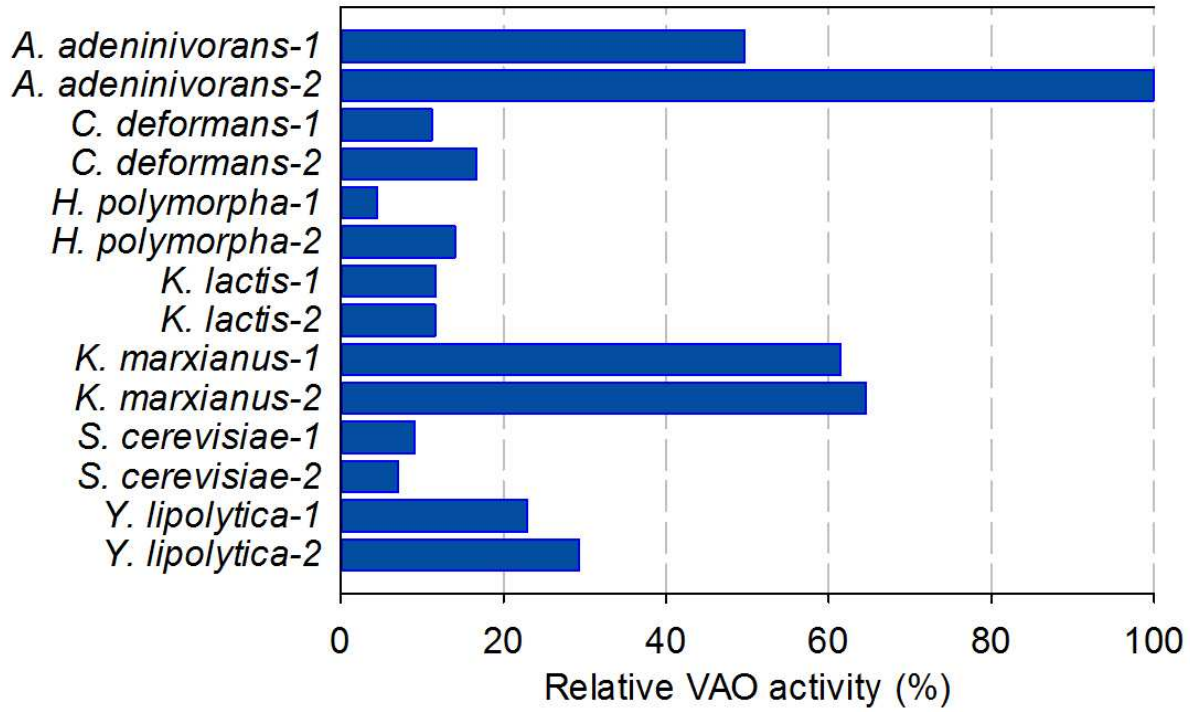

Fig. 3.2: Comparison of VAO activity in different yeasts transformed with the yeast casette of the pKM118 vector with *PsVAO* cloned (Smit *et al*., 2011). Biomass concentrations were the same (10% wet weight/v) for all biotransformations.

Kluyvermyces marxianus is characterized as a homothallic hemiascomycetous yeast that is pyhlogenetically related to *S. cerevisiae.* This yeast has received GRAS (Generally Regarded As Safe) as well QPS (Qualified Presumption of Safety) status in the United States and the European Union, respectively. This means that there are very few restrictions on its application and this greatly enhances its potential for application in the biotechnology sector (Lane and Morrissey, 2010). Although *K. marxianus* is primarily seen as biotechnologically important for the production of its native enzymes such as inulinase, as a biocatalyst for the production of various aromatic compounds as well as for ethanol production, it is slowly coming into its own as host for the production of various heterologous proteins. Some of the proteins that have been successfully expressed include lactate dehydrogenase for lactate production, thermostable endo-β-

1,4-glucanase, cellobiohydrolase and β-glucosidase for the production of ethanol from cellulose (Fonseca *et al*., 2008). One of the major limitations of *K. marxianus* has been the lack of availability of a proper genome sequence. Researchers thus rely upon the partial genome sequence of strain CBS 712 as well as comparison to the completed genome sequence of *K. lactis*.

Due to its increasing popularity as an expression host, a variety of molecular tools have recently become available for *K. marxianus,* making it a viable choice as a heterologous expression host (Lane and Morrissey, 2010). These tools include plasmids, selectable markers and promoters (Fig 3.3). Autonomously replicating plasmids are commonly used for cloning into *S. cerevisiae*, but the variety of plasmids currently available for *Kluyveromyces* spp. is limited to only a few. It is also possible to transform *S. cerevisiae* vectors into *K. marxianus.* Since long-term vector stability is a problem with autonomously replicating plasmids, chromosomal integration of DNA is regarded as a more reliable approach.

Two different systems are used for targeting DNA integration into the chromosome. The first is homologous recombination of linear DNA with flanking regions that are homologous to the genomic DNA. This directs the new DNA fragment into a specific genomic locus. The second system is mediated by the Ku70/Ku80 heterodimer and termed non-homologous end joining (NHEJ), resulting in the random integration of DNA into the genome (Lane and Morrissey, 2010).

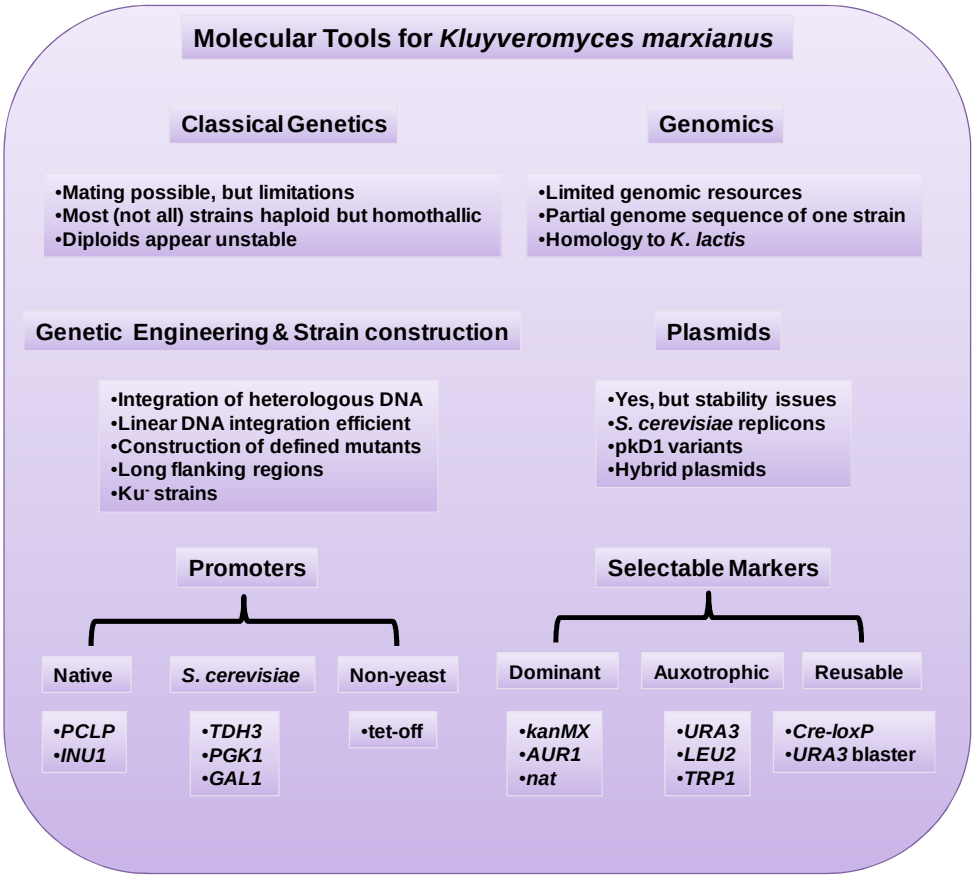

Fig 3.3: Molecular tools and resources for *K. marxianus*. A short summary of the key molecular reagents that are available, or required, to facilitate molecular genetics and strain improvement in *K. marxianus* (Taken from Lane and Morrissey, 2010).

A custom vector for use in *K. marxianus* has also been constructed by Dr. M. Labuschagné. This vector is similar to the wide-range vector pMK118 but contains a geneticin resistance marker (*KanMX*) for positive selection on G418 once cloned into *K. marxianus* and expression of the cloned gene is driven by the native inulinase promoter (Fig 3.4).

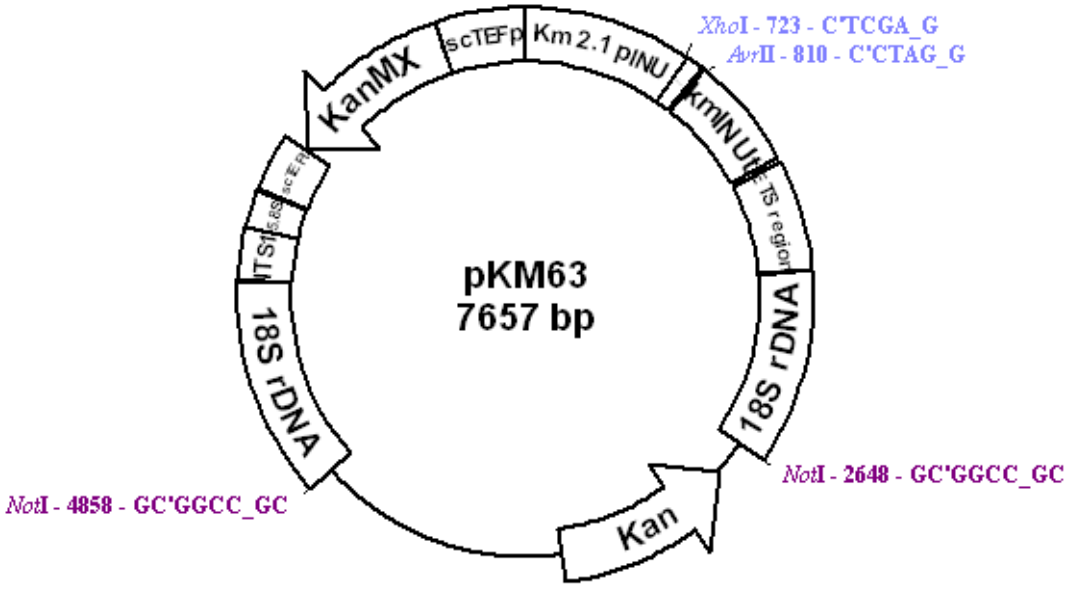

Fig 3.4: The pKM63 vector constructed by Dr. M. Labuschagné, contains the inulinase (Km 2.1 *pINU*) promoter as well as the inulinase terminator (*kmINUt*) from *K. marxianus*, kanamycin (*Kan*) resistance gene for *E. coli* subcloning and geneticin (*KanMX*) resistance marker for cloning into *K. marxianus*.

Arxula adeninivorans (*Blastobotrys adeninivorans*) is a dimorphic yeast with remarkable thermotolerant properties. A Siberian strain of this yeast is capable of growing at 48 ºC without previous adaptation to higher temperatures (Wartmann *et al*., 2002; Wartmann and Kunze, 2000; Böer *et al*., 2007). It is also halotolerant, as well as osmotolerant.

A host of molecular tools have also become available for this yeast (Fig. 3.5). Auxotrophic strains are mostly used for genetic transformation. The majority of genetic tools and methods that are used in the transformation of *Arxula* strains form part of the CoMed and Xplor wide-range vector systems (Steinborn *et al*., 2006; Böer *et al*., 2009). Furthermore, strains have been engineered that co-express two or more heterologous genes (Böer *et al*., 2007).

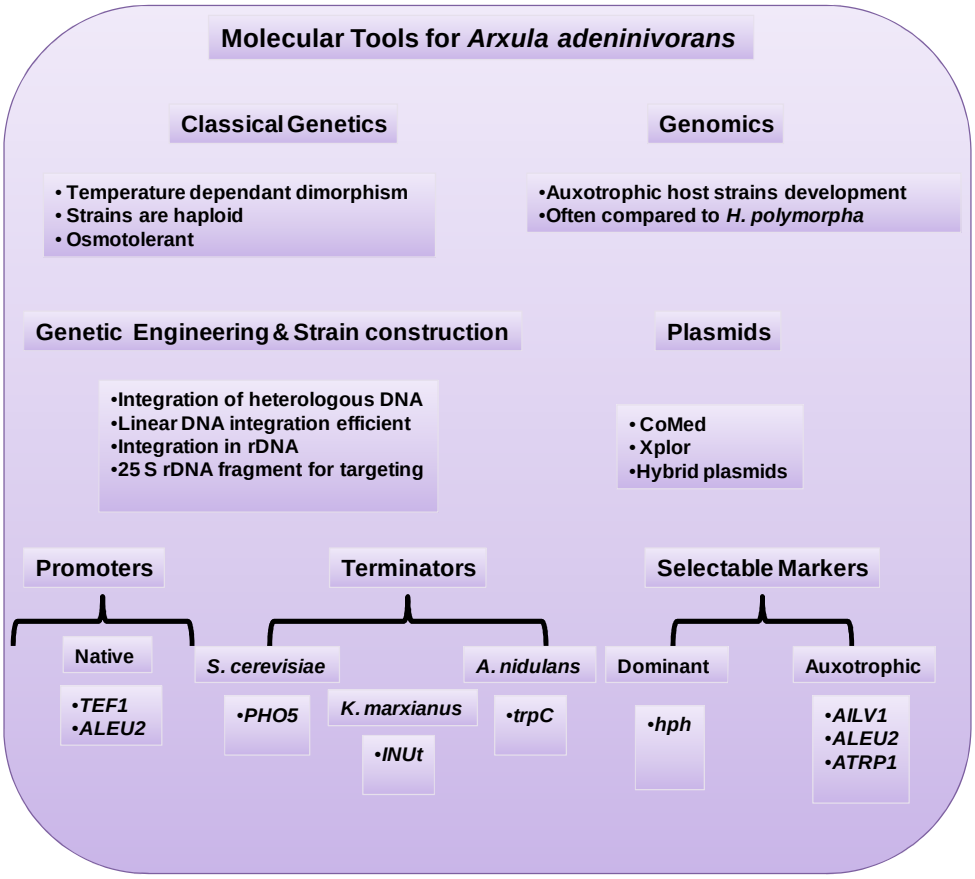

Fig 3.5: Molecular tools and resources for *A. adeninivorans*. A short summary of the key molecular reagents that are available, or required, to facilitate molecular genetics and strain improvement in *A. adeninivorans*.

With the above points in mind the aim of this study was to investigate heterologous expression of the *VAO* from *P. simplicissimum* and the *VAO* from *F. moniliforme* MRC 6155 in *K. marxianus* and *A. adeninivorans*. Given the initial promising results obtained with *K. marxianus* it was decided to also test the custom vector pKM63 for the expression of *PsVAO* and *FmVAO*. Whole cell biotransformation of vanillyl alcohol and eugenol as well as activity in cell-free extracts were evaluated.

3.2 Materials and Methods

3.2.1 Culture conditions for cultivation of *K. marxianus* and *A. adeninivorans*

Cultivation in liquid media was, unless stated otherwise, performed in 25 ml YP_2D_2 broth (see below) in 250 ml Erlenmeyer flasks for pre-cultures and 50 ml YP_2D_2 broth in 500-ml Erlenmeyer flasks for main cultures on a rotary shaker at 180 rpm and 30℃. Shake flasks were inoculated with pre-cultures grown for 24 h.

YP_2D_2 contained (per litre water): 20 g glucose, 20 g peptone and 10 g yeast extract with 20 g agar added for solid medium.

3.2.2 Cloning of the *VAO* gene from *F. moniliforme* into pKM63 and transformation of pKM63 vectors carrying *VAO* genes from *F. moniliforme* and *P. simplicissimum* into *K. marxianus* UOVS Y 1185

All restriction enzymes, DNA modifying enzymes and molecular weight markers were obtained from Fermentas. Oligonucleotides were obtained from Bioneer. PCR amplification was performed using Kapa High Fidelity DNA Polymerase (Kapa Biosystems). PCR and gel-band purification was performed using the Biospin gel extraction kit (Separation Scientific). Sub cloning of PCR products was performed using the pSMART vector (Lucigen) (Chapter 2, Section 2.2.8, Table 2.4). The cloning of the *VAO* from *P. simplicissimum* into pKM63 was done by Dr. M. Labuschagné in the laboratory of Prof. J. Albertyn at the University of the Free State by using primers PFVAO*Xho* and PRVAO*Avr* (Table 3.1).

Table 3.1: Primers used for amplification of *FmVAO* and *PsVAO* for insertion into the pKM 63 vector.

Primer name	Sequence in 5' to 3' orientation	Restriction sites introduced
FFVAO*SalI*	GCGTCGACATGGCTACTGTTAATCCTCTTGT	*SalI*
FRVAO*AvrII*	CGCCTAGGCTACAGTTTTGTAGAGCGCTGTGG	*AvrII*
PFVAO*XhoI*	GCCTCGAGATGTCCAAGACACAGGAATTCAG	*XhoI*
PRVAO*AvrII*	CGCCTAGGTTACAGTTTCCAAGTAACATGAC	*AvrII*

Specific primers, FFVAO*SalI* and FRVAO*AvrII* (Table 3.1) were designed to PCR amplify the *VAO* gene from *Fusarium moniliforme* from the cDNA insert cloned into pET28b(+) (Chapter 2) (initial denaturation for 2 min at 94 ℃; followed by 30 cycles of 94 ℃ for 30 s, 54 ℃ for 30 s, 72 ℃ for 1.5 min, and a final e longation of 72 ℃ for 10 min). The PCR product was cloned into the pSMART vector and transformed into *E. coli* XL10-Gold (Chapter 2, Section 2.2.8, Table 2.5) according to the method described by Inoue *et al*., 1990, followed by DNA sequencing of two independent clones. The plasmid was isolated from *E. coli* XL10-Gold and purified using the Biospin plasmid isolation kit (GE Health) according to manufacturer's specifications. The purified plasmid containing the correct insert was treated with *Sal*I and *Avr*II. The purified pKM63 vector was treated with *Xho*I and *Avr*II. *Sal*I to give a 5' overhang that was compatible with the 3' overhang created by *Xho*I digestion of the pKM63 vector. After incubation at 37 ºC for an hour, the restriction digest mixtures were subjected to gel electrophoresis and purified from the gel using a Biospin gel purification kit according to manufacturer's instructions. The obtained purified fragments were ligated using T4 DNA ligase to join the compatible ends and transformed into *E. coli* XL10-Gold for subcloning purposes. A colony PCR was used and the result was visualized using gel electrophoresis in order to confirm positive insertion of the *VAO* fragment.

pKM63 Plasmids containing the two *VAO* genes were isolated from *E. coli* XL10-Gold using the Biospin plasmid isolation kit (GE Health) according to manufacturer's instructions. Isolated plasmids were treated with *Not*I in order to linearise the backbone. The linearised plasmid fragments were transformed into *K. marxianus* UOVS Y 1185 using the method described by Chen *et al*., 1997. *K. marxianus* was incubated in 5 ml test tubes containing YP_2D_2 broth for 16 h to 24 h at 25 ºC and the OD_{600} was measured. Once the OD has reached 0.6, cells were centrifuged (10 000 x *g*) for 1 min and the pellet resuspended in 100 µl one step buffer. A (see below). Plasmid preparations (1 µl, more than 1 ng DNA) were added for each transformation. The mixture was thoroughly vortexed. Thereafter the mixture was incubated at 39 ºC for 60 min. Transformation mixture was plated out on YP_2D_2 plates containing geneticin (G418) (72 µg/ml) from Sigma Aldrich. Plates were incubated for 48 h at 25 ºC.

One step buffer contained: PEG 4000 (45%), 0.1 M lithium acetate pH 6.0, 25 µg/100 µl single stranded carrier DNA and 100 mM dithiothreitol (DTT). Carrier DNA was prepared according to a method by Gietz *et al*., 1992.

3.2.3 Cloning of *FmVAO* into pKM118 and transformation of pKM118 vectors carrying *PsVAO* and *FmVAO* into *A. adeninivorans* UOVS Y 1220

To clone *FmVAO* into *A. adeninivorans*, the *FmVAO* fragment was released from the pET28b(+) (Chapter 2, Section 2.2.8, Table 2.4) plasmid by digestion with *Nhe*I and *Hin*dIII. The pKM118 plasmid to be used for cloning was digested using *Avr*I and *Xho*I.

After enzyme digestion, the mixtures were subjected to gel electrophoresis and the fragments with the appropriate sizes were excised and purified from the gel using a BioSpin gel purification kit (GE Heatlh) according to manufacturer's instructions. After gel purification, blunting of both the linearised plasmid and the fragment (~ 0.5-4µg DNA) was done using T4 polymerase (NEB) according to manufacturer's instructions.

The reaction mixture was incubated for 20 min at 11 ºC and heat inactivated for 10 min at 75 ºC.

The plasmid (~ 10 µg) was dephosphorylated using 1 µl (4 U) antarctic phosphatase (NEB). Thereafter the reaction mixture was incubated for 1 h at 37 ºC followed by heat inactivation at 60 ºC for 10 min. The fragment was ligated overnight into the plasmid using T4 ligase (Fermentas) according to manufacturer's instructions. The ligation mixture was transformed into *E. coli* XL10-Gold and plated out on LB plates containing kanamycin (30 µg/ml). The plates were incubated overnight at 37 ºC and the resultant colonies were screened using colony PCR. To ensure correct orientation a forward primer based on the *yITEF* promoter in the pKM118 plasmid and the reverse primer of the *VAO* gene were used in a PCR reaction. If the insert was in the correct orientation a band of ~ 1.8 Kb was expected.

pKM118 Plasmids containing *PsVAO* and *FmVAO* were purified and linearised with *Not*I before transforming into *A. adeninivorans* UOVS Y 1220. *A. adeninivorans* UOVS Y 1220 cells were made competent and transformed using a method adapted from Lin-Cereghino *et al*., 2005). Cultures (5 ml) were grown in multiple test tubes overnight (12 h) in YP_2D_2 at 30 ºC. The following day the overnight cultures were diluted to an OD_{600} of 0.15 – 0.2 in 50 ml YP_2D_2 in 500 ml shake flasks. Cultures were incubated at 30 ºC until an OD_{600} of 1 was reached. The cultures were centrifuged at 5000 x *g* at room temperature and the supernatant was poured off. The pellet was resuspended in 9 ml ice cold BEDS solution (see below) supplemented with 1 ml DTT (1.0 M). The cell suspension was incubated for 5 min at 30 ºC on a shaking incubator after which the culture was centrifuged (5000 x *g*) at room temperature. The cells were resuspended in 1 ml BEDS solution without DTT. The competent cells were now ready for transformation. Alternatively the cells were, at this stage stored at -80 ºC and used later.

