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Cloning, expression and characterization of a novel type of feruloyl esterase from *Fusarium oxysporum* in *Pichia pastoris*

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Abstract

Lately, many articles concerning feruloyl esterases (FAEs) have been published, especially after the mapping of certain genomes. There is a growing interest in using these enzymes at a larger (industrial) scale, due to their broad range of potential. One of the areas where FAEs have been proved to be particularly valuable is within the production of bioethanol.

An enzyme, possibly being a new feruloyl esterase from the fungus *Fusarium oxysporum* was successfully cloned and expressed in *Pichia pastoris*. This new enzyme has been demonstrated to show esterase activity. However to validate weather it really is a feruloyl esterase will require further investigations.

Keywords

Feruloyl esterase, *Fusarium oxysporum*, gene cloning, *Pichia pastoris*, expression

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Sammanfattning

Feruloylesteras är namnet på ett enzym (dvs ett protein som katalyserar en specifik kemisk reaktion) som genom att bryta en esterbindning har förmågan att frigöra ämnet ferulasyra från de långa sockerartskedjorna som utgör viktiga komponenter i den generella strukturen hos växternas cellväggar. Ferulasyra kan bland annat användas för att framställa vanillin och vaniljsyra, ämnen som ger vaniljsmak. Detta gör enzymet intressant från industriell synvinkel, inte bara på grund av förmågan att frigöra denna värdefulla molekyl, men också på grund av dess viktiga roll vid själva nedbrytningen av växtcellväggarna, den största källan av förnyelsebar energi på jorden.

Ett av de områden där feruloylesteraser har visat sig vara speciellt värdefulla är inom produktionen av bioetanol. Svampen *Fusarium oxysporum* är för detta ändamål ytterst intressant då den inte bara bär på gener som kodar för feruloylesteras utan även gener för att framställa de enzymer som behövs för att utföra en därefter följande fermentering.

Detta examensarbete har gått ut på att klona en gen från *Fusarium oxysporum* som kodar för feruloylesteras och sedan uttrycka den i jästen *Pichia pastoris* och på så vis kunna studera dess enzymatiska aktivitet.

Examensarbete 20 p i programmet Molekylär bioteknik

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Introduction

Feruloyl esterases (EC 3.1.1.73), also known as *ferulic acid esterases* (FAE), *cinnamic acid esterases*, or *cinnamoyl esterases*, represent a subclass of the *carboxylic ester hydrolases*, a group of enzymes that release hydroxycinnamic acids (such as ferulic acid (FA)) and their dimmers, from common polysaccharides found in the cell walls of all plants, such as hemicelluloses and pectins ([1], [2], [6]).

Figure 1 illustrates how hydroxycinnamic acids, esterified with arabinoxylans, are linked to the hemicellulose fibers either as sidechains or as crosslinks. The job of the feruloyl esterases is to break these ester bonds.