Cells were transformed *via* heat shock as follows: Linearised plasmid DNA (1 µl) was mixed into the competent cell suspension. To this was added: 1.4 ml of a 40% polyetheleneglycol (PEG) solution made up in 200 mM bicine, pH 8.3 (Lin-Cereghino *et al*., 2005). The transformation mixture was incubated for 60 min at

30 ºC after which the cells were heat shocked at 42 ºC for 10 min followed by a 1 h recovery at 30 ºC. The cells were centrifuged for 1 min and resuspended in 150 mM NaCl (final concentration) made up in 10 mM bicine (final concentration), pH 8.3 and again centrifuged for 1 min (Lin-Cereghino *et al*., 2005). This step was repeated one more time after which the cells were ready to be plated out. The transformation mixture was plated out on YP_2D_2 plates containing hygromycin (40 µg/ml). The plates were incubated for 48 h at 30 ºC and the resultant colonies were screened using eugenol as a substrate in a biotransformation experiment. Formation of a yellow color indicated biotransformation of eugenol and thus successful integration of the DNA carrying VAO (Priefert *et al*., 1999).

BEDS solution contained: 10 mM Bicine-NaOH, pH 8.3, 3% v/v ethylene glycol, 5% v/v dimethylsulfoxide (DMSO) and 1 M sorbitol.

3.2.4 Whole cell biotransformation studies with *K. marxianus* strains

K. marxianus strains were precultured in YP_2D_2 broth containing G418 (72 µg/ml) at 37 ºC for 24 h. This preculture (1% v/v) was inoculated into a 500 ml Erlenmeyer flask containing YP_2 (20 g peptone and 10 g yeast extract per liter) with sucrose (2% w/v) and G418 (72 µg/ml). Cultures were incubated for 48 h at 30 ºC. After 48 h vanillyl alcohol 1% w/v or eugenol 1% v/v was added as substrate and samples (500 µl) were taken at 0 h, 0.5 h, 1.5 h,1 h, 2 h, 16 h and 48 h respectively..

For GC analysis samples (500 µl) were extracted twice with 300 µl ethyl acetate containing 1,2-dodecanediol (0.25 % w/v) as internal standard. The samples were vortexed for 5 minutes and the phases were separated by centrifugation (10 000 x *g* for 10 min). The extracts were combined.

For UV analysis samples were extracted with 1 ml ethyl acetate and the extracted samples were diluted appropriately with ethyl acetate and three 200 µl aliquots of each sample were transferred to a UV microtiter plate. Ethyl acetate was used to blank the spectrophotometer. The formation of vanillin was followed at 340 nm and the formation

of coniferyl alcohol at 320 or 300 nm. The concentrations of the products formed were calculated using the following extinction coefficients obtained from standard curves of vanillin and coniferyl alcohol dissolved in ethyl acetate: vanillin ε_{340} = 0.33 mM^{-1} cm^{-1} and coniferyl alcohol ε_{300} = 2.78 mM^{-1} cm^{-1} or at ε_{320} = 0.39 mM^{-1} cm^{-1}). The path length was 0.596 cm.

3.2.5 Whole cell biotransformation studies with *A. adeninivorans* strains

A. adeninivorans strains were pre-cultured in YP_2D_2 broth for 24 h at 30 ºC. Precultures (500 µl, 1% v/v) were transferred to 50 ml YP_2D_2 broth in 500 ml Erlenmeyer flasks. The cultures were incubated at 30 ºC for 48 h. Cell cultures were harvested by centrifugation (5000 x *g* for 5 min) and washed with 50 mM Tris-HCl buffer. The wet weights were determined and cells were resuspended (10 % wet weight/v) in 50 mM Tris-HCl buffer pH 8.

Cell suspensions (10 ml) were aliquoted into 100 ml Erlenmeyer shake flasks. Eugenol (1% v/v; 65 mM) or vanillyl alcohol (1% w/v; 64 mM) was added to these cell suspensions.

Samples (500 µl) were taken at 0 h, 1 h, 2 h, 3 h, 6 h, 10 h and 24 h intervals and 600 µl ethyl acetate was added to these samples in microcentrifuge tubes. Samples were vortexed for 10 min and then centrifuged at 10 000 x *g* for 10 min. The organic layer was transferred to a new tube and assayed for product formation.

Biomass (dry weight) determinations were done in triplicate: Samples (2 ml) were centrifuged at 10 000 x *g* for 10 min in pre-weighed microcentrifuge tubes. The samples were dried overnight at 100 ºC and the weight was recorded the following morning.

3.2.6 Analysis of ethyl acetate extracts

For TLC (Thin Layer Chromatography) analysis, samples were spotted on Alugram Sil G/UV_{245} TLC plates (Merck) containing a fluorescent indicator. Plates were developed using a mobile phase consisting of hexane (Merck), diethylether (Merck) and formic acid (Merck) in a 60:35:5 ratio. Plates were visualized under short wavelength UV-light.

For GC (Gas Chromatography) analysis samples were extracted with ethyl acetate containing 1, 2-dodecanediol (0.25% w/v), as internal standard. GC analysis of samples was carried out on a Hewlett Packard 5890 Series II gas chromatograph equipped with a flame ionization detector (FID) and a CP-Wax CB column (Chrompack) measuring 30 m x 0.53 mm x 1 µm. GC conditions were as follows: initial oven temperature was held at 120 ℃ for 5 min, increasing at 10 ℃/min to a f inal temperature of 250 ℃, held for 12 min. Flow through the column was at 6 ml/min hydrogen with a split ratio of 1:50. The temperature of the detector (FID) was 280 ℃.

For UV assays samples were diluted appropriately with ethyl acetate and three 200 µl aliquots of each sample were transferred to a UV transparent microtiter plate. Ethyl acetate was used to blank the spectrophotometer. The formation of vanillin was followed at 340 nm and the formation of coniferyl alcohol at 320 or 300 nm. The concentrations of the products formed were calculated using the following extinction coefficients obtained from standard curves of vanillin and coniferyl alcohol dissolved in ethyl acetate: vanillin ε_{340} = 0.33 mM^{-1} cm^{-1} and coniferyl alcohol ε_{300} = 2.78 mM^{-1} cm^{-1} or at ε_{320} = 0.39 mM^{-1} cm^{-1}). The path length was 0.596 cm.

3.2.7 Assay with crude cell-free extracts

To obtain cell free extracts, the cell cultures (100 ml) were harvested by centrifugation (5000 x *g* for 5 min) and washed with 50 mM Tris-HCl buffer at pH 8. Hereafter cells were ruptured with a One Shot Cell Disrupter System (Constant Cell Disruption

Systems) at 32 kpsi and passed through twice. The cell debris was separated from the crude extract by centrifuging at 12 000 x *g* for 45 min.

The protein content for all crude extracts was determined by using the Bradford method. Activity assays were performed with 40 μg protein added to give 200 μl reaction mixtures. Stock solutions in ethanol (0.1 M) were prepared for eugenol and vanillyl alcohol. All assays were done in triplicate.

Reactions were performed at 30 ºC. 50 mM Tris-HCl pH 8 containing 1 mM of substrate was used as blank. Coniferyl alcohol formation (ε_{300} = 7.4 mM^{-1} cm^{-1}) and vanillin formation (ε_{340} = 8.7 mM^{-1} cm^{-1}) was read every 0.5 min for 15 min. The path length of a 200 µl sample in the microtiter plate was 0.596 cm.

3.2.8 Fractionation of *A. adeninivorans* cell-free extracts

Cells were grown for 48 h at 30 ^{o}C and then harvested by centrifugation at 4 ºC and 4000 x *g* for 10 min. Cells were lysed with a One Shot Cell Disrupter System (Constant Cell Disruption Systems) at 32 kpsi and passed through twice. Fractionation of cell-free extracts was done as described by Stephanova and Topouzova (2001). The resultant lysate was centrifuged at 4 ºC and 1000 x *g* for 10 min. The supernatant was transferred to an ultracentrifuge tube. It was then centrifuged at 12 000 x *g* for 30 min to obtain the mitochondrial fraction (pellet). The supernatant was again transferred into a fresh tube and centrifuged at 80 000 x *g* for 2 h. The pellet obtained is the microsomal fraction. The supernatant is the soluble fraction.

3.3 Results and Discussion

3.3.1 Cloning of the *VAO* from *F. moniliforme* MRC 6155 into the pKM63 vector and pKM118 vector

In order to clone the *VAO* genes from *F. moniliforme* and *P. simplicissimum* into the pKM63 vector, the necessary restriction sites were introduced using specific primers in a PCR reaction (Fig 3.6). The bands were purified and subcloned into the pSMART vector and then transformed into *E. coli* XL10-Gold. Plasmid was purified and digested with *Xho*I and *Avr*II in the case of the *VAO* from *P. simplicissimum* or *Sal*I and *Avr*II in the case of the *VAO* from *F. moniliforme* MRC 6155.

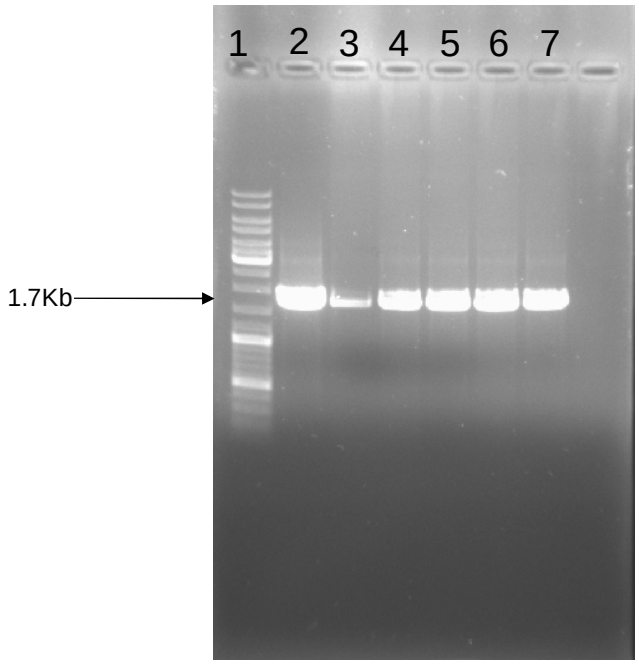

Fig 3.6: Successful amplification of the *FmVAO* insert with additional restriction sites for insertion into the pKM 63 vector Lane 1: DNA molecular weight ladder (GeneRuler 1 kb DNA ladder, Fermentas), lanes 2-7: *F. moniliforme VAO* amplicon.

The *VAO* inserts were ligated into the pKM63 vector using T4 DNA ligase and transformed into *E. coli* XL10-Gold. Transformants were screened using PCR to check for the presence of inserts (Fig 3.7). The "yeast cassettes" were excised from plasmids with correct inserts by *Not*I digestion and then transformed into *K. marxianus*.

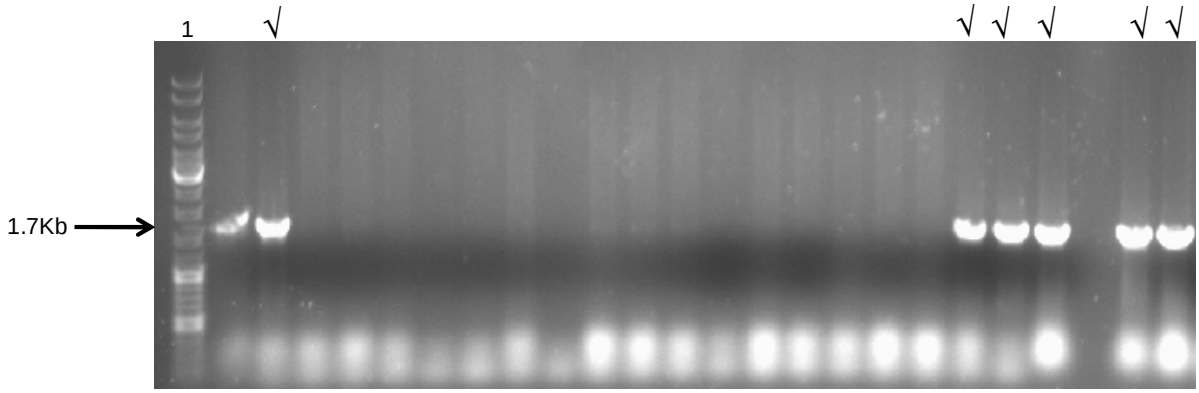

Fig 3.7: Colony PCR for putative *VAO* gene successfully integrated into the pKM63 vector. Positive PCR results are indicated with a √ . Lane 1: DNA molecular weight ladder

Sequencing of the pSMART construct of the *F. moniliforme VAO* confirmed the mutations introduced during cDNA synthesis (Chapter 2). The *K. marxianus* strains containing the mutated *FmVAO* gene (*FmutVAO*) were unfortunately already created by the time the sequencing results were received. It was decided not to repeat the cloning and transformation into *K. marxianus* with the corrected *FmVAO* but to clone the corrected *FmVAO* only into the pKM118 vector for transformation into *A. adeninivorans*.

For cloning into pKM118 *FmVAO* fragments were excised from pET28b(+) containing the corrected *FmVAO* gene (Chapter 2) with *Nhe*I and *Hind*III and blunted as described in section 3.2.3. The newly ligated pKM118 plasmid containing *FmVAO* was sub cloned into *E. coli* XL10-Gold. In order to verify positive insertion and correct orientation a colony PCR was done and the result visualized with gel electrophoreses. Twenty eight

colonies were screened and multiple clones displaying a ~1.8 Kb band where chosen for transformation into *A. adeninivorans* (Fig 3.8).

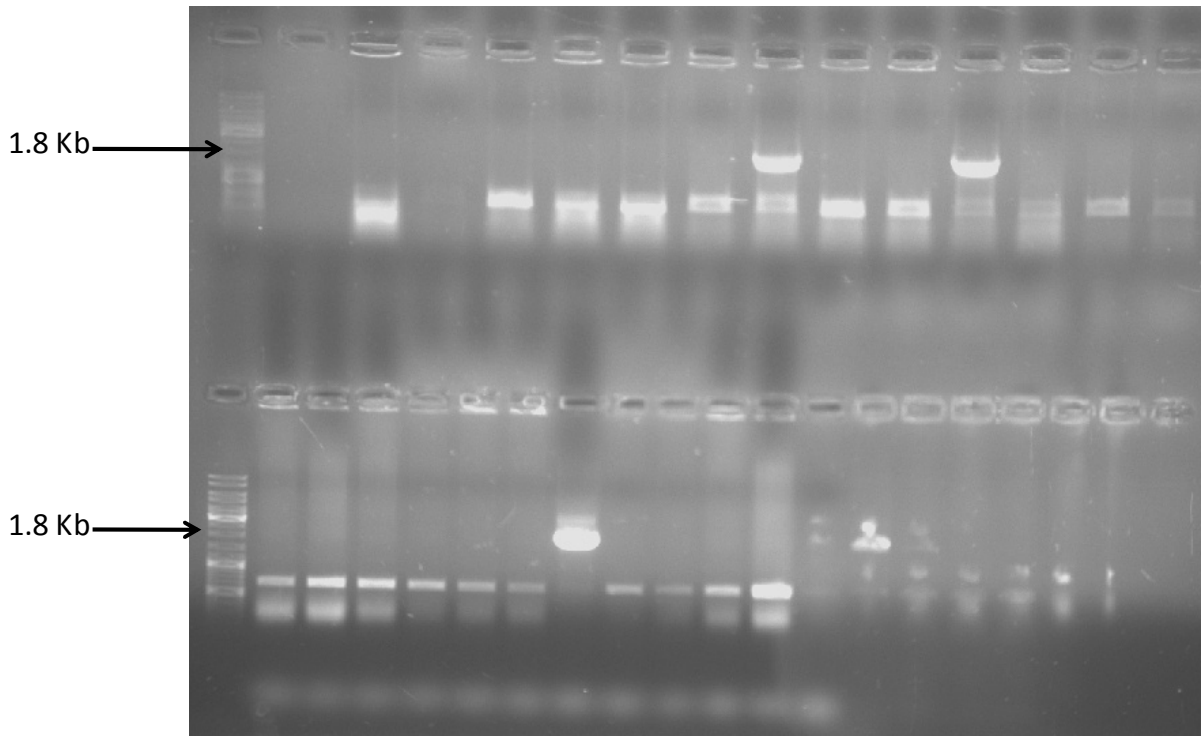

Fig 3.8: Colony screening by PCR was used to identify clones carrying pKM118 with *FmVAO* inserted in the correct orientation. If the fragment was present and in the correct orientation a fragment of approximately 1.8 Kb was expected.

3.3.2 Whole cell biotransformation studies using *K. marxianus* with *PsVAO* cloned

To assess the production of *P. simplicissimum* PsVAO, by *K. marxianus* vanillyl alcohol (1% w/v) or eugenol (1% v/v) were added as substrates after 48 h of incubation. Samples were taken regularly for 48 h and analysed using GC and TLC. A *K. marxianus* strain containing an empty pKM63 vector served as negative control.

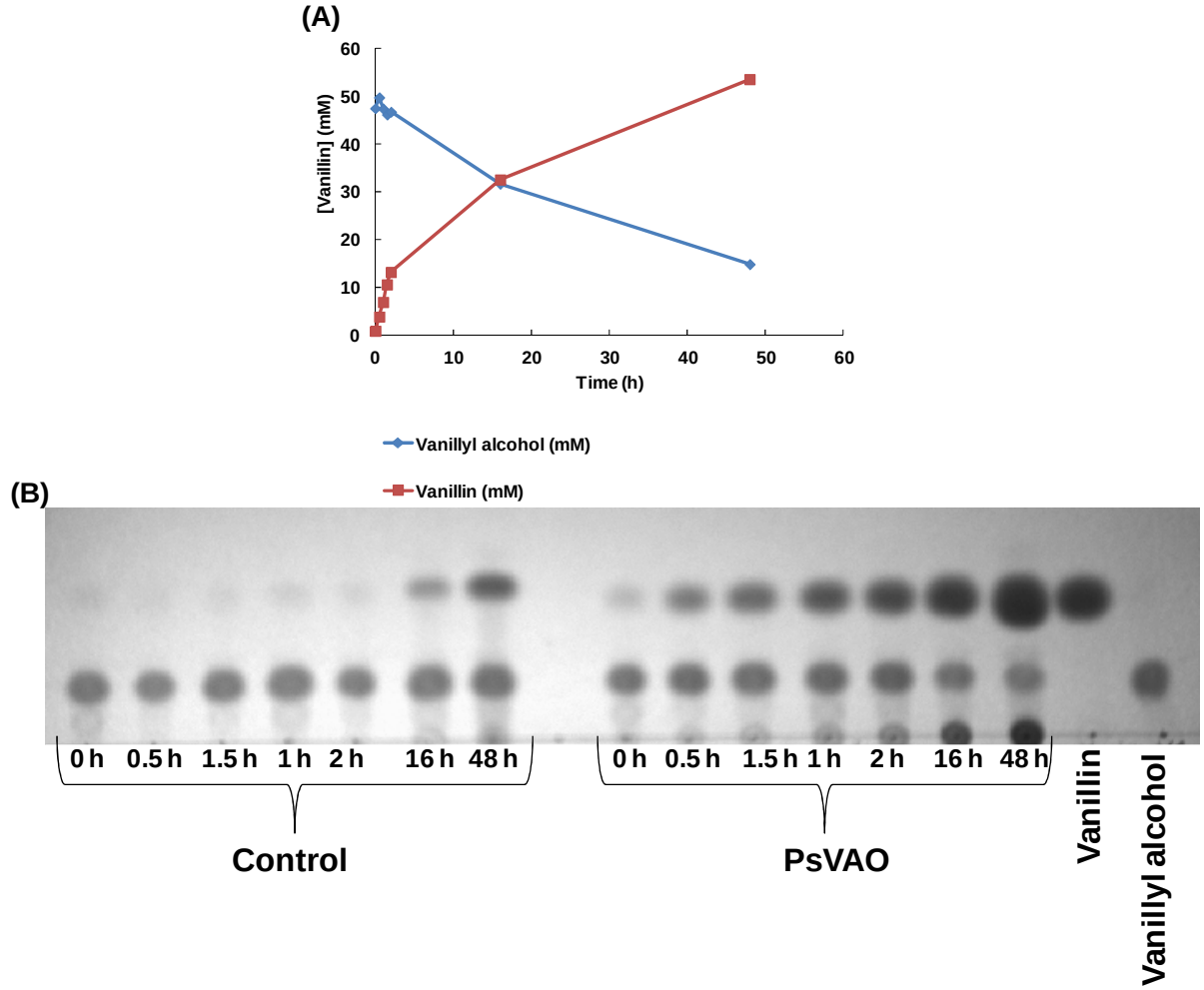

Fig 3.9: Whole cell biotransformation of vanillyl alcohol by *K. marxianus* expressing *PsVAO*. Vanillyl alcohol (64 mM) was added to cultures grown for 48 h. Ethyl acetate extracts were analysed with GC (A) and TLC (B).

Initial analysis showed 89% conversion of vanillyl alcohol to vanillin after 48 h to give 53.4 mM vanillin (Fig 3.9). TLC analysis of these samples corroborated the above mentioned results (Fig 3.9). Background activity could also be observed in the control strain of *K. marxianus*, which accumulated a significant amount of vanillin after 48 h of incubation (Fig 3.9).

The biotransformation of eugenol to form coniferyl alcohol happened much faster than the vanillyl alcohol biotransformation with 87% conversion (~57 mM) to coniferyl alcohol after only 16 h of incubation (Fig 3.10).

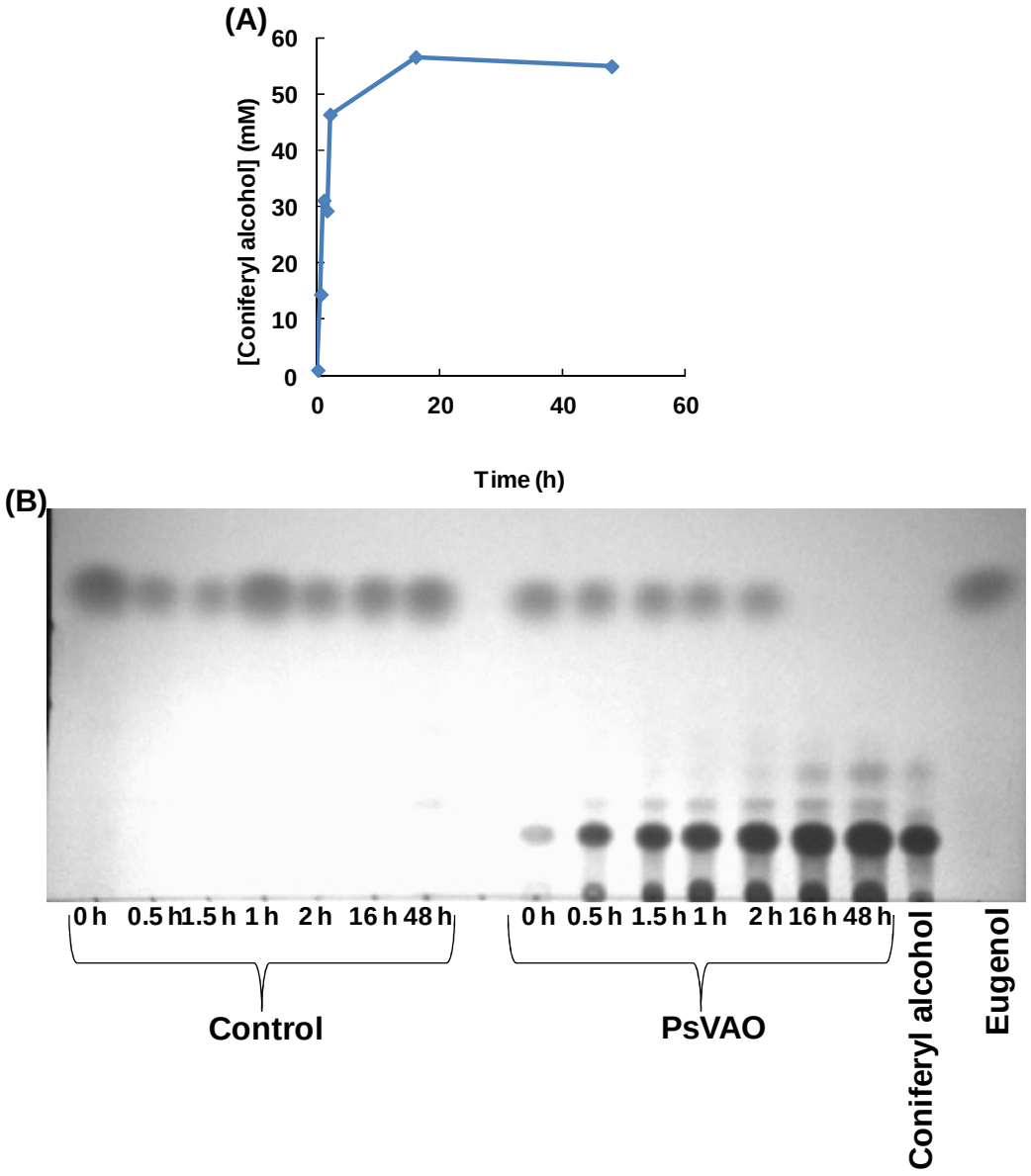

Fig 3.10: Whole cell biotransformation of eugenol by *K. marxianus* expressing *PsVAO*. Eugenol (65 mM) was added to cultures grown for 48 h. Ethyl acetate extracts were analysed with GC (A) and TLC (B).

Analysis of the above samples by TLC revealed similar results. Product formed extremely fast and some product was already visible on the plate after 0.5 h. The TLC also revealed complete utilization of the substrate after 16 h of incubation (Fig 3.10). GC analysis revealed that the conversion to coniferyl alcohol was high (~ 57 mM), resulting in an 87% conversion to this product (Fig 3.10). The TLC analysis showed the formation of a second as yet unidentified product forming after 0.5 h and a third product after 2 h, which explains why 65 mM was not accumulated even though 65 mM eugenol was depleted as substrate (Fig 3.10). GC and TLC analysis showed that no coniferyl alcohol was formed by the control strain.

3.3.3 Whole cell biotransformation studies using *K. marxianus* strains with *PsVAO* and *FmutVAO* cloned

Due to the previous success with the biotransformation of eugenol by *K. marxianus* producing PsVAO, it was decided to repeat the biotransformation experiment and this time include higher concentrations of eugenol (2% and 10% v/v). After it was shown that *FmutVAO* had been successfully transformed into *K. marxianus*, it was also included in this experiment to test for expression, to confirm that it was also, after expression in yeast inactive as observed with *E. coli* (Chapter 2). The concentration of coniferyl alcohol in ethyl acetate extracts was determined spectrophotometrically at 320 nm.

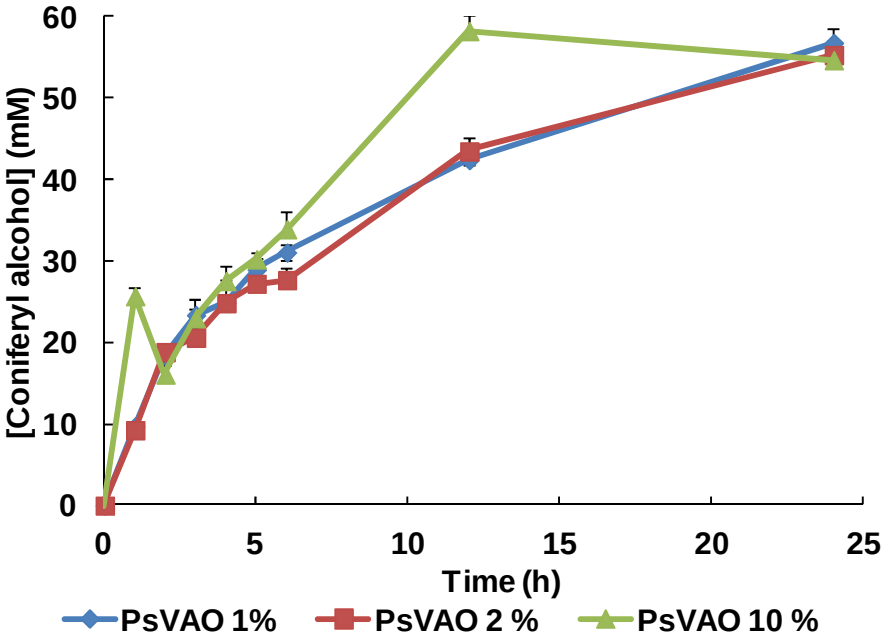

Fig 3.11: The formation of coniferyl alcohol from eugenol as substrate by *K. marxianus* producing PsVAO during a whole cell biotransformation. Three different eugenol concentrations were evaluated; 1% v/v, 65 mM; 2 % v/v, 120 mM and 10% v/v, 650 mM. Samples were extracted with ethyl acetate and formation of coniferyl alcohol measured spectrophotometrically at 320 nm.

Results from these biotransformations conducted with *K. marxianus* producing PsVAO revealed that when 1% (v/v) eugenol was added as substrate the maximum concentration of coniferyl alcohol that could be accumulated after 24 h was 56 mM coniferyl alcohol translating to 87% conversion (Fig 3.11). Although conversion of product was once again observed with PsVAO produced by *K. marxianus,* it appeared that conversion to product took slightly longer than was previously reported. Previously an 87% conversion to coniferyl alcohol was achieved in just 16 h (Section 3.3.2). It now took 24 h to achieve the same type of result. At approximately the same time it was demonstrated at the UFS that the vectors integrated into the rDNA of *K. lactis* and *K. marxianus* were unstable (Smit *et al*., 2011). In the case of *K. marxianus* only 41% of transformants with the hygromycin resistance gene cloned remained resistant to

hygromycin after 24 h growth in YPD medium without hygromycin. The *K. marxianus* strain with *PsVAO* cloned had been stored for eight weeks at -80 °C. The lower activity of *PsVAO* in *K. marxianus* after storage can thus probably be ascribed to these stability issues. Because *K. marxianus* transformants were unstable no further work was done with this yeast.

When comparing the results obtained with different eugenol concentrations it was noted that initial activity during the first six hours was not drastically affected by substrate concentration indicating that even at 1% v/v eugenol, activity was not limited by substrate concentration (Fig 3.11). It also indicated that enzyme activity was not inhibited by eugenol concentrations of up to 10% (v/v) i.e. 650 mM. However, although more coniferyl alcohol was detected in the biotransformation with 10% eugenol after 12 h than in the ones with 1 and 2% (v/v) eugenol (58 mM vs. 42 mM and 43 mM), the final product concentration in all three biotransformations was the same (56 mM, 55 mM, 55 mM for 1%, 2% and 10% eugenol respectively) (Fig 3.11). This might either be because the enzyme activity was in the end inhibited by the high substrate or product concentrations or it might more likely be due to a problem with the extraction or assay, since in later work, after some optimization of extractions, up to 200 mM coniferyl alcohol was recovered from a biotransformation of 650 mM eugenol by *A. adeninivorans* producing PsVAO (Smit *et al*., 2011).

Biotransformations using the *K. marxianus* with *FmutVAO* cloned did not reveal any significant coniferyl alcohol formation at any of the substrate concentrations used, indicating that the *FmutVAO* has not been successfully expressed or is simply not active when expressed in *K. marxianus.* These results confirmed the negative results obtained with *E. coli* and supported the decision to correct and reclone the *FmVAO* cDNA.

3.3.4 Whole cell biotransformation using *A. adeninivorans* expressing *FmVAO* and *PsVAO*

Whole cell biotransformations were carried out using resting cells of *A. adeninivorans*. Vanillyl alcohol (64 mM) or eugenol (65 mM) was added as substrate and samples were

taken at 0 h, 1 h, 2 h, 3 h, 6 h, 10 h and 24 h intervals. Samples were extracted with ethylacetate and analyzed with TLC and UV at 340 nm for the formation of vanillin and at 300 nm for the formation of coniferyl alcohol.

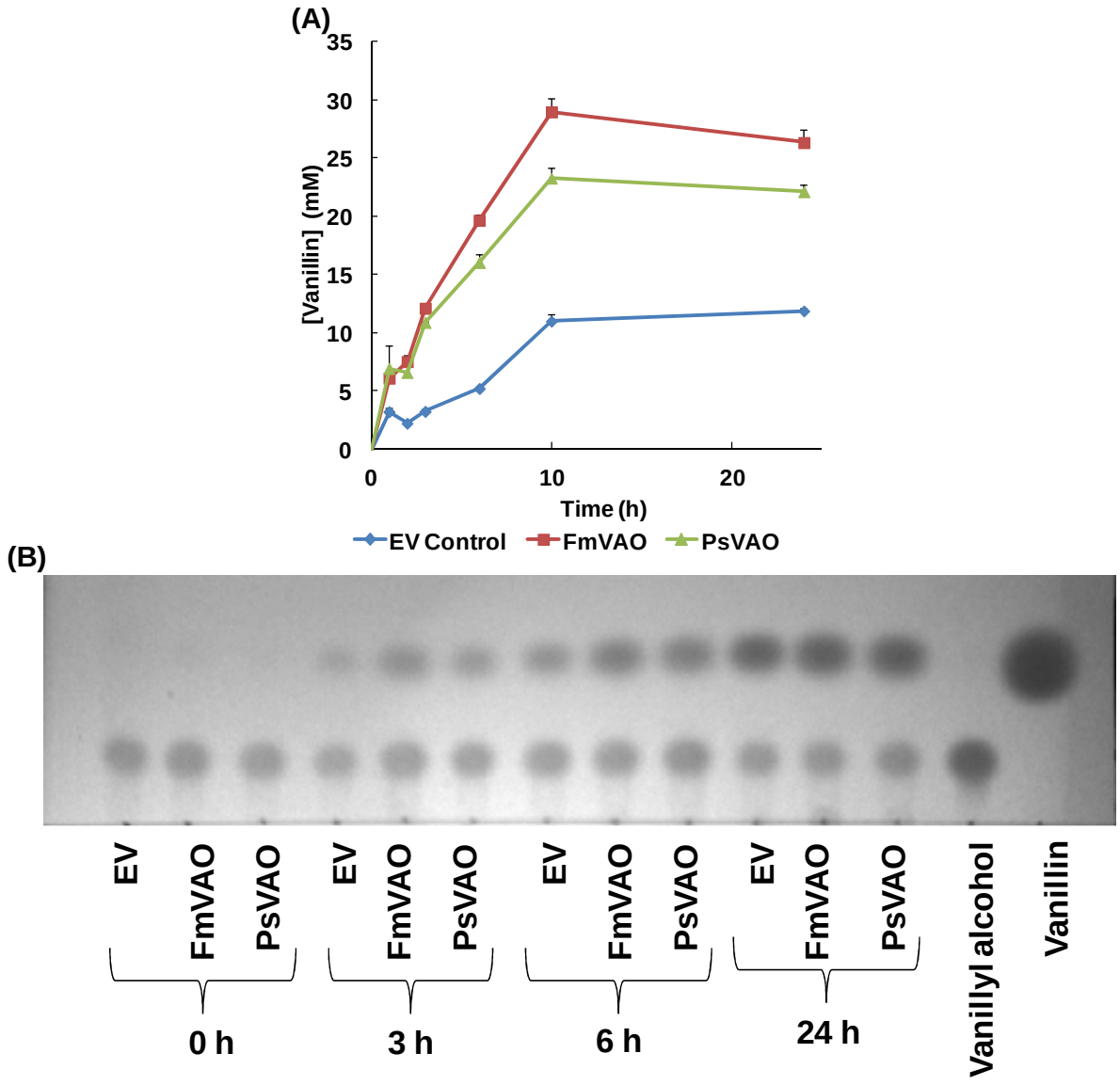

Fig 3.12: Biotransformation of vanillyl alcohol (64 mM) by *A. adeninovorans* producing FmVAO or PsVAO. (A) UV assay of ethyl acetate extracts at 340 nm to detect

vanillin formation. (B) TLC analysis:). A negative control strain containing an empty vector (EV) was also included.

TLC and UV analysis showed that vanillin formation was already evident after 3 h of incubation with the strains expressing *PsVAO* and *FmVAO* and that vanillyl alcohol conversion with both strains was approximately the same. Even with the negative control strain some vanillin formation could already be observed after 3 h of incubation, although this activity was lower than with the two strains expressing *PsVAO* and *FmVAO* (Fig 3.12). After 24 h of incubation it appeared that vanillyl alcohol was with all three strains still present at approximately the same concentration as in the 0 h samples (Fig 3.12). The UV results showed that the maximum amounts of vanillin accumulated after 10 h incubation by the strains expressing *PsVAO* and *FmVAO* were 23 and 29 mM, respectively. The specific vanillyl-alcohol oxidase activities calculated over the first 6 h for *A. adeninivorans* strains expressing *PsVAO* and *FmVAO* were 1.3 and 1.8 U/g dry weight, respectively (Table 3.2). In *E. coli* PsVAO activity had been slightly higher than FmVAO activity.

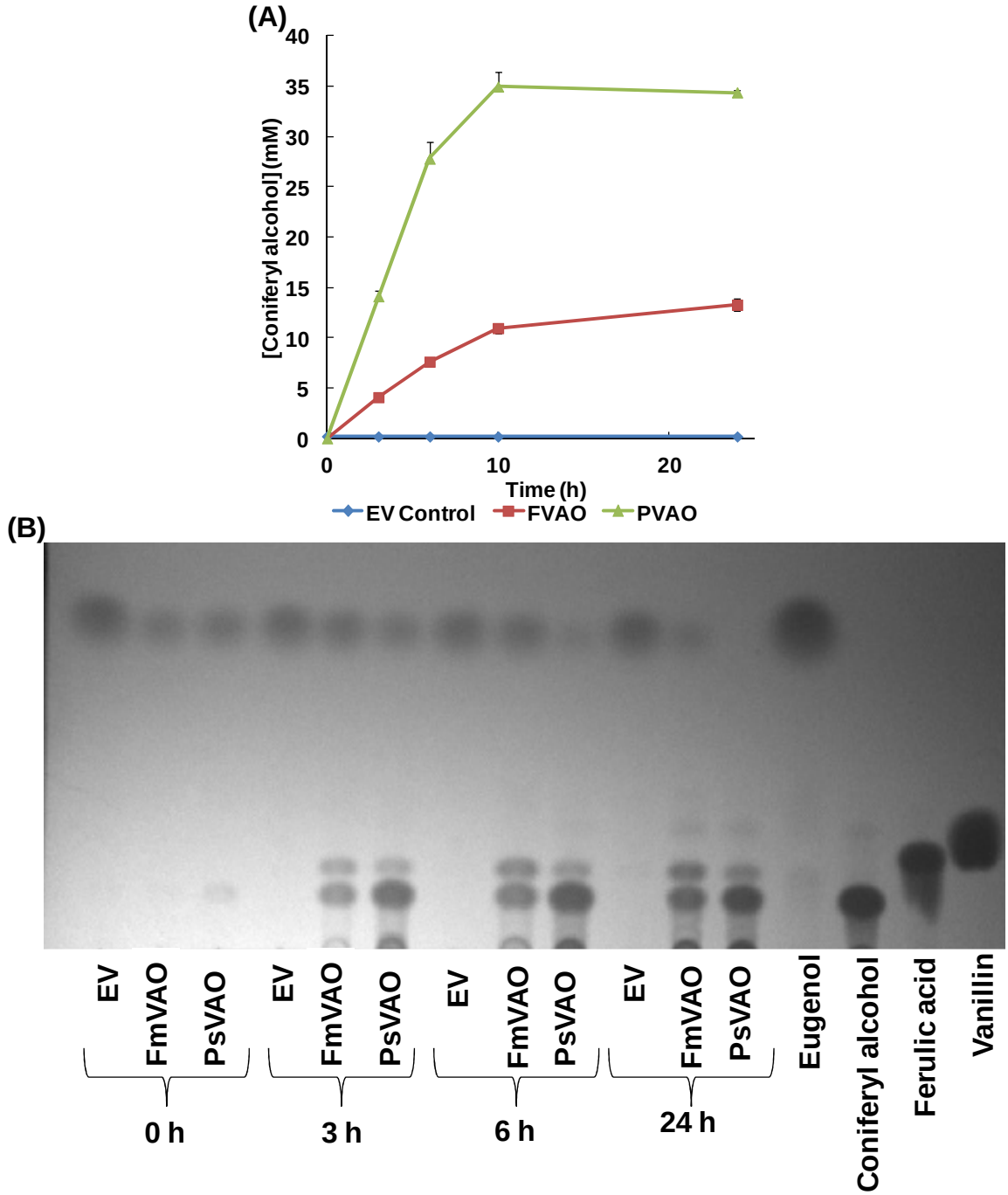

Fig 3.13: Biotransformation of eugenol (65 mM) by *A. adeninovorans* strains producing FmVAO or PsVAO. (A) UV assay of ethyl acetate extracts at 300 nm to coniferyl alcohol formation. (B) TLC analysis of ethyl acetate extracts. A negative control strain (EV) containing an empty vector was also included.

TLC analysis of samples from whole cell eugenol biotransformations showed that coniferyl alcohol was formed after 2 h incubation by both the *PsVAO* and *FmVAO* expressing strains. The *PsVAO* expressing strain formed more coniferyl alcohol than the *FmVAO* expressing strain. A second product, possibly ferulic acid, could also be observed with both strains after 3 h incubation. The eugenol was almost depleted after 6 h in the biotransformation with the strain containing *PsVAO*. After 24 h a third product, possibly vanillin, appeared in the reactions with both strains (Fig 3.13). It seems that after the initial reaction in which eugenol is converted to coniferyl alcohol by both PsVAO and FmVAO, the native enzymes of the yeast are oxidizing it further resulting in the formation of ferulic acid, which can then be converted to vanillin (Overhage *et al*., 2003). A relatively large amount of product could also be observed at the origin of the TLC. These are most likely the larger coniferyl alcohol polymers. These coniferyl alcohol polymers, together with 2 other building blocks, *p*-hydroxycoumaryl and sinapyl alcohol, form lignin (Reale *et al*., 2010). The control showed no background activity thus proving that the products formed are due to the eugenol substrate and the cloned *VAO* genes. *K. marxianus* had formed less of these coniferyl alcohol byproducts and recovery of coniferyl alcohol from the whole cell biotransformations with *K. marxianus* was better. The highest coniferyl alcohol concentration observed with *K. marxianus* was 55 mM, while with *A. adeninivorans* it was only 35 mM. Also with *E. coli* much less byproducts were formed with measured coniferyl alcohol concentrations (70 mM) even slightly exceeding the expected concentration for 100% conversion.

Table 3.2: Comparison of the specific whole cell activities obtained with *E. coli* Bl21(DE3) pLysS-RARE2 and *A. adeninivorans* producing PsVAO and FmVAO. Whole cell biotransformations were carried out with eugenol and vanillyl alcohol as substrates.

		E. coli*		***A. adeninivorans***	
		Dry biomass concentration g/L	**Specific activity U/g dry weight**	**Dry biomass concentration g/L**	**Specific activity U/g dry weight**
PsVAO	Eugenol	3.7	11.0	29.6	2.6
	Vanillyl alcohol	3.7	6.1	29.6	1.3
FmVAO	Eugenol	2.8	2.2	30.5	0.7
	Vanillyl alcohol	2.8	5.7	30.5	1.8

*Results from Chapter 2

The specific eugenol oxidase activities calculated from coniferyl alcohol concentrations determined spectrophotometrically for samples taken during the first 6 h were 2.6 U/g dry weight for the strain expressing *PsVAO* and 0.7 U/g dry weight for the strain expressing *FmVAO* (Table 3.2). Using coniferyl alcohol concentrations as a measure of eugenol oxidase activity of course in this case slightly underestimates the real activity, since coniferyl alcohol is not the only product produced. As previously observed in *E. coli* eugenol oxidase activity with whole cells *of A. adeninivorans* producing PsVAO was at least double the vanillyl-alcohol oxidase activity, while vanillyl-alcohol oxidase activity with FmVAO was almost three times higher than the eugenol oxidase activity. Thus as with whole cells of *E. coli,* PsVAO clearly preferred eugenol as substrate while FmVAO preferred vanillyl alcohol. Specific activities with whole cells of *E. coli* expressing *PsVAO* was approximately 4 times higher than with *A. adeninivorans* expressing *PsVAO*, while in the strains expressing *FmVAO* specific activities with *E. coli* was approximately 3 times higher than with *A. adeninivorans*.

3.3.5 VAO activity in crude cell-free extracts of *A. adeninivorans* expressing *FmVAO* and *PsVAO*

The crude cell-free extracts were used to monitor the specific activity of PsVAO and FmVAO produced by *A. adeninivorans*. A continuous assay was used and vanillyl alcohol (1 mM) and eugenol (1 mM) was used as substrates. The formation of vanillin was followed at 340 nm and the formation of coniferyl alcohol was followed at 300 nm using a microtiterplate reader. All assays were done in triplicate.

Comparison of the activities obtained with PsVAO and FmVAO when vanillyl alcohol was used as substrate (Table 3.3) revealed that the activities were almost the same, namely 0.045 U/mg protein for FmVAO and 0.040 U/mg protein for PsVAO. Activity of PsVAO was the same for both substrates, whereas FmVAO again, as with whole cells, preferred vanillyl alcohol as substrate. PsVAO gave a specific activity of 0.040 U/mg protein when eugenol was used as substrate in comparison with FmVAO which gave only 0.015 U/mg protein.

Comparison of the VAO activity in cell-free extracts of *A. adeninivorans* and *E. coli* BL21(DE3)pLysS-RARE2 indicated that specific activities were better in *A. adeninivorans* than in *E. coli* (Table 3.3). This was opposite from with whole cells, where specific activity of the VAOs in *E. coli* was higher.

Table 3.3: Comparison of the specific activity in cell-free extracts of *E. coli* Bl21(DE3)pLysS-RARE2 and *A. adeninivorans* producing PsVAO and FmVAO. Crude protein extracts were assayed for VAO activity using eugenol
(1 mM) and vanillyl alcohol (1 mM) as substrates.