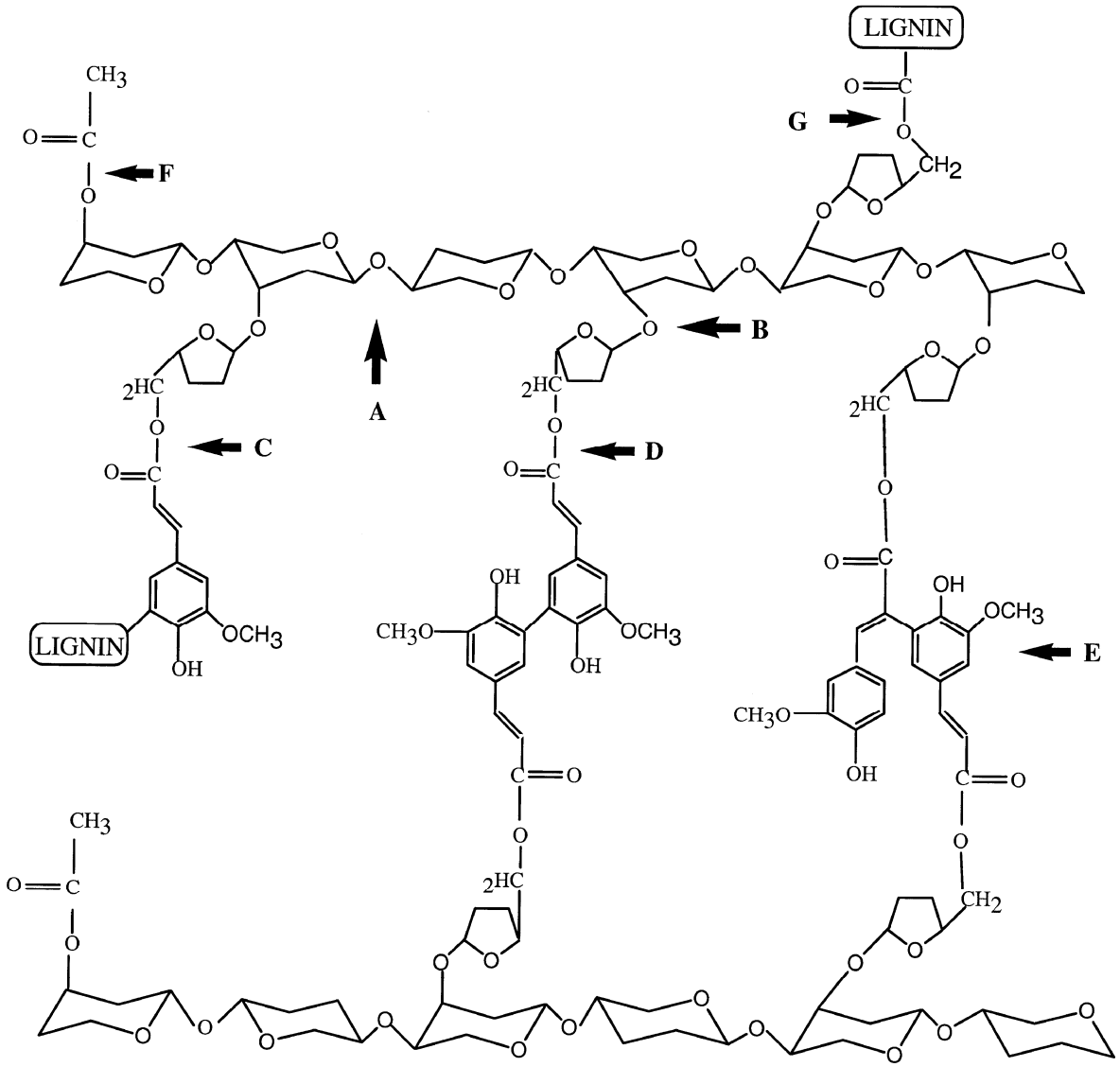

Fig. 1: Structure of arabinoxylan, the main component of the hemicellulose, found in plant cell walls. **A**: *β*-(1-4) linkage in xylan backbone, **B**: Xylose-arabinose linkage, **C**: 5-O-feruloyl lignin, **D**: 5-O-diferuloyl group (5-5'linked dimer), **E**: 5-O-diferuloyl group(8-5'dimer), **F**: 3-O-acetyl group, **G**: arabinose-lignin. [**3**]

For a complete degradation of this biomass component (lignin-carbohydrate complex), it is necessary, on one hand, enzymes capable of cleaving the **main xylan backbone** (called xylanases); and, on the other hand, enzymes assisting the xylanases by breaking down the **crosslinks** and **sidechains** of the arabinoxylan. Thus, feruloyl esterases play a very important role: to increase the accessibility of degrading enzymes to lignocellulose fibers, which will determine their subsequent hydrolysis.

Reaction mechanism of FAE

The primary structural analysis of FAEs has shown that these enyzmes possess an *Asp/His/Ser catalytic triad* at their active site. This means that the FAE-catalyzed reaction is very similar to the hydrolytic action of serine proteases, lipases, and other esterases, a reaction which is taking place by mean of a covalent acylenzyme intermediate, as illustrated in figure 2.