		E. coli*		***A. adeninivorans***	
		Protein concentration mg/ml	**Rate U/ mg protein**	**Protein concentration mg/ml**	**Rate U/ mg protein**
PsVAO	Eugenol	200	0.030	200	0.040
	Vanillyl alcohol	400	0.028	200	0.041
FmVAO	Eugenol	200	0.005	200	0.015
	Vanillyl alcohol	400	0.018	200	0.045

*Results from chapter 2.

In the case of *PsVAO*, the activity increased from 0.030 U/mg protein in *E. coli* to 0.040 U/mg protein in *A. adeninivorans* when eugenol was used as substrate and from 0.028 U/mg protein to 0.041 U/mg protein when vanillyl alcohol was used. With FmVAO the activity increased three fold from 0.005 U/mg protein in *E. coli* to 0.015 U/mg protein in *A. adeninivorans* when eugenol was used as substrate and almost three fold from 0.017 U/mg protein in *E. coli* to 0.045 U/mg protein in *A. adeninivorans* when vanillyl alcohol was used (Table 3.3). Specific PsVAO activities obtained with cell free extracts of *A. adeninivorans* were 1.5 times higher than with *E. coli*, while FmVAO activities were almost three times higher. *FmVAO* thus benefited more from expression in *A. adeninivorans* than *PsVAO*, confirming the potential that yeast expression holds for the study of new enzymes from filamentous fungi and other eukaryotes. The specific vanillyl-alcohol oxidase activities obtained with *A. adeninivorans* were, however, still 10 times less the activity obtained by Benen *et al*. (1998) when they expressed *PsVAO* in *Aspergillus niger* under the wild type promoter of *PsVAO*. They obtained 0.484 U/mg protein after induction with anisyl alcohol and 0.346 U/mg protein after induction with

veratryl alcohol. Studies are currently in progress in our group to gain a better understanding of the *TEF* promoter that is driving expression in *A. adeninivorans* and to further improve expression of *PsVAO* and *FmVAO*.

3.3.6 Fractionation of cell-free extracts from *A. adeninivorans* strains containing *PsVAO* or *FmVAO* to determine localization of the protein

Fraaije *et al*. (1998) demonstrated that wild type PsVAO is located in the peroxisomes and cytosol of *P. simplicissimum* and they argued that the WKL-COOH tripeptide sequence at the C-terminus sufficiently resembles the SKL-COOH consensus sequence of the well-known peroxisomal targeting sequence type I (PTS1) to facilitate targeting of some of the PsVAO to the peroxisomes. The C-terminal tripeptide sequence of FmVAO is TKL-COOH and might also be able to target some of the FmVAO to the peroxisomes.
In order to determine where the protein is localized in *A. adeninivorans*, crude cell-free extracts were subjected to differential centrifugation. The different fractions (12 000 x *g* pellet, 80 000 x *g* pellet and 80 000 x *g* supernatant) were loaded onto a SDS-PAGE gel and later visualized using Coomassie Brilliant Blue as a stain. It was expected that the 12 000 x *g* pellet should contain mitochondria and peroxisomes and the 80 000 x *g* pellet microsomes while the 80 000 x *g* supernatant should then contain only soluble protein (Stephanova and Topouzova, 2001). The centrifugation speeds used to separate mitochondria and peroxisomes from microsomes might not have been sufficient, since the protein bands in all samples from the 12 000 x *g* and 80 000 x *g* pellets appeared to be almost the same. According to studies done with *S. cerevisiae* isolation of mitochondria and peroxisomes require centrifugation at 20 000 to 30 000 x *g* while isolation of microsomes require centrifugation at 300 000 x *g* (Wiederhold *et al*., 2010). Both the 12 000 x *g* and 80 000 x *g* pellets we obtained can thus be expected to contain mitochondria and peroxisomes. The expected protein sizes for PsVAO and FmVAO should be between 55kDa and 70 kDa. After visualization no discernable bands could be seen in this size range that could be ascribed to FmVAO or PsVAO in any of the fractions. To determine if the FAD cofactor could be detected a second SDS-

PAGE gel was run and soaked in acetic acid before exposing to UV-light. No fluorescence could be observed with any of the test crude extracts (Fig 3.14). The SDS analysis was done using 10 µg protein from each fraction and this might not be enough protein to detect fluorescence.

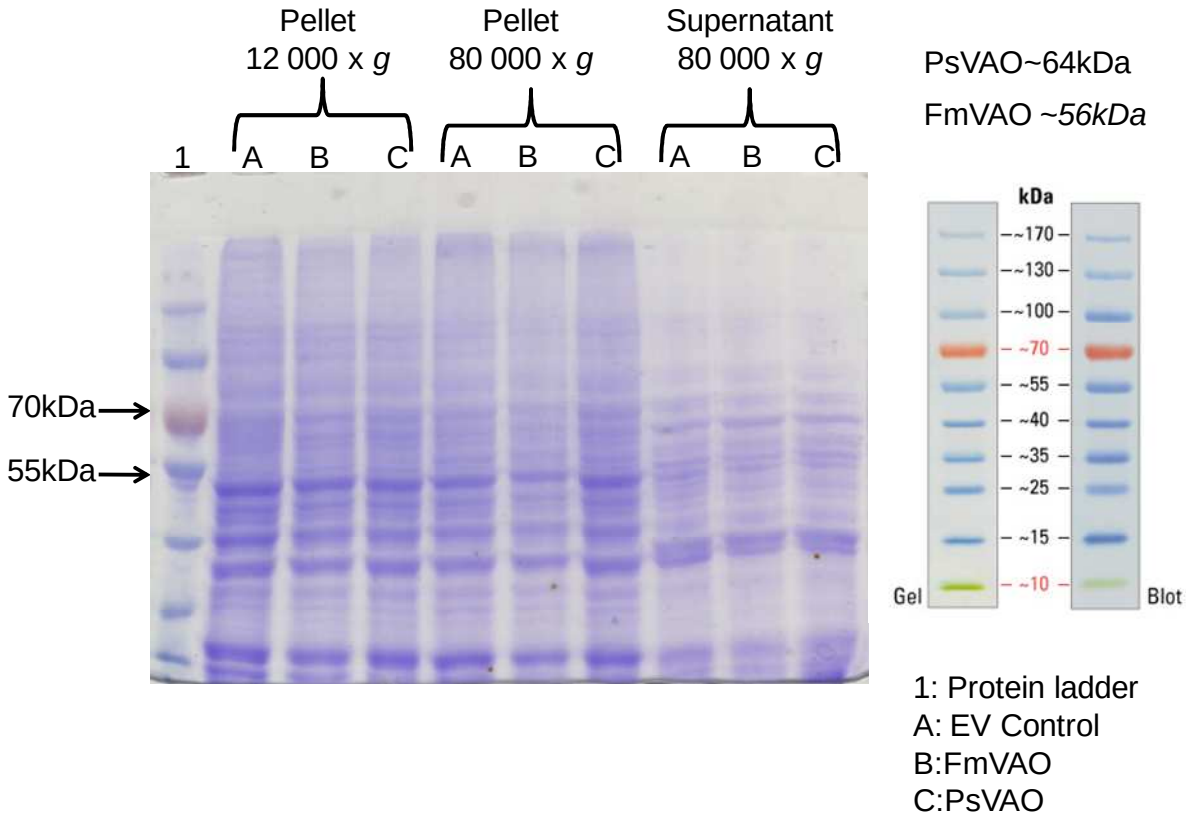

Fig 3.14: SDS-PAGE gel visualizing the different fractions from cell-free extracts of *A. adeninivorans* containing PsVAO, FmVAO or the empty vector (EV Control). 10 µg protein was loaded in each lane. The expected protein size for *PsVAO* is ~64 kDa and for FmVAO it is ~56kDa.

These results were similar to those obtained previously when these genes were expressed in *E. coli* (Chapter 2). The expression of these genes is apparently still too low to be visualized with SDS-PAGE gels.

VAO activity assays carried out with the different fractions showed no activity in the 12 000 x *g* and 80 000 x *g* pellets, indicating that the VAOs were not present in the peroxisomes of *A. adeninovorans*. It thus seems that *A. adeninivorans* does not recognize the unusual PTS1 sequences recognized by *P. simplicissimum* and *F. moniliforme.* The vanillyl-alcohol oxidase activities in the soluble fractions were 0.009 U/mg prt for PsVAO and 0.012 U/mg prt for FmVAO. These activities were four times lower than the activities observed with the total crude extract in a previous experiment.

3.4 Conclusion

Benen *et al*. (1998) obtained 0.017 U/mg protein vanillyl-alcohol oxidase activity when expressing *PsVAO* in *E. coli* TG2 containing the pIM3972 plasmid carrying the *PsVAO* gene, while we obtained 0.028 U/mg when *E. coli* Bl21(DE3)pLysS-RARE2 was transformed with the pET28b(+) vector containing the *PsVAO* gene. Compensating for rare codons thus did not improve expression dramatically and it was still not possible to detect discernable VAO bands of either PsVAO or FmVAO on SDS-PAGE gels. When *A. niger* NW156-T10 was transformed with the pIM3971 plasmid carrying PsVAO up to 0.484 U/mg protein vanillyl alcohol oxidase activity was obtained (Benen *et al*., 1998). Dr. M. Labuschagné had at the UFS used PsVAO to test a newly developed wide range expression vector, pKM118, to test expression in seven different yeasts (unicellular fungi) namely *A. adeninivorans*, *C. deformans*, *H. polymorhpa*, *K. lactis*, *K. marxianus*, *S. cerevisiae* and *Y. lipolyitca* (Smit *et al*., 2011). He found with whole cell biotransformations that two of the yeasts, *A. adeninivorans and K. marxianus*, performed much better than the others. In this study *K. marxianus* and A*. adeninivorans* were further evaluated for the expression of *PsVAO* and *FmVAO*.

PsVAO and *FmutVAO*, the mutated *VAO* gene from *F. moniliforme* MRC 6155, were cloned into *K. marxianus* using the pKM63 vector which contains 18S rDNA fragments from *Kluyveromyces marxianus* for genomic integration, a geneticin resistance marker and the native inulinase promoter of *K. marxianus* to drive expression of the cloned

gene. Strains containing the *FmutVAO* gene formed no coniferyl alcohol in whole cell eugenol biotransformations, but strains containing the *PsVAO* gene initially produced 47 mM coniferyl alcohol within 2 h when 65 mM eugenol was added directly to 48 h cultures. In a follow up experiment carried out 8 weeks later with cultures stored at -80 ºC it took 12 h to produce 43 mM coniferyl alcohol. At approximately the same time Labuschagné demonstrated that the vectors integrated into the rDNA of *K. lactis* and *K. marxianus* were unstable (Smit *et al*., 2011) and it was concluded that the dramatic drop in activity of PsVAO in *K. marxianus* can probably be ascribed to instability. Because *K. marxianus* transformants were unstable no further work was done with this yeast. Genetic engineering of *K. marxianus* is currently in progress at the UFS to improve stability of transformants.

PsVAO and *FmVAO* were cloned into *A. adeninivorans* using the pKM118 vector which also integrates into the 18S rDNA, but contains a hygromycin resistance marker and the *Yarrowia lipolytica TEF* promoter to drive expression of the cloned gene. Comparison of the specific activities in cell free extracts of both *FmVAO* and *PsVAO* expressed in *A. adeninivorans* and *E. coli* revealed that expression in the yeast increased the activity in cell-free extracts significantly, with FmVAO production benefiting more from expression in *A. adeninivorans*. The vanillyl-alcohol oxidase activity of FmVAO more than doubled from 0.018 U/mg protein in *E. coli* to 0.045 U/mg protein in *A. adeninivorans*, while the eugenol oxidase activity tripled from 0.005 U/mg protein to 0.015 U/mg protein. Both the vanillyl-alcohol oxidase and eugenol oxidase activities of PsVAO increased from 0.028 U/mg protein in *E. coli* to 0.04 U/mg protein in *A. adeninivorans*. Differential centrifugation of cell free extracts showed that both PsVAO and FmVAO activity could only be detected in the soluble fraction, but it was still not possible to detect discernable VAO bands on SDS page gels.

One problem with the use of *A. adeninivorans* strains expressing PsVAO for whole cell eugenol biotransformations is that recovery of coniferyl alcohol after complete conversion of eugenol ranged between only 54% (this study) and 76% (M.J. Maseme, personal communication). TLC analysis of whole cell biotransformations revealed that

several other products were formed from eugenol. These products were also observed on TLC with *K. marxianus* PsVAO, but in lower concentrations. Comparison of R_f values of these products with those of authentic ferulic acid and vanillin indicated that the additional products with higher R_f values might be ferulic acid and vanillin. If conditions can be identified for induction of the enzymes responsible for vanillin production by *A. adeninivorans*, strains expressing *PsVAO* can be used for the synthesis of "natural" vanillin from eugenol, a cheaper starting material than ferulic acid, which is currently used for the synthesis of vanillin.

Some products also remained at the origin of the TLCs. These products are most likely polymers of coniferyl alcohol. Coniferyl alcohol polymers form naturally in plants to form the macromolecular structure lignin. These polymerizations cannot be studied *in vivo* and relies mostly on *in vitro* experiments. Coniferyl alcohol can for instance be polymerized in the presence of enzymes like laccase or in the presence of hydrogen peroxide and peroxidase. It is quite likely that such reactions can be catalyzed by endogenous enzymes of *A. adeninivorans*.

Eugenol oxidase activity of 2.6 U/g dry weight was observed in whole cell biotransformations with PsVAO produced in *A. adeninivorans*. Whole cell eugenol oxidase activities with *PsVAO* expressed in *E. coli* was 11 U/g dry weight. In the *E. coli* experiments biomass concentrations were however 10 times lower than in the *A. adeninivorans* experiments and only 65 mM eugenol was used. It has already been demonstrated in later studies that *A. adeninivorans* strains producing PsVAO can convert up to 650 mM eugenol in whole cell biotransformations to yield 200 mM coniferyl alchol. Experiments still need to be done to test *E. coli* strains expressing *PsVAO* under conditions of high biomass and substrate concentrations, before it can be decided which of *E. coli* or *A. adeninovorans* strains expressing *PsVAO* are the best whole cell biocatalysts.

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CHAPTER 4

General discussion and conclusion

This project raised a number of questions/issues which require some discussion.

4.1 To synthesize or to amplify a gene

Genome and metagenome sequencing has made available vast amounts of sequence data that can be easily accessed through various databases. This gives us access to a massive pool of potential new biocatalysts. Tools for sequence comparison (BLAST) can be used to search for similar sequences in these databases. After obtaining the results from such a search two routes can be followed in order to express the gene in a recombinant host. RNA can be isolated from the host and cDNA can be synthesized or the gene can be synthesized directly by making use of the various companies that provide such services. Genes can be ordered and delivered within five to ten days making it time saving. At current 2012 prices of around $0.35/bp it would cost only around $600 to have the *FmVAO* gene synthesized and it seems unthinkable that we had chosen to clone the gene from mRNA. However, when this project started in 2007 gene synthesis was about four times more expensive and at around $1.25/bp, the cost would have been approximately $2100. This amount was still considered expensive and when mRNA isolation and cDNA synthesis, which can often be a bottle neck, was successful at the first attempt we thought we had made the correct decision. It was very unfortunate that so many mutations were introduced during cDNA synthesis and in retrospect gene synthesis would still have been the better, cheaper option.

However, by starting with the wild type organism we learnt that the VAO in *Fusarium moniliforme* is an active enzyme and induced by the same inducers that induce expression in *Penicillium simplicissimum.* We also learnt that the VAO genes from *Fusarium moniliforme* strains from different continents (North America and South Africa) are 98% identical. If researchers will only have genes synthesized and never go back to the wild type organisms this type of knowledge will be lost. On the other hand one can

still separately investigate expression conditions and amplify and sequence several genes from different strains in parallel studies.

4.2 Why clone the gene rather than study the enzyme from the wild type organism

In this study we have investigated expression of *PsVAO* and *FmVAO* in *E. coli* as well as in two yeasts. The expression levels achieved, after much work, were of the same order as obtained with wild type *F. moniliforme, i.e*. 0.028 U/mg protein in *E. coli* and 0.040 U/mg protein in *A. adeninivorans* versus 0.036 U/mg protein in *F. moniliforme*. In this case one might reason that we might as well have continued to study and purify the FmVAO in cell-free extracts of *F. moniliforme*. Expressing a protein with a His-tag (in this case it was possible to express both *FmVAO* and *PsVAO* with N-terminal His-tags) usually offers the advantage of purification with Ni^{2+}-affinity chromatography. However, two attempts to purify FmVAO *via* this route have been unsuccessful (results not reported). The most important advantage offered by heterologous expression of the *FmVAO* was that expression was reproducible. With *F. moniliforme* growth and expression was often unpredictable.

4.3 Which cloning system to use for heterologous expression

There are today almost too many *E. coli* strains available for heterologous expression, many created to overcome common problems with heterologous expression of specific types of proteins, such as the presence of rare codons in a gene or toxicity of some recombinant proteins to the host. However *PsVAO* expression in three different *E. coli* strains using three different vectors gave very similar results. Benen *et al*., (1998) obtained 0.017 U/mg protein vanillyl-alcohol oxidase activity in cell free extracts of *E. coli* TG2 expressing *PsVAO* under its own promoter, while Overhage *et al*. (2003) obtained 0.02 U/mg protein with *E. coli* XL1-Blue(pSK*vao*mP*cal*Am*cal*B) with *PsVAO*

cloned under a *lac* promoter using IPTG induction. In this study we obtained 0.028 U/mg protein when we expressed PsVAO in *E. coli* Bl21(DE3)pRARE2 carrying *PsVAO* in the pET28b(+) vector which has the same promoter system and also require IPTG induction. These expression levels are too low to detect the protein on SDS PAGE gels, but was still sufficient for Benen *et al*. (1998) to purify the recombinant *PsVAO* and for others to create and study mutants of PsVAO (van den Heuvel *et al*., 1998; van den Heuvel *et al*., 2000a; van den Heuvel *et al*., 2000b). Interestingly the highest production of PsVAO has been obtained by Overhage *et al*. (2006) with *PsVAO* cloned into the hybrid plasmid pRLE6SK*vaom* which was subsequently transformed into *E. coli* XL 1-Blue and the gram-positive bacterium *Amycolatopsis* sp. HR167. With this vector they obtained in cell-free extracts of *E. coli* 7.1 U/mg of protein vanillyl alcohol oxidase activity and in *Amycolatopsis* sp . HR167 1.1 U/mg of protein.

Benen *et al.* (1998) also used *A. niger* NW156-T10 transformed with the pIM3971 plasmid carrying *PsVAO* for PsVAO expression. In this plasmid *PsVAO* expression is controlled by its own promoter and it was strongly induced by veratryl alcohol and anisyl alcohol. Because multiple copies of the vector (25 - 30 copies) were introduced very high expression levels of 0.484 U/mg protein vanillyl-alcohol oxidase activity was obtained. The use of a wide range yeast expression vector, pKM118, allowed comparative expression of *PsVAO* in seven different yeasts which are unicellular or dimorphic fungi. Two of these yeasts *Kluyveromyces marxianus* and *Arxula adeninivorans* gave in whole cell biotransformations of eugenol activities that were double the activities observed with the other yeasts (Smit *et al*,. 2011). *K. marxianus* transformants, which initially gave the best results, were unfortunately not stable and the focus turned to *A. adeninivorans*. Both PsVAO and FmVAO activities obtained with cell-free extracts of *A. adeninivorans* strains expressing the respective genes were higher than obtained with *E. coli i.e*. vanillyl-alcohol oxidase activities increased from 0.028 and 0.018 U/mg protein in *E. coli* to 0.041 and 0.045 U/mg protein in *A. adeninivorans*. It was interesting that *FmVAO* expression benefited more from using *A. adeninivorans* as host, even though it has fewer rare codons than *PsVAO*. Vectors using the same elements as pKM118 namely rDNA integration and the hygromycin

resistance marker have been shown to give 1 - 2 copies integrated into the chromosome (Gellissen *et al*., 2005). It was observed that with two copies of the expression casette integrated or when in later work two copies of the *PsVAO* gene were cloned into pKM118, activity in whole cell biotransformations doubled (Smit *et al*., 2011). The use of vectors that will give integration of high copy numbers thus can further improve *VAO* expression. Optimization studies giving a better understanding of the *TEF* promoter and of the requirements for *VAO* expression can also further improve expression levels.

4.4 To use whole cells or cell-free enzymes as biocatalysts

Fraaije *et al*., (1995) claimed that eugenol concentrations should preferably be lower than 10 mM for PsVAO catalyzed biotransformations using dead cells or cell-free enzyme preparations. Studies using PsVAO for the initial conversion of eugenol to coniferyl alcohol which is then to be further converted to ferulic acid and ultimately vanillin required the use of viable whole cells (Overhage *et al*., 2003; Overhage *et al*., 2006). Because of the extreme toxicity of eugenol, eugenol concentrations were kept below 0.01% v/v (2.6 mM) in these studies. Overhage *et al*., (2003) used *E. coli* XL1-Blue(pSK*vao*mP*cal*Am*cal*B), which also expresses two dehydrogenases to convert eugenol to coniferyl alcohol and then to ferulic acid. By feeding eugenol at rates that maintained the concentration below 0.01% v/v they were able to produce 14.7 g/L (76 mM) ferulic acid within 30 h in 30-liter scale fermentation. With 50 ml cultures in shake flasks they obtained 8.6 g/L (44 mM) ferulic acid within 15 h. Later Overhage *et al*. (2006) cloned *PsVAO* into the gram-positive bacterium *Amycolatopsis* sp. HR167. Using resting cells of this strain they produced within 16 h 4.7 g/L (26 mM) coniferyl alcohol from 26 mM eugenol added step-wise. With *K. marxianus* producing PsVAO we obtained 56 mM coniferyl alcohol from 650 mM eugenol added in one addition. No further increase in coniferyl alcohol was observed after 24 h, this might have been due to product inhibition, but could also have been due to problems with product extraction. In later work up to 200 mM coniferyl alcohol was

recovered from biotransformations of 650 mM eugenol by *A. adeninivorans* producing PsVAO (Smit *et al*., 2011). Even *E. coli* Bl21(DE3)pRARE2 producing PsVAO completely converted 65 mM eugenol within 30 h. In these *E. coli* experiments biomass concentrations were 10 times lower than in the *A. adeninivorans* experiments and higher substrate concentrations were not tested. PsVAO catalyzed conversion of eugenol to coniferyl alcohol evidently does not require viable cells. Cell-free extracts and *E. coli* containing PsVAO still need to be tested at high substrate concentrations to determine whether the use of yeasts hold any specific benefit when high substrate concentrations are employed.

Notable in the whole cell biotransformations of eugenol with *A. adeninivorans* expressing *PsVAO* and *FmVAO* were the accumulation of additional products - most likely ferulic acid and possibly vanillin or vanillic acid. Specifically with the strain producing FmVAO, which displayed lower eugenol oxidase activity, proportionately more ferulic acid was formed. With *A. adeninivorans* the further oxidation of coniferyl alcohol apparently did not require viable cells. These observations should be followed up to rigorously identify the products and to determine whether conditions can be found which enhance the expression of these endogenous oxidizing enzymes. These enzymes can also be isolated and studied in more detail.

4.5 Why search for more fungal VAOs

Members of the bigger VAO flavoprotein family share a conserved FAD binding domain and most members have a covalently bound FAD. FAD linkages involve different residues in the FAD binding domain and different atoms of the FAD, but linkage of the 8a-methyl group of the flavin isoalloxazine ring to a histidine residue occurs most often (Leferink *et al*., 2008). Members of the bigger VAO family catalyze a diversity of different reactions *via* oxidase and dehydrogenase mechanisms. Enzymes catalyzing oxidation of 4-alkyl- and 4-allylphenols form only a small branch on the bigger VAO family tree (Leferink *et al*., 2008). Despite PsVAO being the protein on which this bigger family is based (Fraaije *et al*., 1998), there were at the beginning of this project only two

confirmed fungal VAOs, those from *P. simplicissimum and Byssochlamys fulva*, and only the gDNA and cDNA sequences of the one from *P. simplicissimum* were available. BLAST searches with *FvVAO* delivered 72 sequences of VAO-like proteins. A phylogenetic analysis of these sequences showed that PsVAO and FvVAO (and thus FmVAO) belong together with only 10 other fungal sequences to a cluster of proteins that, with two exceptions, have the conserved residues that are critical for the VAO reaction mechanism. The question arises whether more of these putative VAOs and/or the two exceptions should be cloned and characterized. There is also a second cluster of fungal VAO-like proteins that lack the acidic residue (Asp170 in PsVAO) that is critical for catalysis and covalent flavinylation. What will we learn from the characterization of these enzymes?

FmVAO still needs to be purified and rigorously characterized but there are already strong indications that its substrate specificity and thus binding pocket is different from that of PsVAO. It is probably the first true vanillyl-alcohol oxidase which prefers vanillyl alcohol as substrate. Both PsVAO and the VAO from *B. fulva* in fact prefer eugenol as substrate. In whole cell biotransformations of ethylphenol FmVAO also yielded more product, although it was also subject to what is probably inhibition by 4-vinylphenol. PsVAO is subject to severe inhibition by 4-vinylphenol, a minor product formed from ethylphenol (van den Heuvel *et al*., 1998). More in depth study of FmVAO (i.e. kinetic studies with purified enzyme; 3D-structure modelling on PsVAO; crystallization and X-ray structure determination) might bring a better understanding of the substrate binding pocket that might in turn assist us in the development of better 4-alkylphenol hydroxylases with higher activity and that will not be subject to such severe product inhibition. It is of course also possible that one of the other eight putative VAOs of which the sequences are already available might be an excellent 4-alkylphenol hydroxylase.

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SUMMARY

There are currently only two confirmed fungal vanillyl-alcohol oxidases (VAOs), one from *Penicillium simplicissimum* (here called PsVAO) and one from *Byssochlamys fulva*. Only the gene sequence of PsVAO is available. *Fusarium* spp. was targeted as a source of more VAOs, because they are plant pathogens known for production of lignolytic enzymes and utilization of aromatic compounds. BLAST searches of the databases of the Fungal Genome Initiative of the Broad Institute using PsVAO as query supported this choice. The predicted protein (called FvVAO) of one hit, gene number FVEG 03424 from *Fusarium verticillioides,* shared 63% amino acid identity with PsVAO and grouped with PsVAO in a phylogenetic analysis.

Seven *Fusarium* strains from three species *F. verticilliodes* (synonym *Fusarium moniliforme*), *Fusarium graminearum* and *Fusarium oxysporum* were investigated for VAO activity. *F. moniliforme* MRC 6155 consistently displayed VAO activity in cell-free extracts with 0.036 U/mg protein obtained after veratryl alcohol induction. Primers based on the *FvVAO* gene were used to amplify the VAO gene (called *FmVAO*) from *F. moniliforme* MRC 6155 from both genomic DNA and mRNA. Comparison of the genomic sequences of *FvVAO* and *FmVAO,* which both have the same four introns*,* revealed a total of 42 nucleotide differences while the deduced amino acid sequences differed by seven amino acids. The sequences of the new *FmVAO* were submitted to GenBank (NCBI), accession number JQ410355.

Both *PsVAO* and *FmVAO* were cloned into the pET28b(+) vector adding N-terminal His-tags and expressed in *E. coli* BL21(DE3)pRARE2. Using this strain to compensate for rare codons improved the expression of *PsVAO* but it was still not possible to detect discernable VAO bands of either PsVAO or FmVAO on SDS-PAGE gels. Comparison of substrate specificity of PsVAO and FmVAO in assays done with cell free extracts and whole cell biotransformations revealed that FmVAO preferred vanillyl alcohol as substrate and can thus be regarded as a "true" vanillyl-alcohol oxidase - possibly the first. Vanillyl-alcohol oxidase activities of PsVAO and FmVAO in cell-free extracts were respectively 0.028 and 0.018 U/mg protein, while eugenol oxidase activities were 0.030 and 0.005 U/mg protein. In whole cell biotransformations of vanillyl alcohol, specific activities of PsVAO and FmVAO were respectively 6.1 and 5.7 U/g dry weight, while with eugenol as substrate activities were 11.0 and 2.2 U/g dry weight. In

whole cell biotransformations FmVAO showed higher activity with ethylphenol, again indicating its different substrate specificity.

PsVAO was also cloned and expressed in the yeasts *Kluyveromyces marxianus* and *Arxula adeninivorans* while FmVAO was also cloned and expressed in *A. adeninivorans*. The *K. marxianus* vector pKM63 which gave excellent but unstable expression in *K. marxianus* contains 18S rDNA fragments from *K. marxianus* for genomic integration, a geneticin resistance marker and the native inulinase promoter of *K. marxianus* to drive expression of the cloned gene. The wide range vector pKM118 used for cloning into *A. adeninivorans* only differs from pKM63 in that it contains a hygromycin resistance marker and uses the *Yarrowia lipolytica TEF* promoter to drive expression of the cloned gene. Comparison of the specific activities in cell free extracts of both *FmVAO* and *PsVAO* expressed in *A. adeninivorans* and *E. coli* revealed that expression in the yeast increased the activity in cell-free extracts, with *FmVAO* benefiting more from expression in *A. adeninivorans*. The vanillyl-alcohol oxidase activity of FmVAO in *A. adeninivorans* was 0.045 U/mg protein and the eugenol oxidase activity, 0.015 U/mg protein. Both the vanillyl-alcohol oxidase and eugenol oxidase activities of PsVAO in *A. adeninivorans* were 0.04 U/mg protein. Differential centrifugation of cell free extracts showed that both PsVAO and FmVAO activity could only be detected in the soluble fraction.

Keywords: Fungi, vanillyl-alcohol oxidase, phylogenetic analysis, gene, rare codon, heterologous expression, specific activity, *Fusarium*

OPSOMMING

Daar is tans slegs twee bevestige vanilliel-alkohol oxidases (VAOs) afkomstig van swamme, een van *Penicillium simplicissimum* (genoem PsVAO) en een van *Byssochlamys fulva*. Slegs die geen volgorde van PsVAO is beskikbaar. *Fusarium* spp. is geteiken as 'n bron van meer VAOs, want hulle is plantpatogene wat bekend is vir die produksie van lignien afbreekbare ensieme en die produksie van aromatiese verbindings. Die BLAST navrae wat gerig was aan die databasisse van die Swam Genoom Inisiatief van die Broad Institute met PsVAO as navraag ondersteun hierdie keuse. ‘n Voorspelde proteïen (genoem FvVAO), geen aantal FVEG 03424 van *Fusarium verticillioides*,wat so verkry is het ‘n 63% aminosuur identiteit gedeel met PsVAO en was gegroepeer saam met PsVAO in 'n filogenetiese analise.

Sewe *Fusarium* stamme van drie spesies. *F. verticilliodes* (sinoniem *Fusarium moniliforme*), *Fusarium graminearum* en *Fusarium oxysporum* is vir VAO aktiwiteit ondersoek. *F. moniliforme* MRC 6155 het konsekwent VAO aktiwiteit, vanaf 0.036 U/mg proteïen in selvrye fraksies getoon nadat veratryl alkohol bygevoeg is vir induksie. Teiken DNA fragmente wat gebaseer is op die *FvVAO* geen, is gebruik om die *VAO* geen (genoem *FmVAO*) van *F. moniliforme* MRC 6155 van beide DNA en mRNA te amplifiseer. ‘n Vergelyking van die genomiese DNA van *FvVAO* en *FmVAO* het dit aan die lig laat kom dat albei dieselfde vier introns het. Daar was 'n totaal van 42 nukleotied verskille, terwyl die afgeleide aminosuurvergelyking met sewe aminosure verskil het. Die genoomkode van die nuwe *FmVAO* was ingedien by GenBank (NCBI), Aanwysingsnommer: JQ410355.

Beide *PsVAO* en *FmVAO* is gekloon in die pET28b(+) vektor en n-terminale His-etikette is bygevoeg vir uitgedrukking in *E. coli* BL21(DE3)pRARE2. Die spesifieke subspesie is gebruik om te vergoed vir seldsame kodons om dus so uitdrukking van *PsVAO* te verbeter, maar dit was nog nie moontlik om waarneembare VAO bande van, of PsVAO of FmVAO op SDS PAGE gels waar te neem nie. ‘n Vergelyking van die substraat spesifisiteit van PsVAO en FmVAO in toetse wat gedoen is met 'n selvrye fraksies sowel as met heelsel biotransformasies het aan die lig gebring dat FmVAO voorkeur aan vanilliel alkohol as substraat gee en kan dus beskou kan word as 'n "ware" vanilliel-alkohol oxidase - moontlik die eerste.

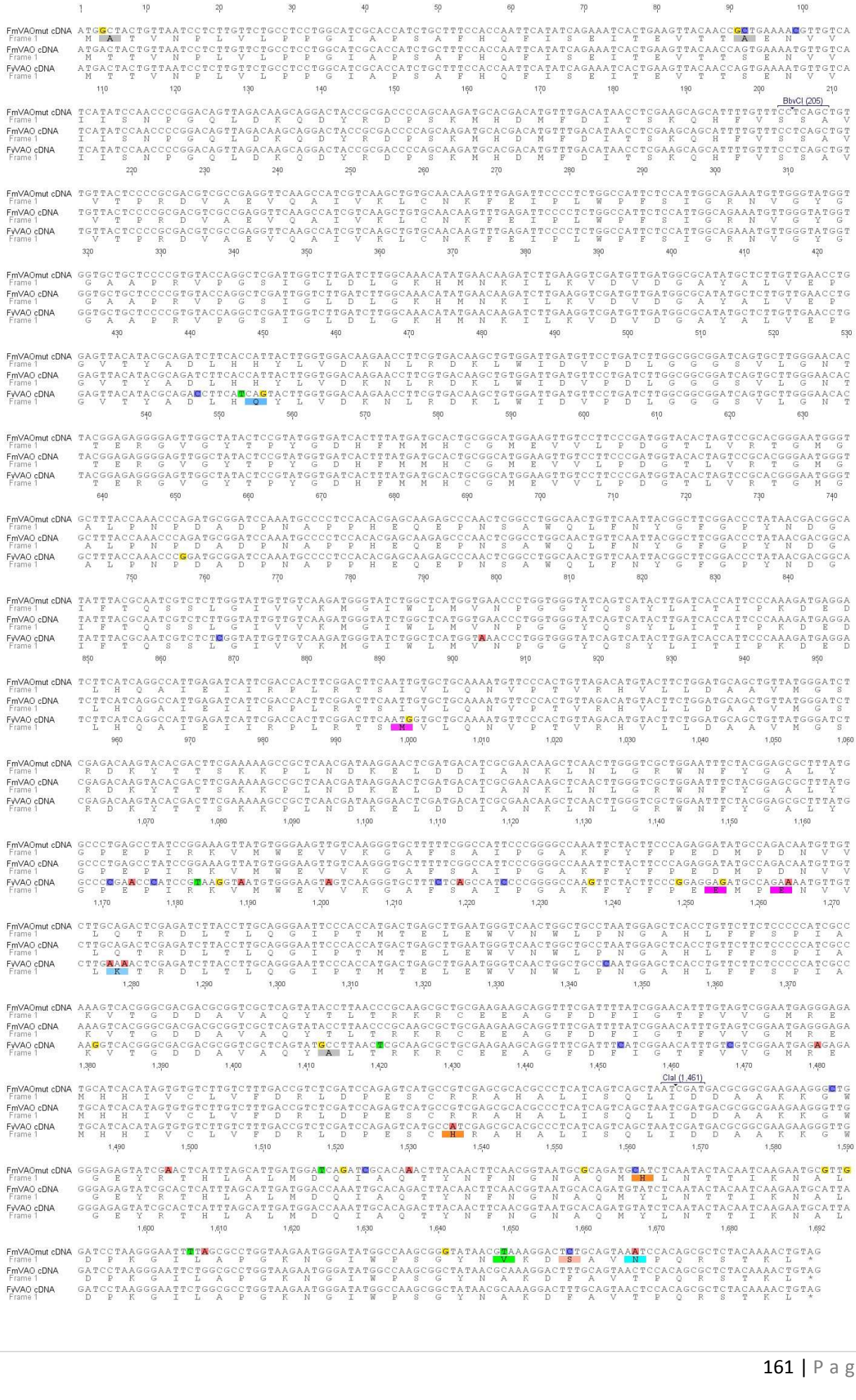
Vanilliel-alkohol oxidase aktiwiteite van PsVAO en FmVAO in selvrye fraksies was onderskeidelik 0,028 en 0,018 U/mg proteïen, terwyl eugenol oksidase aktiwiteit 0,030 en 0,005 U/mg proteïen onderskeidelik was. Heelsel-biotransformasies van vanilliel alkohol het onderskeidelik 6,1 en 5,7 U/g droë gewig gegee vir PsVAO en FmVAO gegee, terwyl die eugenol substraat aktiwiteite, 11,0 en 2,2 U/g droë gewig onderskeidelik was. In heelsel-biotransformasies het FmVAO hoër aktiwiteit met ethylphenol getoon, wat weereens klem lë op die verskil in substraat spesifisiteit.

PsVAO is ook gekloon en uitgedruk in die-giste *Kluyveromyces marxianus* en *Arxula adeninivorans*, terwyl *FmVAO* gekloon en uitgedruk is in *A. adeninivorans*. Die *K. marxianus* vektor pKM63 het uitstekende, maar onstabiele uitdrukking in *K. marxianus* veroorsaak. Dit bevat 18s rDNA fragmente afkomstig vanaf *K. marxianus* vir genomiese integrasie, 'n geneticin weerstands merker en die inheemse inulinase promotor afkomstig van *K. marxianus* vir uitdrukking van die gekloonde geen. Die wye verskeidenheids vektor, pKM118 wat gebruik is vir kloning in *A. adeninivorans* verskil slegs van pKM63 in dat dit 'n hygromycin weerstands merker bevat sowel as die *Yarrowia lipolytica TEF* promotor vir uitdrukking van die gekloonde geen . Vergelyking van die spesifieke aktiwiteite in 'n sel vrye ekstrakte van beide *FmVAO* en *PsVAO* uitgedruk in *A. adeninivorans* en *E. coli* het die lig gebring dat die uitdrukking in die gis die aktiwiteit in die selvrye ekstrak met FmVAO bevoordeel het. Die vanilliel-alkohol oksidase aktiwiteit van FmVAO in *A. adeninivorans* was 0,045 U/mg proteïen en die eugenol oksidase aktiwiteit, 0,015 U/mg proteïen. Beide die vanilliel-alkohol oxidase en eugenol oksidase aktiwiteite van PsVAO in *A. adeninivorans* was 0,04 U / mg proteïen. Differensiële sentrifugering van 'n selvrye ekstrakte het gewys dat beide PsVAO en FmVAO aktiwiteit slegs waargeneem kan word in die oplosbare oplosbare fraksie.

Sleutelwoorde: Swam, vanilliel-alkohol oxidase, filogenetiese analiese, geen, rare kodon, aminosuur kode, heterologiese uitdrukking, spesifieke aktiwiteit, *Fusarium*

Appendix A

Alignment of FvVAO cDNA with the FmutVAO cDNA and the FmVAO cDNA.


FmVAOmut cDNA
FmVAO cDNA
FvVAO cDNA
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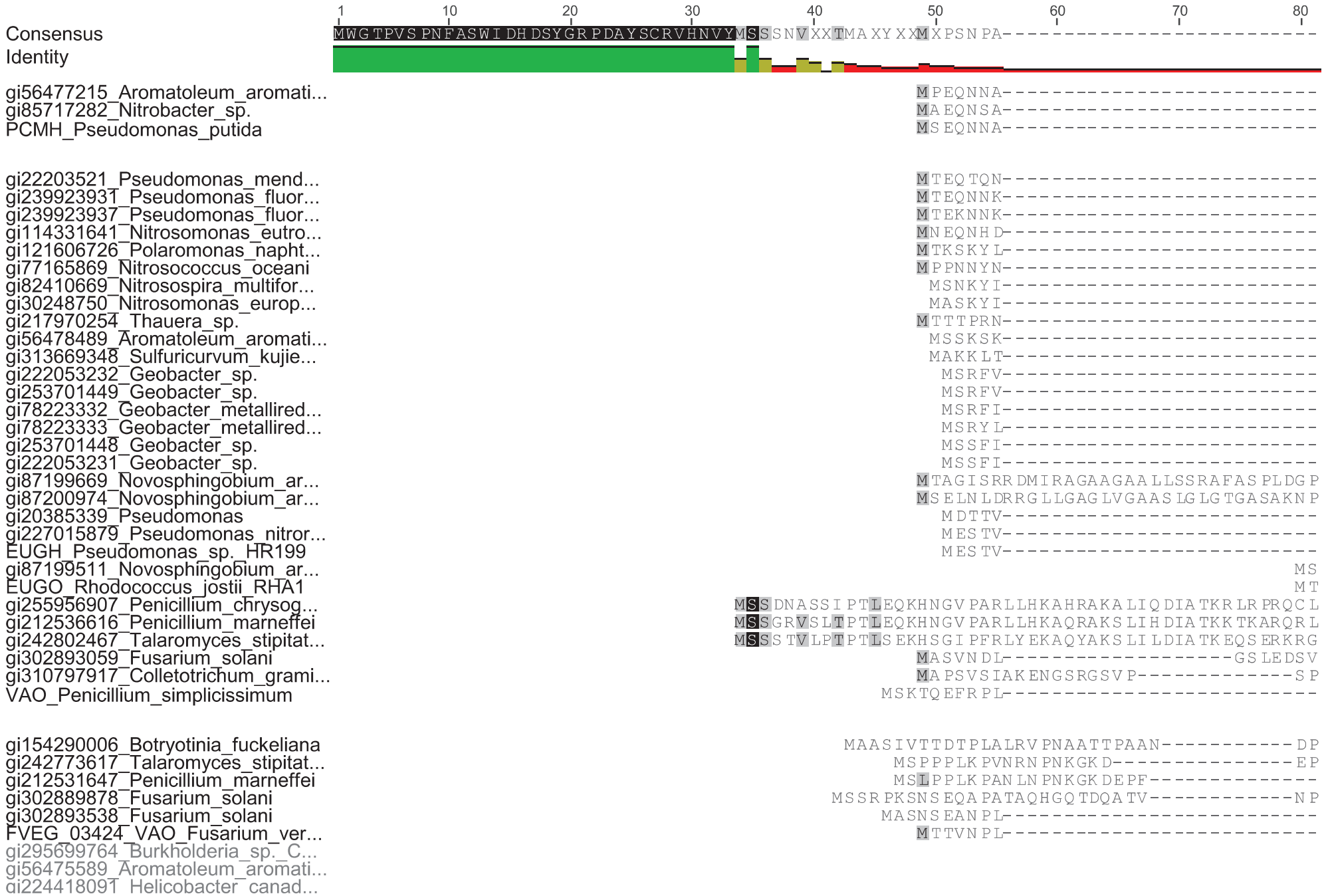
Appendix B