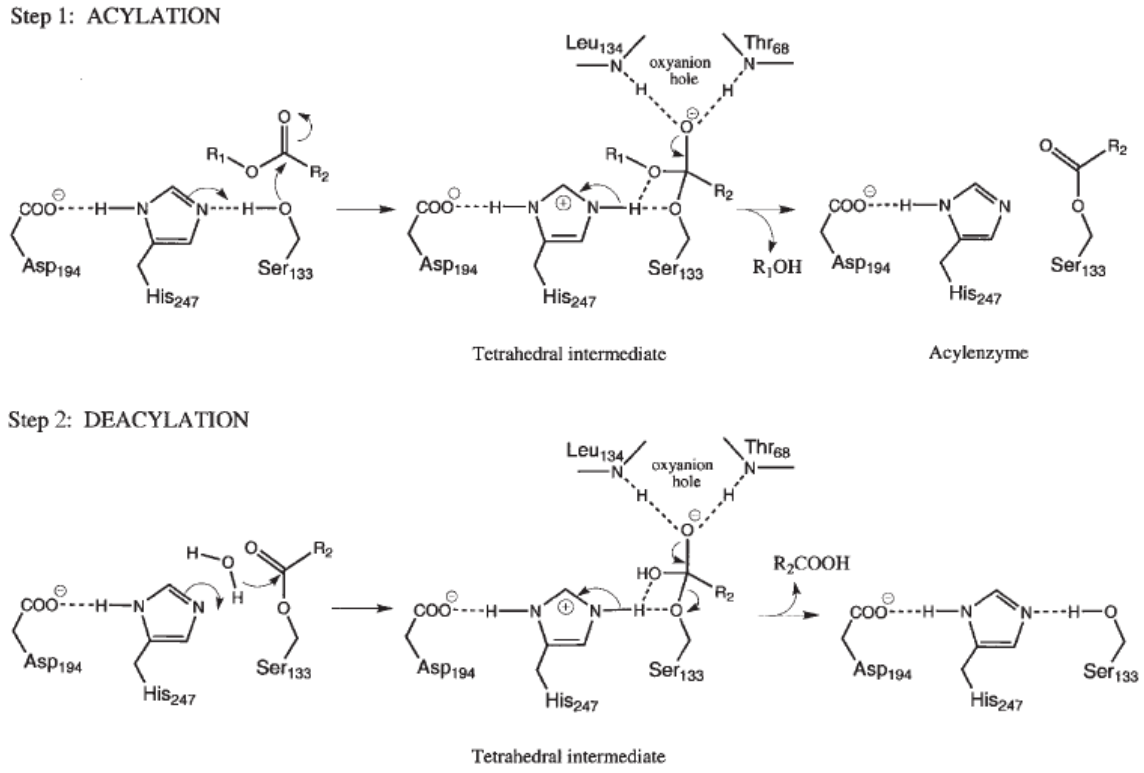

Fig. 2: The proposed two-step mechanism of feruloyl esterases, taking place through an initial acylation step followed by a subsequent deacetylation step [6]

The action mechanism takes place in two steps:

1) The acylation step: the Ser O is activated by the catalytic His and, through a nucleophilic attack on the carboxyl carbon of the substrate, a tetrahedral intermediate is

formed. His-catalyzed protonation of the ester oxygen, is releasing the carbohydrate moiety as the product and also, the acylenzyme complex is formed. In the tetrahedral intermediate, the substrate carbonyl oxygen anion is stabilized by the formation of hydrogen bonds with two main chain NH groups in the “oxyanion anion hole’’.