Seventy two sequences of VAO-like proteins obtained through BLAST searches.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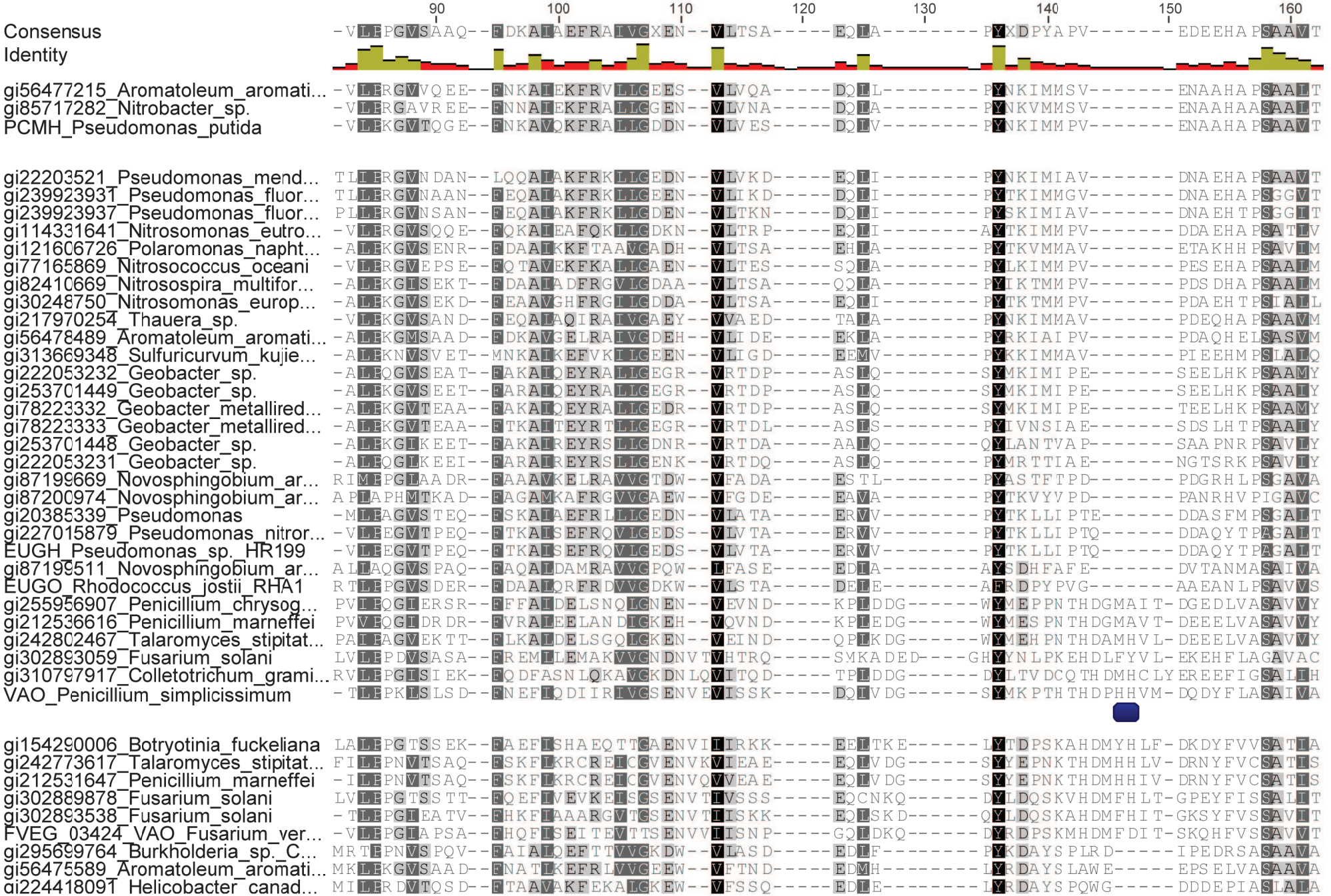
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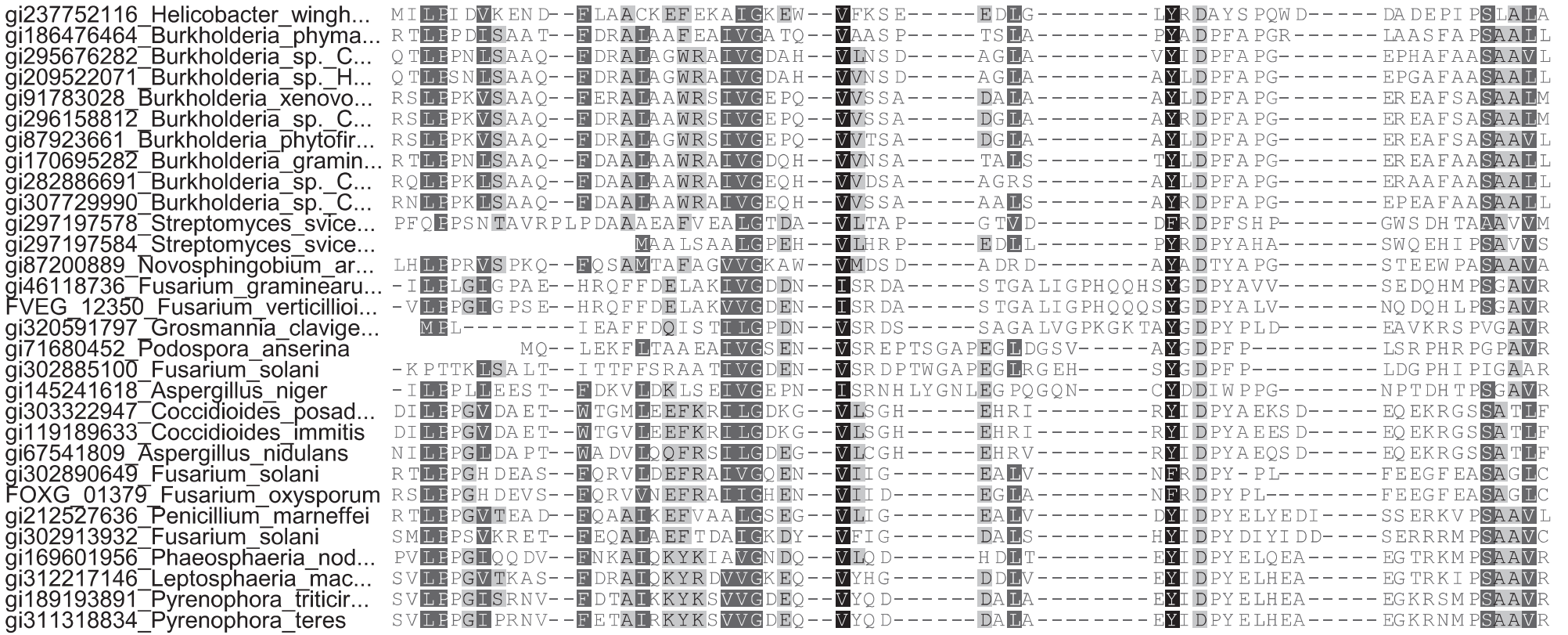
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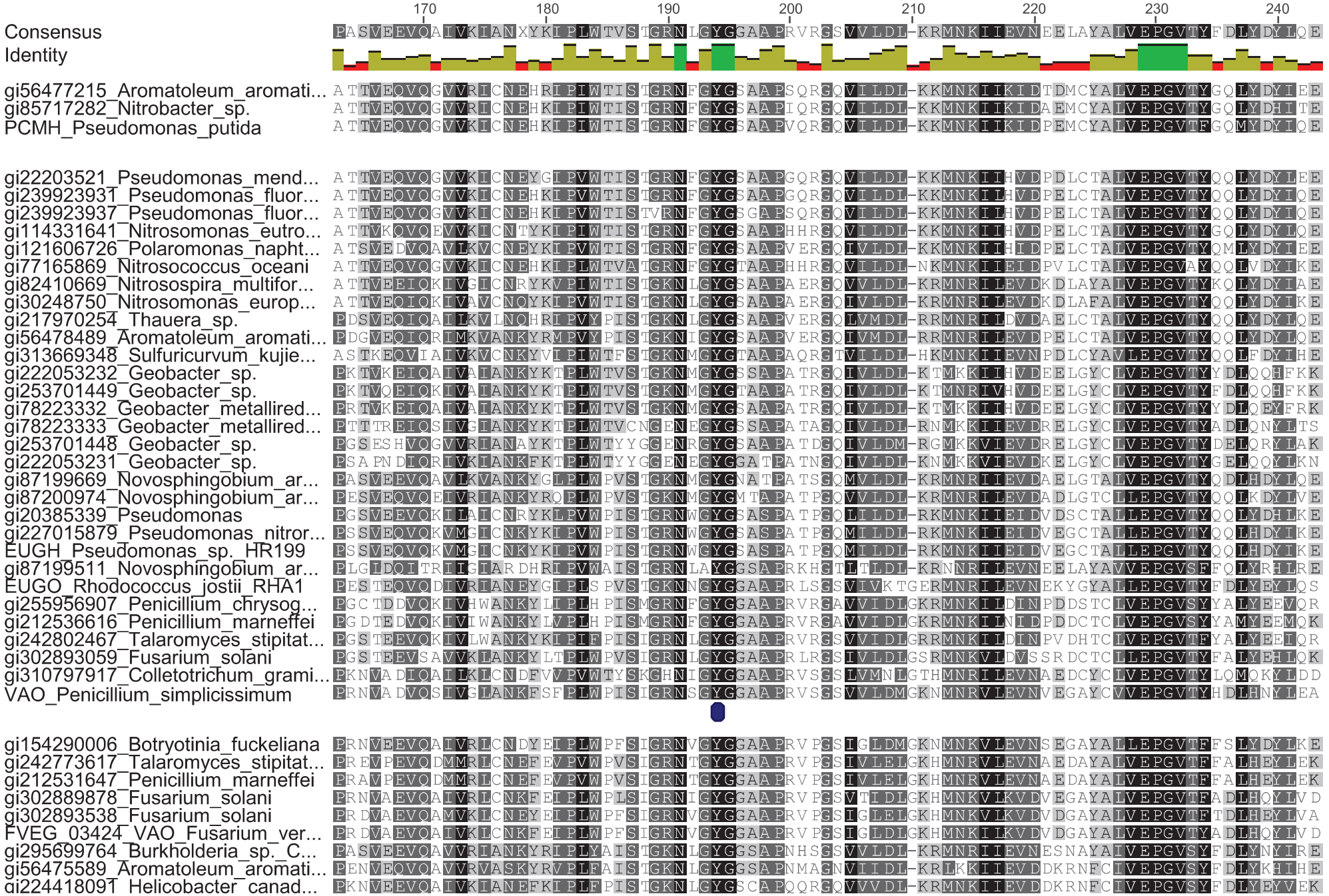
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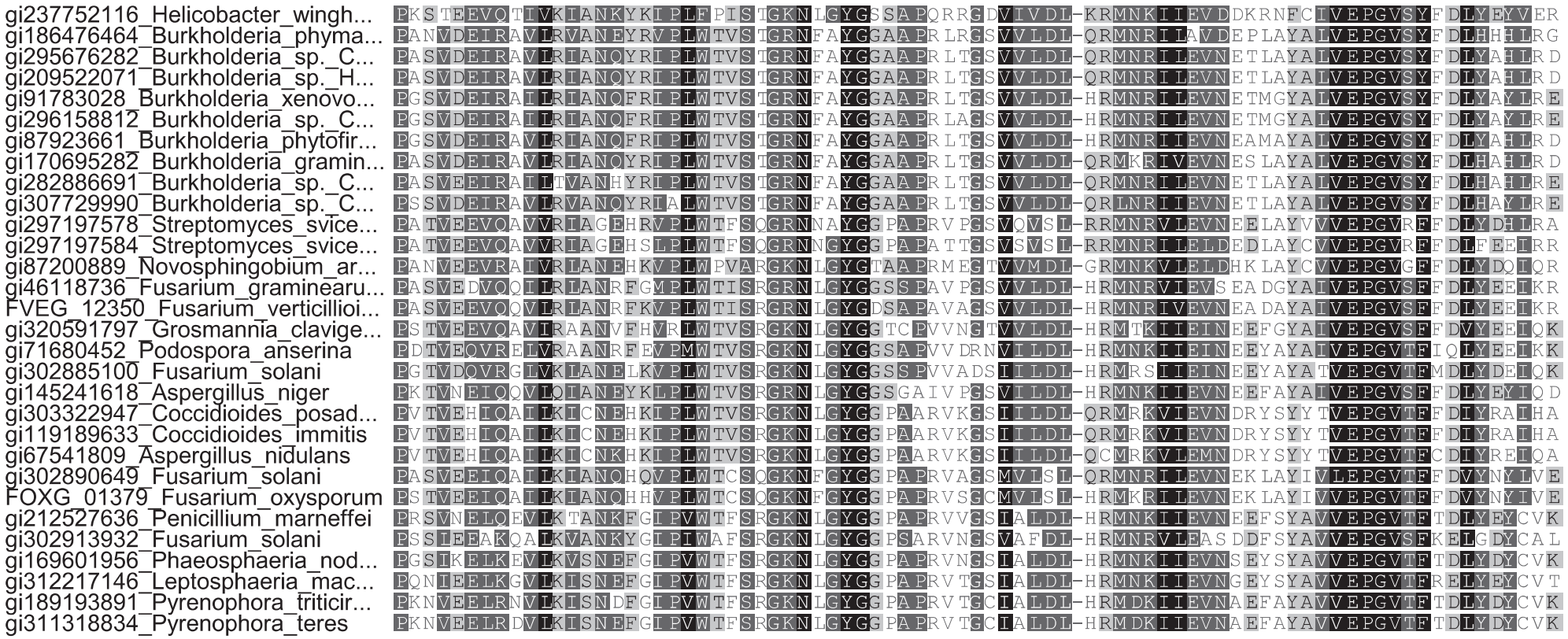
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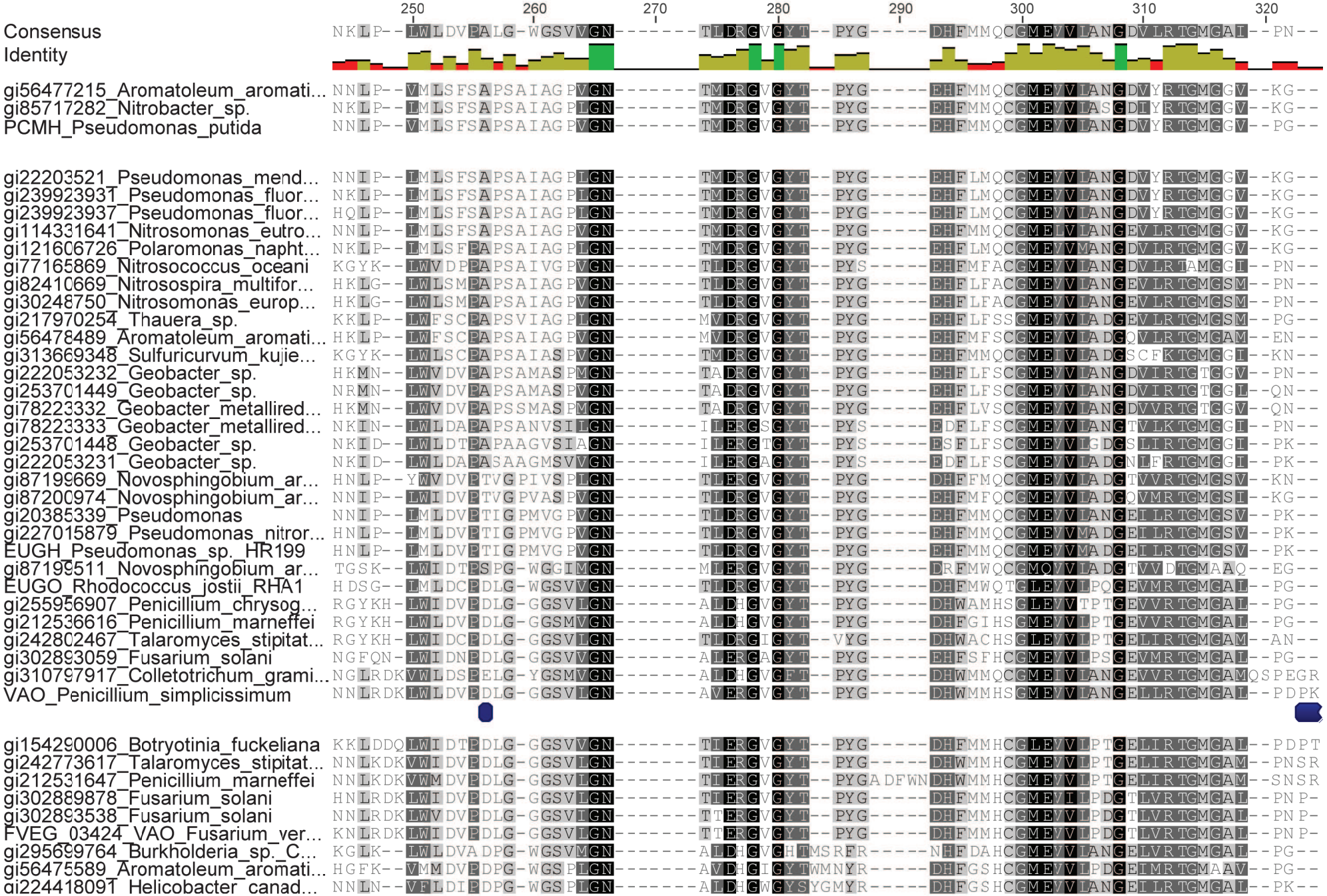
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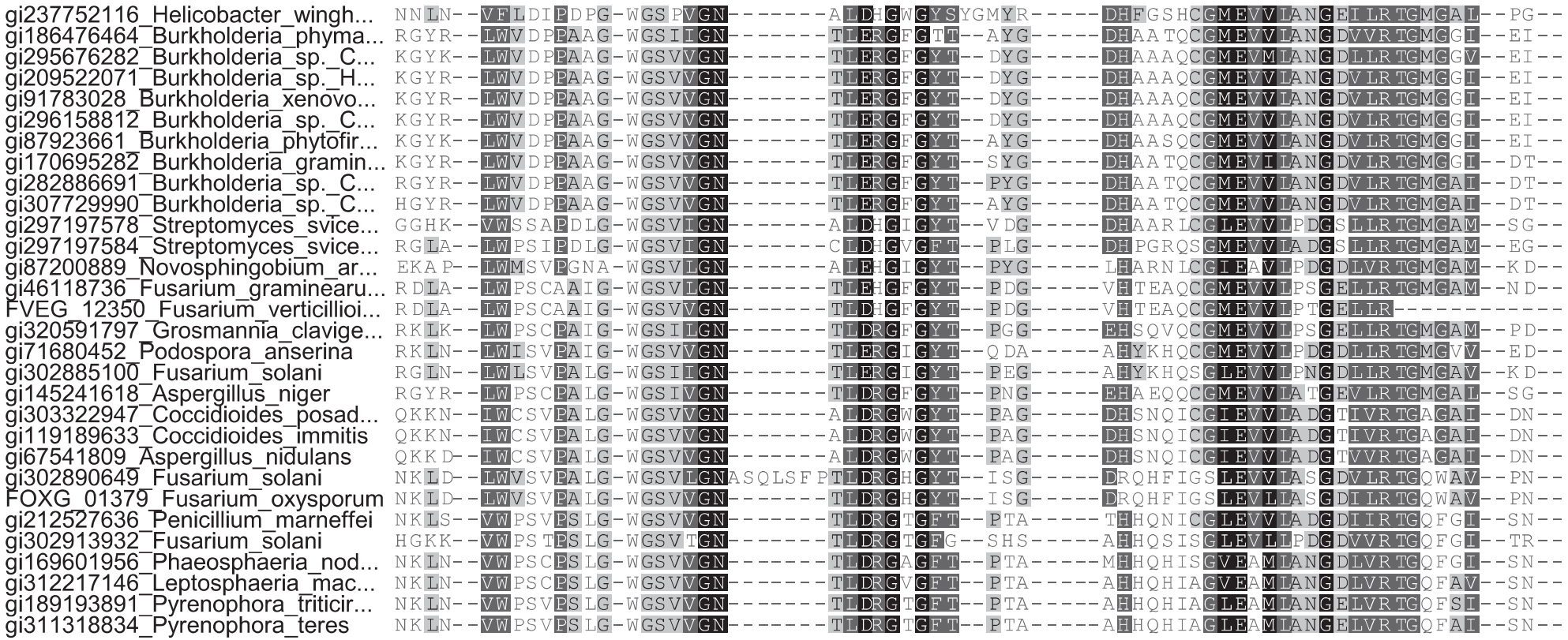
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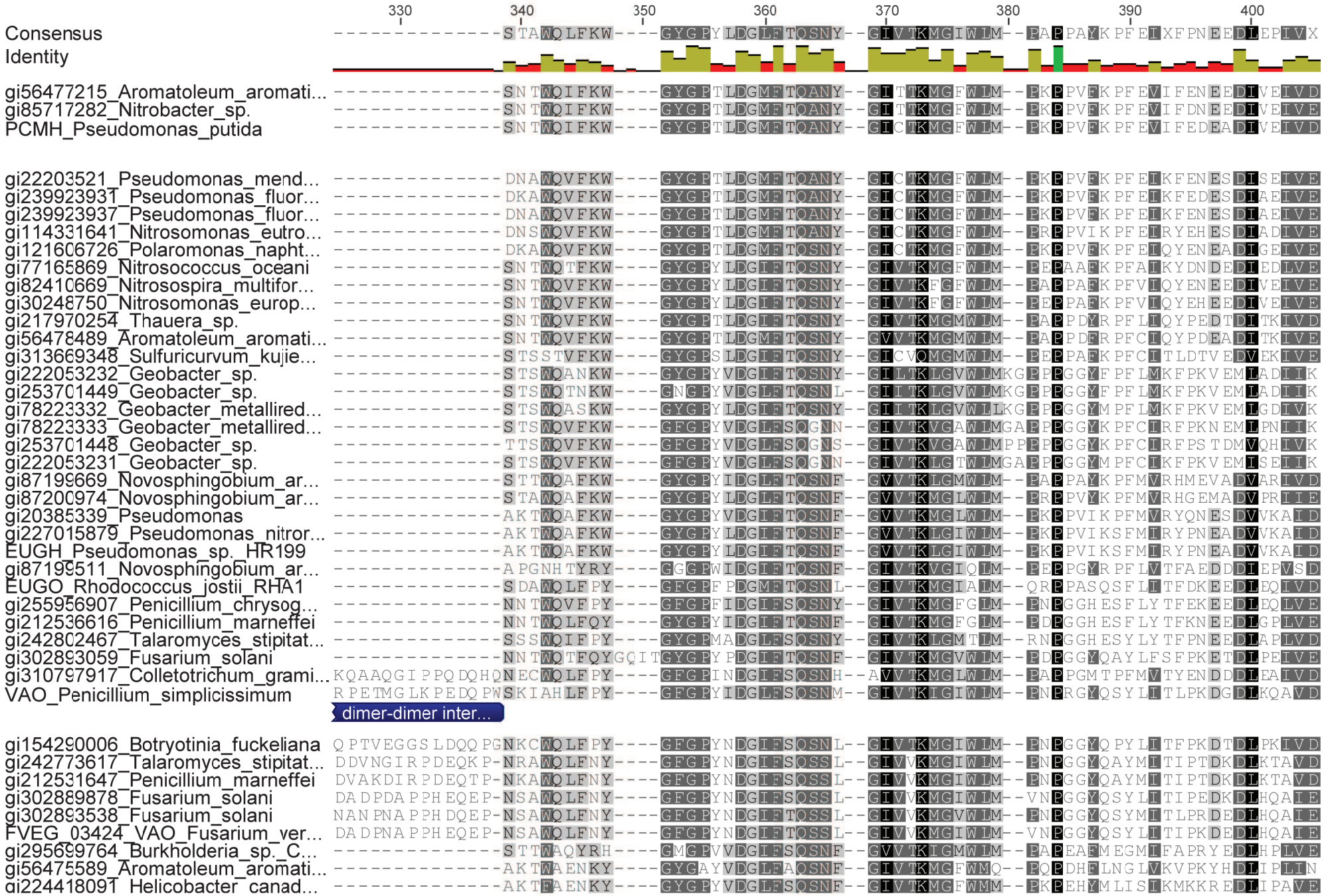
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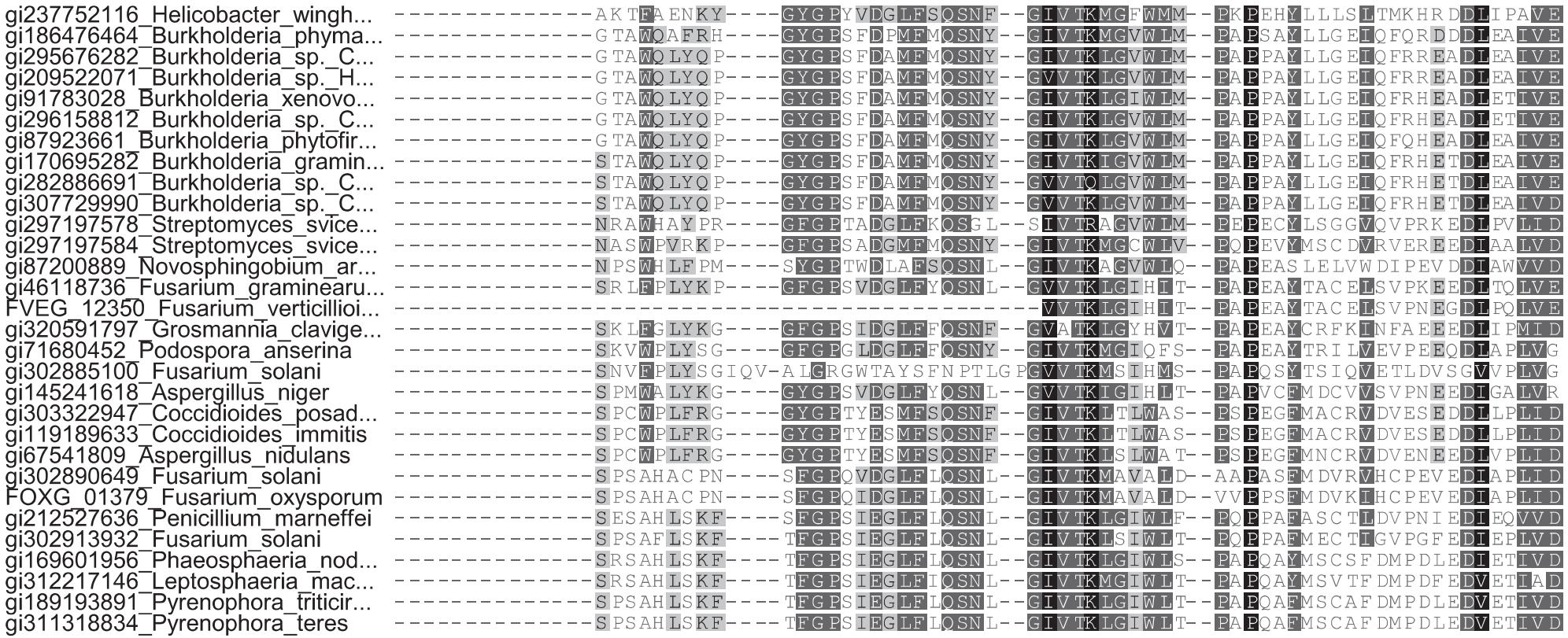
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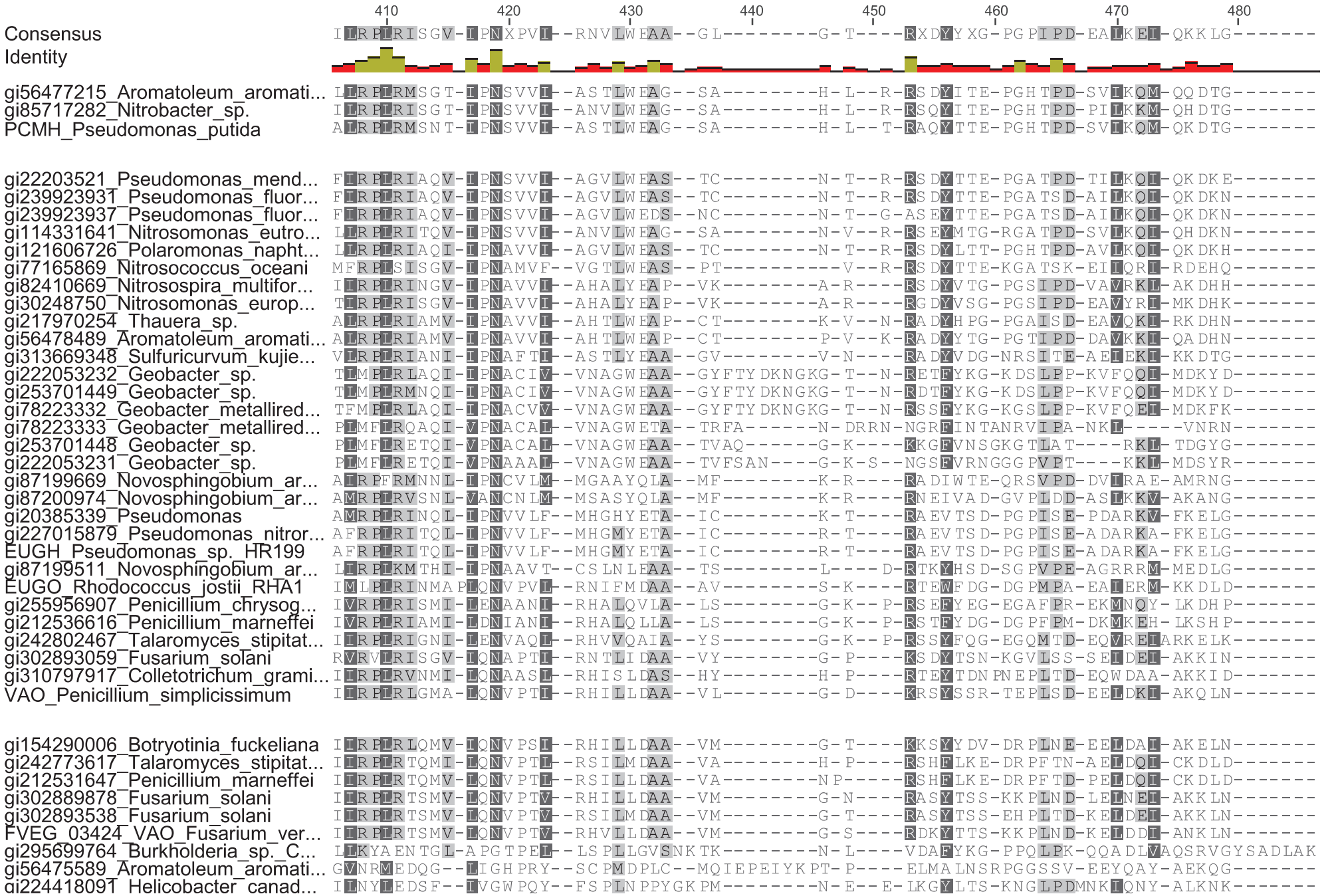
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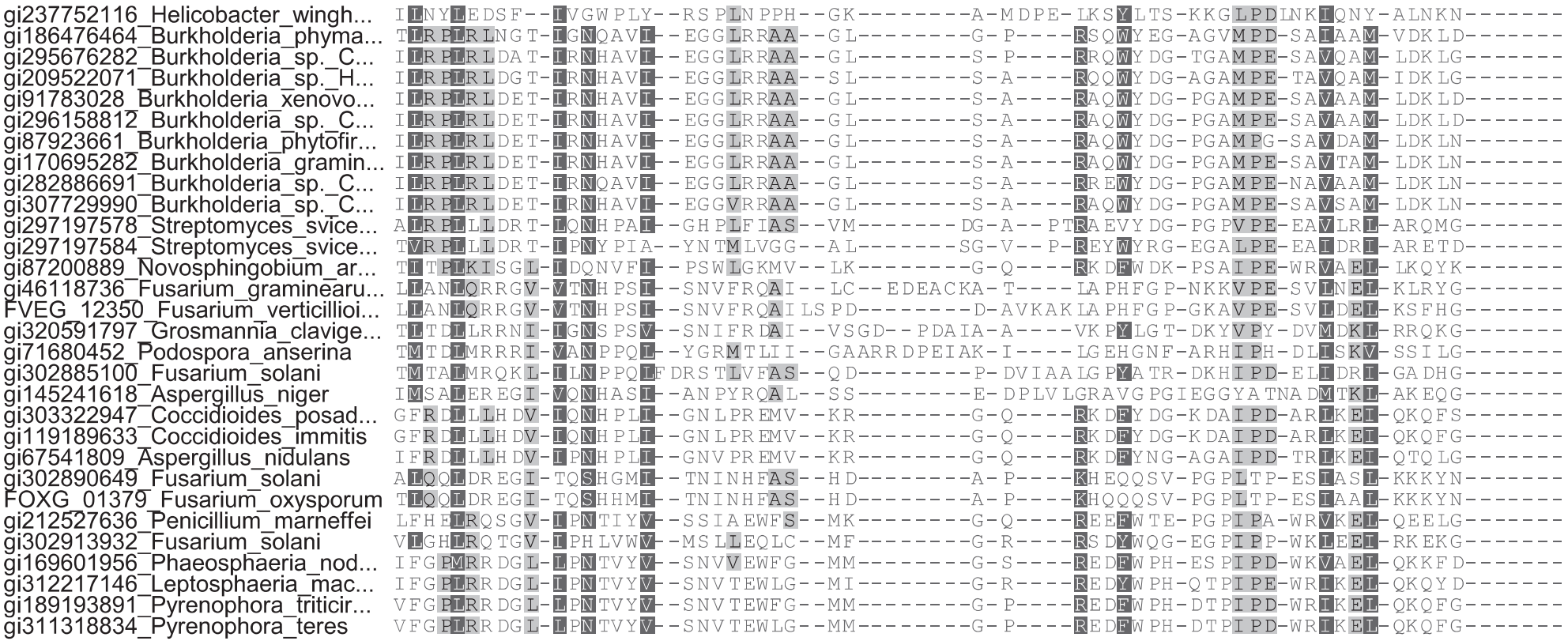
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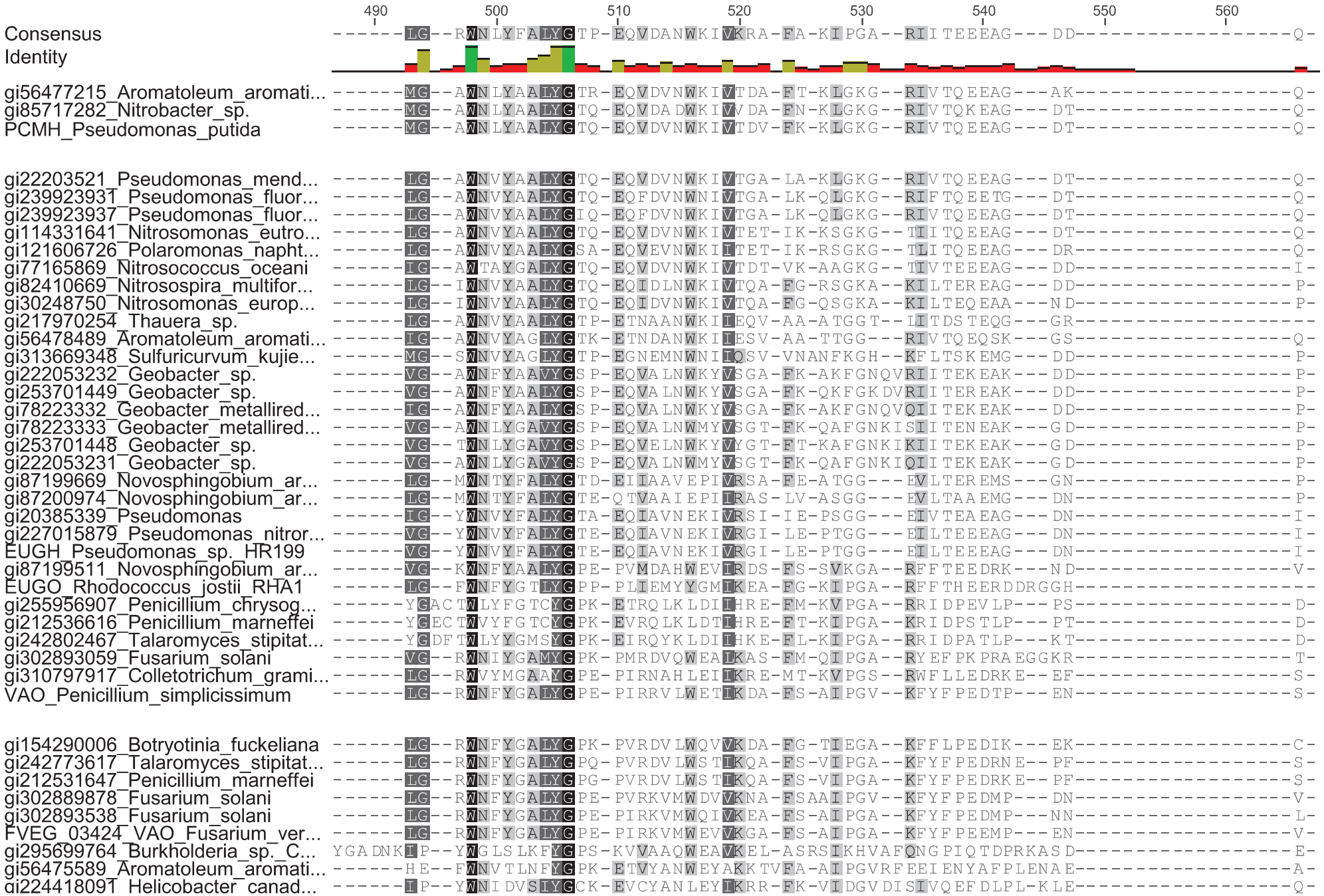
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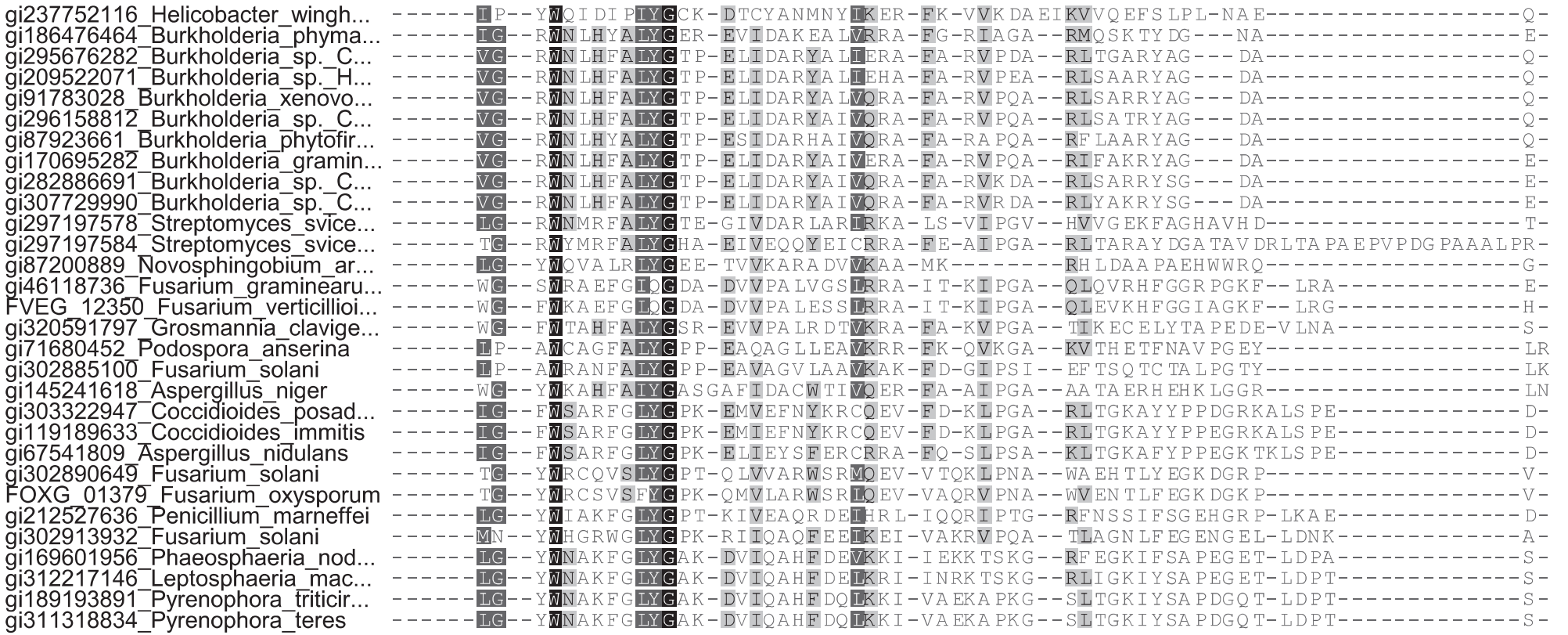
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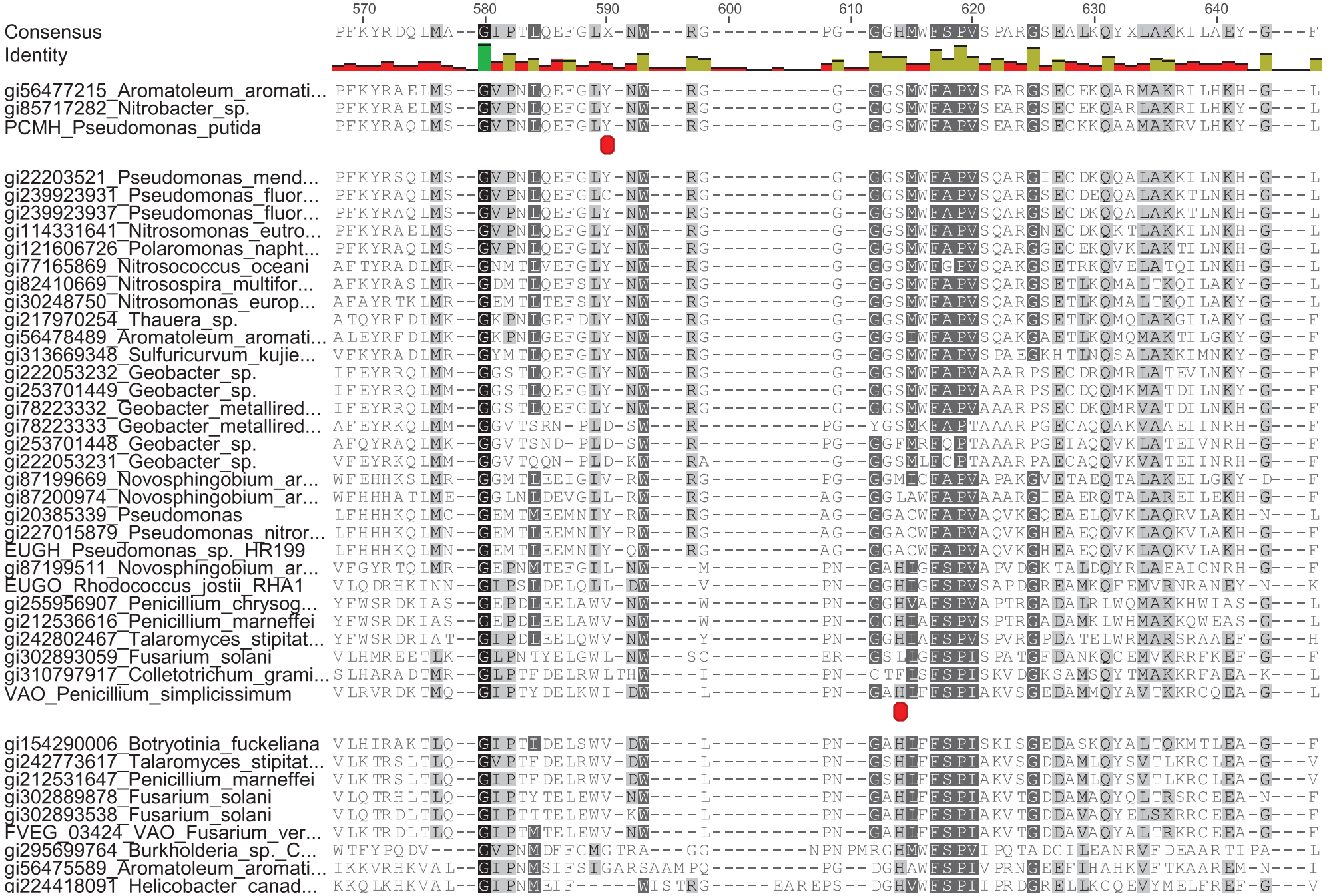
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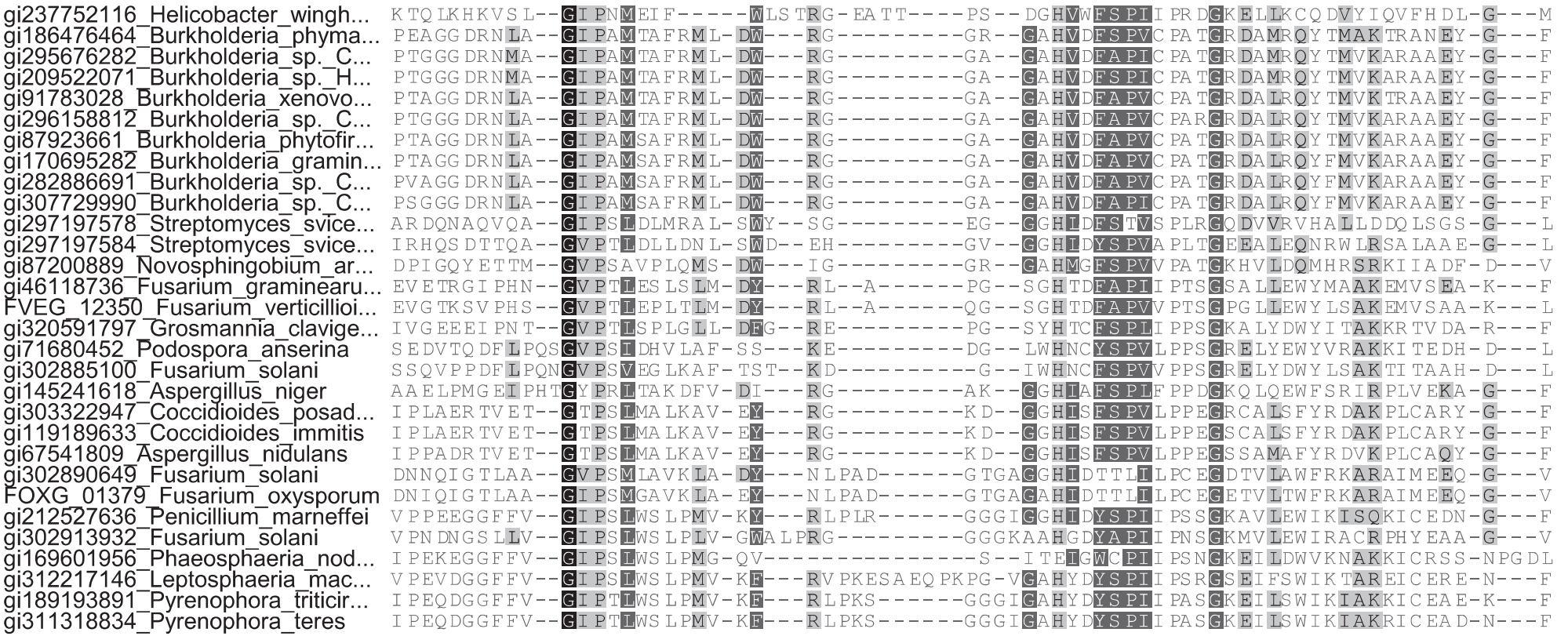
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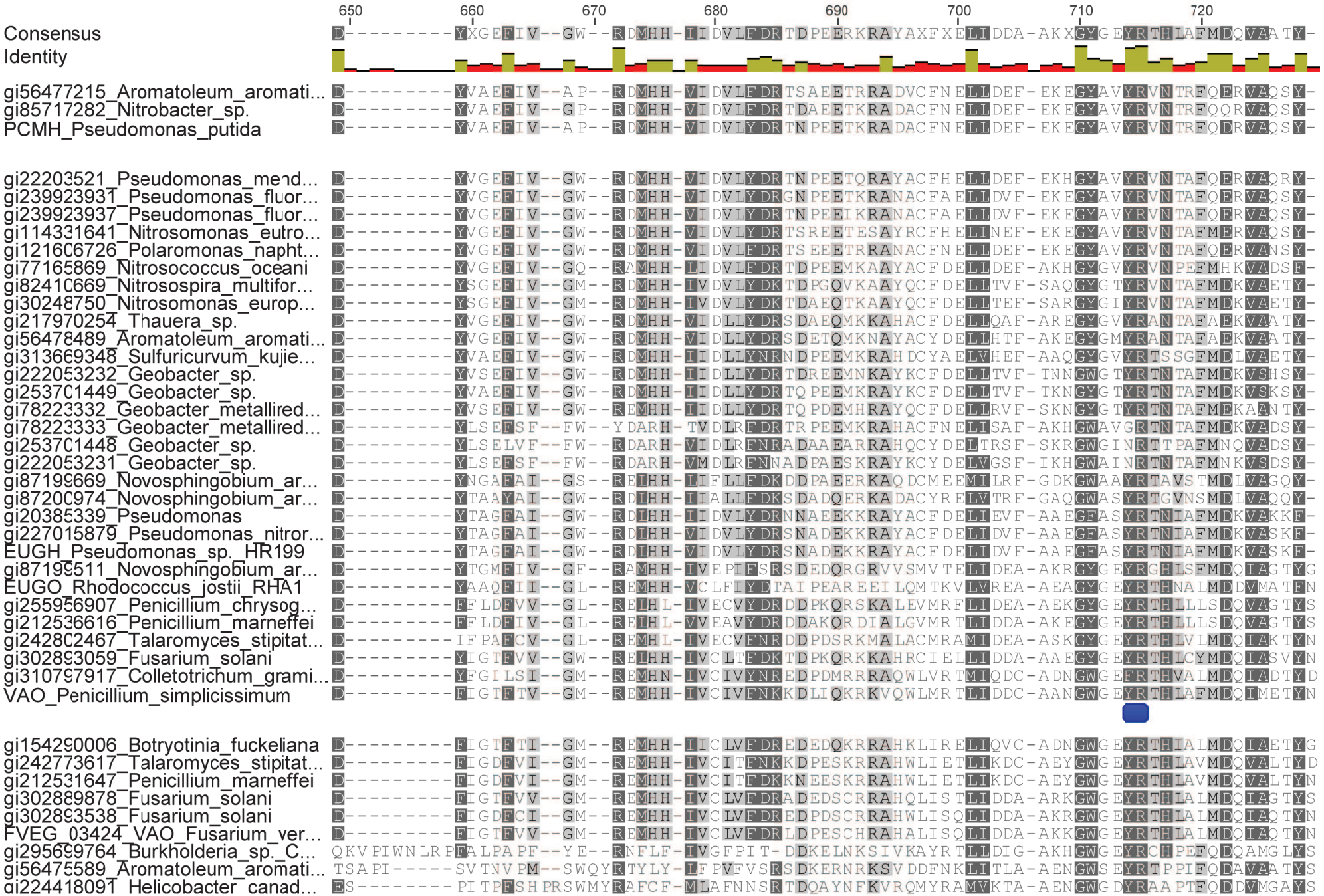
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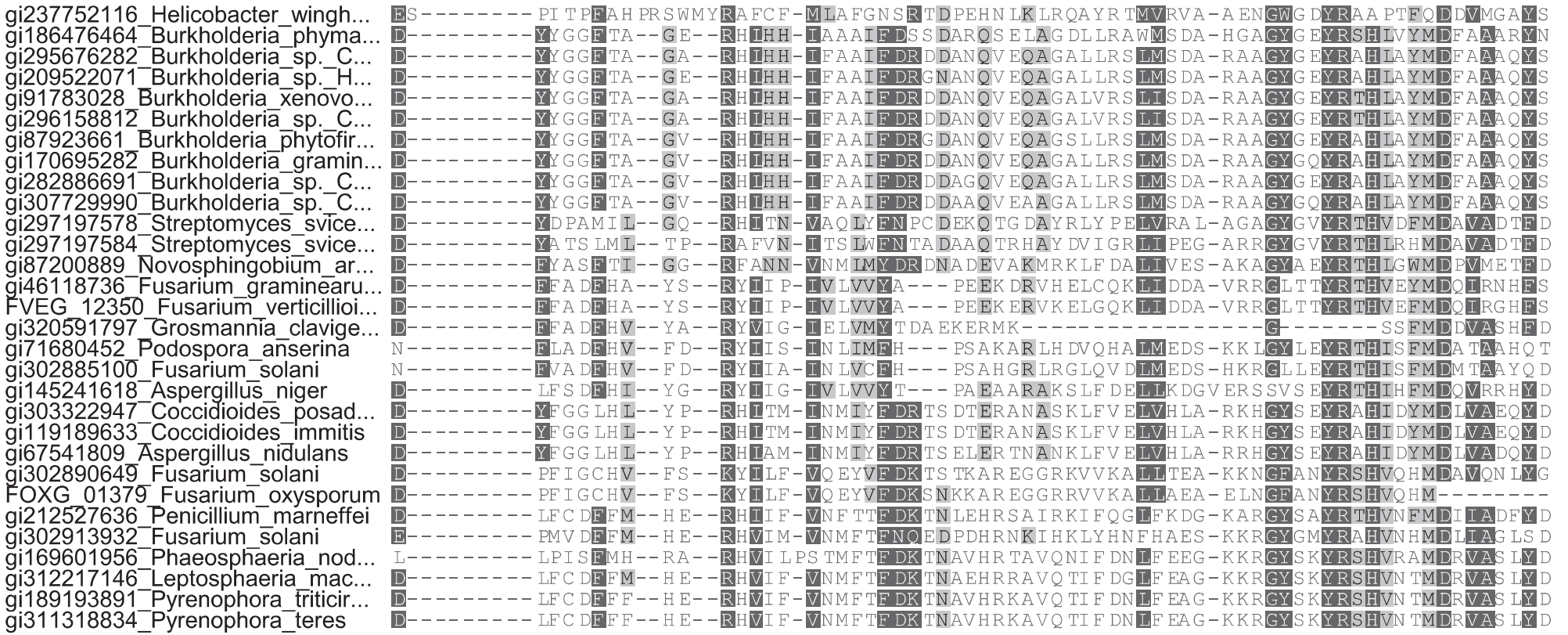
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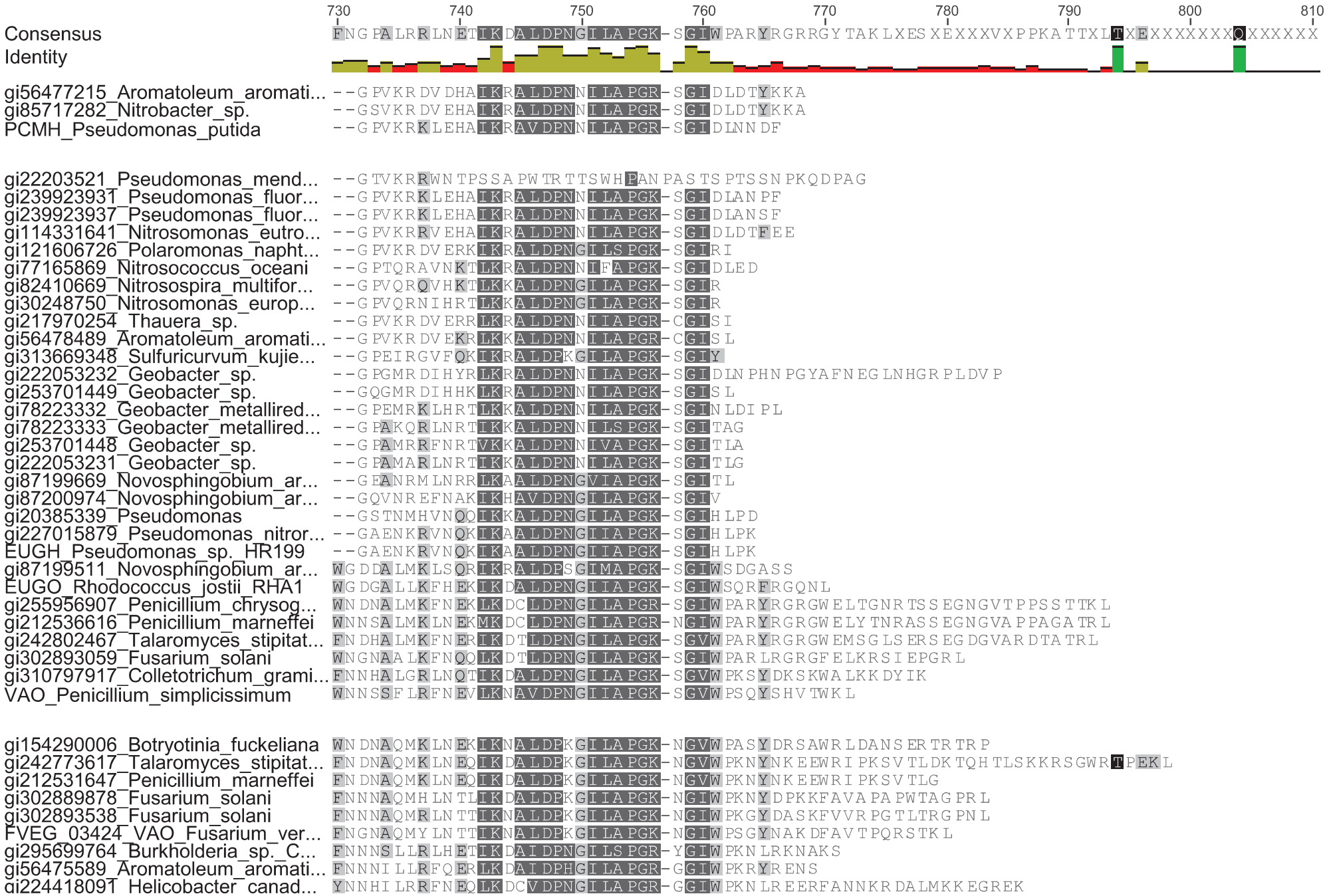
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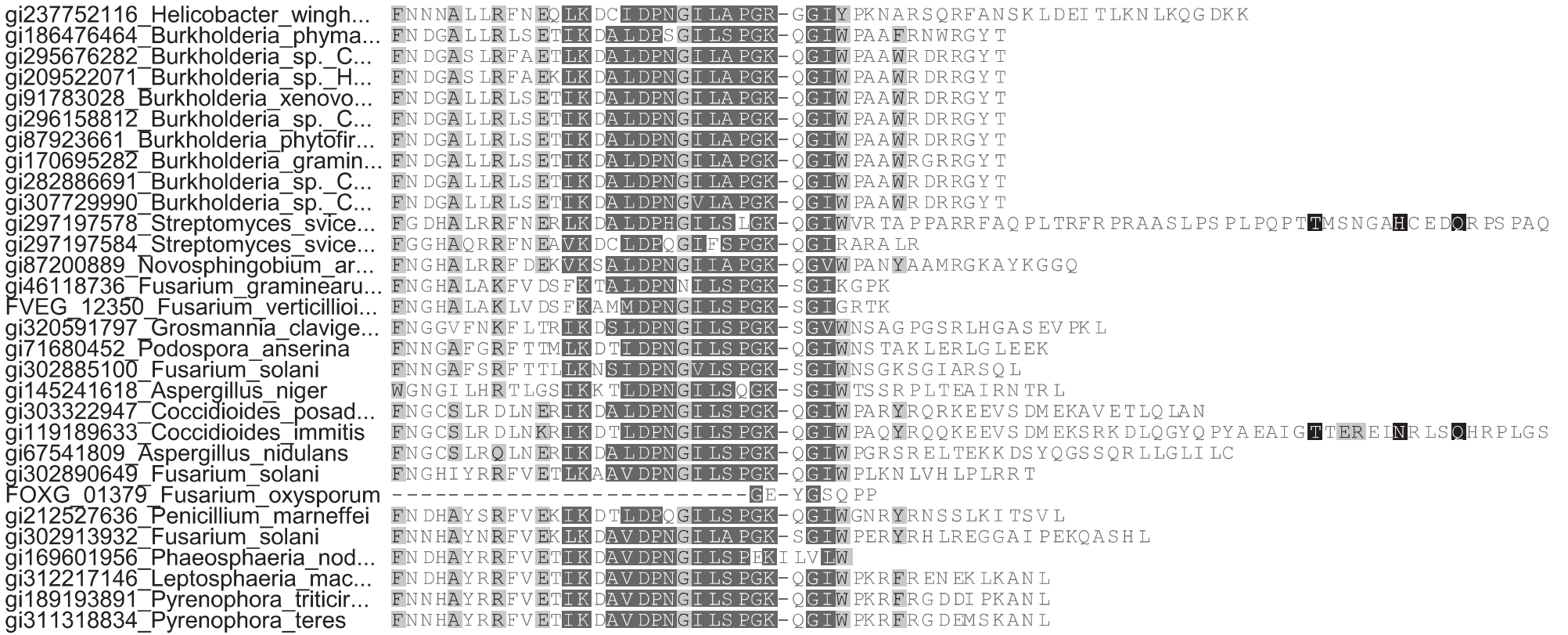
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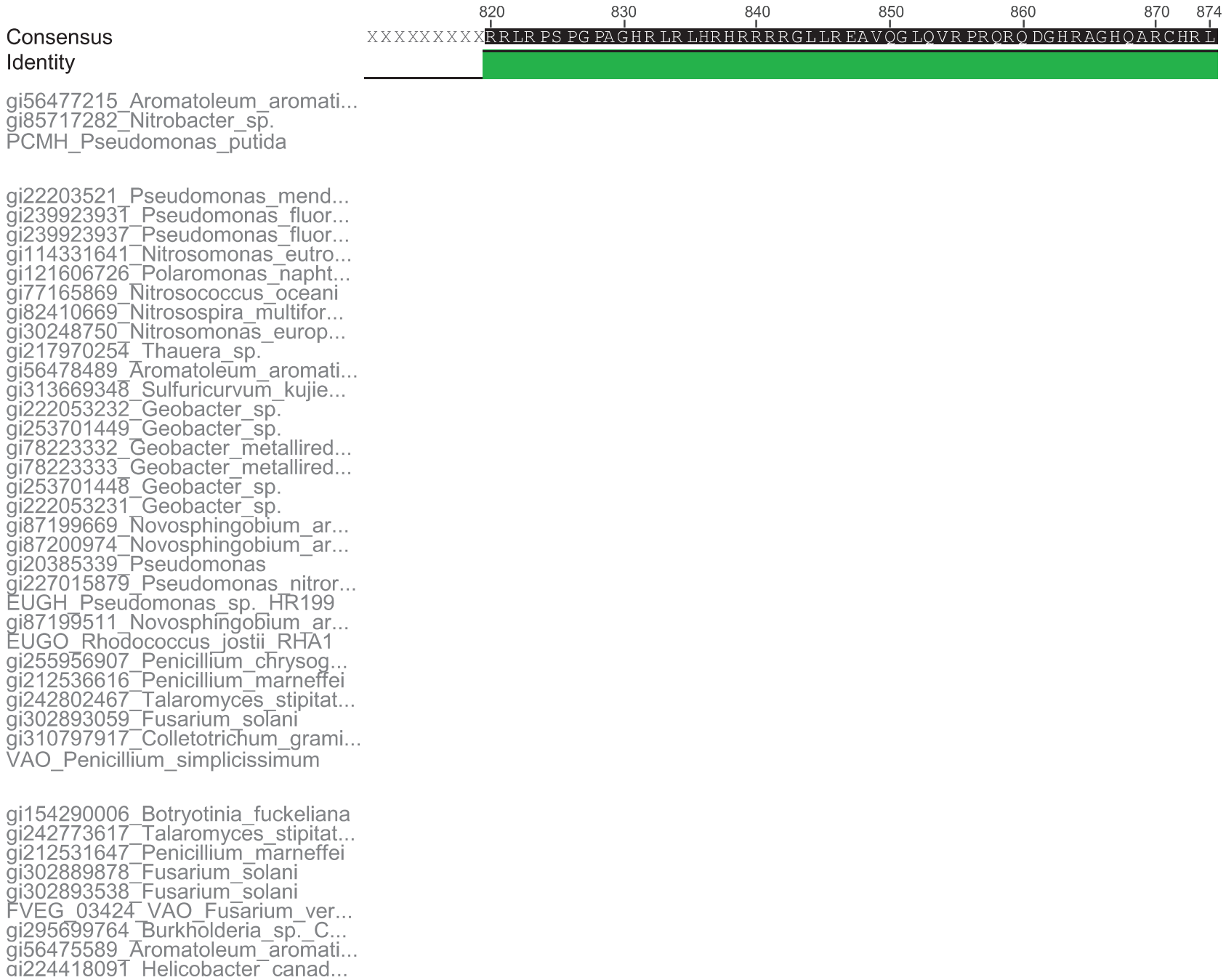
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PCMH_Pseudomonas_putida
gi22203521_Pseudomonas_mend...
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gi114331641_Nitrosomonas_eutro...
gi121606726_Polaromonas_napht...
gi77165869_Nitrosococcus_oceani
gi82410669_Nitrosospira_multifor...
gi30248750_Nitrosomonas_europ...
gi217970254_Thauera_sp.
gi56478489_Aromatoleum_aromati...
gi313669348_Sulfuricurvum_kujie...
gi222053232_Geobacter_sp.
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gi78223332_Geobacter_metallired...
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gi20385339_Pseudomonas
gi227015879_Pseudomonas_nitror...
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gi87199511_Novosphingobium_ar...
EUGO_Rhodococcus_jostii_RHA1
gi255956907_Penicillium_chrysog...
gi212536616_Penicillium_marneffei
gi242802467_Talaromyces_stipitat...
gi302893059_Fusarium_solani
gi310797917_Colletotrichum_grami...
VAO_Penicillium_simplicissimum
gi154290006_Botryotinia_fuckeliana
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gi212531647_Penicillium_marneffei
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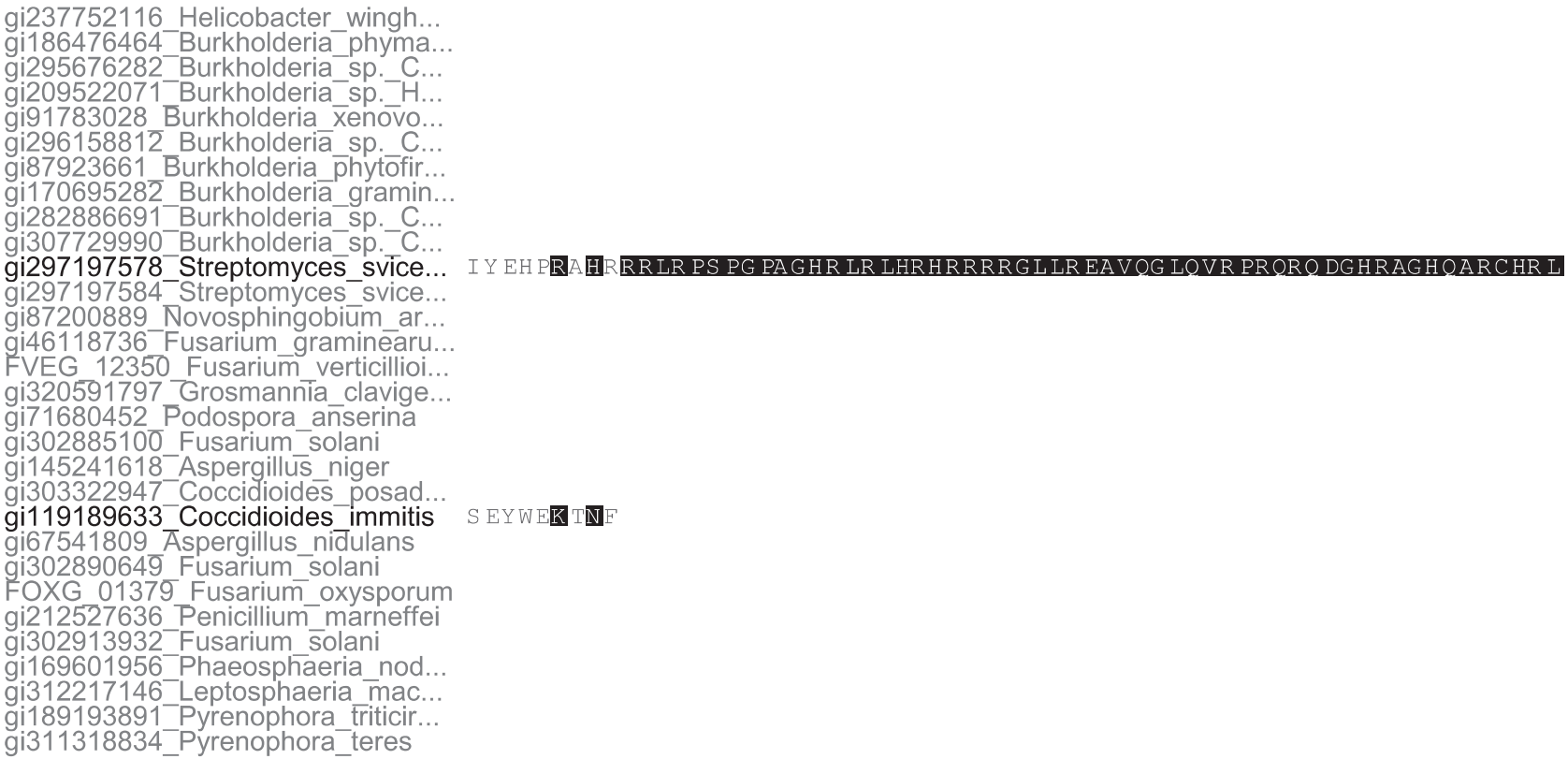
gi295699764_Burkholderia_sp._C...
gi56475589_Aromatoleum_aromati...
gi224418091_Helicobacter_canad...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gi295676282_Burkholderia_sp._C...
gi209522071_Burkholderia_sp._H...
gi91783028_Burkholderia_xenovo...
gi296158812_Burkholderia_sp._C...
gi87923661_Burkholderia_phytofir...
gi170695282_Burkholderia_gramin...
gi282886691_Burkholderia_sp._C...
gi307729990_Burkholderia_sp._C...
gi297197578_Streptomyces_svice... IYEHPRAHRRRLRPSPGPAGHRLRLHRHRRRRGLLREAVQGLQVRPRQRQDGHRAGHQARCHRL
gi297197584_Streptomyces_svice...
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gi46118736_Fusarium_graminearu...
FVEG_12350_Fusarium_verticillioi...
gi320591797_Grosmannia_clavige...
gi71680452_Podospora_anserina
gi302885100_Fusarium_solani
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gi169601956_Phaeosphaeria_nod...
gi312217146_Leptosphaeria_mac...
gi189193891_Pyrenophora_triticir...
gi311318834_Pyrenophora_teres

“A dreamer is one who can only find his way by moonlight, and his punishment is that he sees the dawn before the rest of the world.”

Oscar Wilde