2) The deacylation step: a second tetrahedral intermediate is formed by a nucleophilic attack of a water molecule, by mean of a His-assisted general basis catalysis. The breackdown of the intermediate is caused by the His-catalyzed protonation of the Ser-O; the ferulic acid is released as a product. [6]

The active site doesn’t have the same substrate recognition requirements for all FAEs; the nature of interaction can be mainly hydrophobic, side-chain or solvent mediated with either the polar substituents of the ferulic acid aromatic ring or the second ferulate moiety. The ferulic acid binding pocket of these enzymes is located in a long and narrow cavity on the molecular surface, created by a number of side chains (of the amino acids from the structure of enzyme) for the recognition of the aromatic ring substitutions and the sugar part of the substrate. The distance between the aromatic ring and the ester bond is a determinant factor for the catalytic activity. [6]

Applications of FAEs and ferulic acids

FAEs have a very high potential for industrial applications such as the food, pulp and paper and bio-fuel industries.

One area in which the FAE enzymatic preparations are widely used is the bakery industry. In this context, together with a number of glycanases and oxidases, FAEs have been implicated in the improvement of bread-making quality. Their main effect is in solubilizing the arabinoxylan fraction of the dough, resulting in increased bread volume and an improved quality of the dough. Other applications: increasing the feed conversion efficiency of animal food, clarifying juices, and producing oligosaccharides that are used as functional food additives or alternative sweeteners [4].

Biomass degradation requires synergistic action between feruloyl esterases and several cellulolytic, xylanolytic and/or pectinolytic enzymes. FAE are involved in lignocellulosic network disorganization as ‘‘helper’’ enzymes due to their ability to hydrolyse ester bonds between sugar residues and phenolic compounds and therefore facilitate the access of hydrolases to the backbone of cell wall polymers. Untangling the crosslinks among various carbohydrate polymers increases accessibility of the cellulose and hemicellulose fractions to enzymatic hydrolysis. This can potentially increase the yield of hexose and

pentose sugar in the bioconversion as feedstock for yeast fermentation to biofuel or other value-added chemicals ([4], [6]).

The high specificity of biological conversions presents an interesting alternative to chemical bleaching. Hemicellulases and oxidoreductases such as xylanases and laccases are being used in pulp bleaching to decrease chlorine consumption and increase the final brightness of the pulp. Enzymatic degradation of the hemicellulose-lignin complexes present in pulps leaves the cellulose fibers intact and strongly reduces the amount of bleaching chemicals (chlorine) required. This not only results in a reduction in costs of chemicals but also reduces the environmental problems caused by the use of chlorine ([1], [4]).

A second non-food application of feruloyl esterases is the production of fuel ethanol from renewable lignocellulosic materials. *A. niger* FaeA was used in association with xylanases and laccases for conversion of lignocellulosic biomass to fermentable sugars for the production of bioethanol. In the saccharification step, efficacy of the enzymatic treatment was evaluated by measuring sugar yield with the best results obtained with a combination of FaeA and xylanase treatment ([1]).

The importance of feruloyl esterase also relates to the enzyme product ferulic acid and ferulated oligosaccharides, which have a potential application for food and medicine uses, so that the ferulic acid derivates are used like strong antioxidants and have gel-forming properties.

Ferulic acid can perform several biological functions such as UV absorber, anti-oxidant and anti-inflammatory activity. It is one of the major antioxidant constituents in beer, while its occurrence in orange juice is responsible for the off-flavour formation during storage. FA it has been also shown to possess some activity toward peroxynitrite and oxidized low-density lipoprotein (oxLDL) in vitro. FA has been evaluated in synaptosomes and neuronal cultures exposed to peroxyl, and hydroxyl radical insult via several oxidative stress indexes and may be a promising candidate as an antioxidant in neurodegenerative disorders such as Alzheimer's disease. Any reactive radical colliding with FA easily abstracts a hydrogen atom to form a phenoxy radical. This radical is highly resonance stabilized, since the unpaired electron may be present not only on oxygen but it can be delocalized across the entire molecule.

In fact, the antioxidant activity of phenolic acids is largely due to their chemical structure and the presence of hydroxy groups on the aromatic ring. The presence of two hydroxy groups on caffeic acid compared to one on ferulic acid can therefore explain its higher antioxidant efficiency.

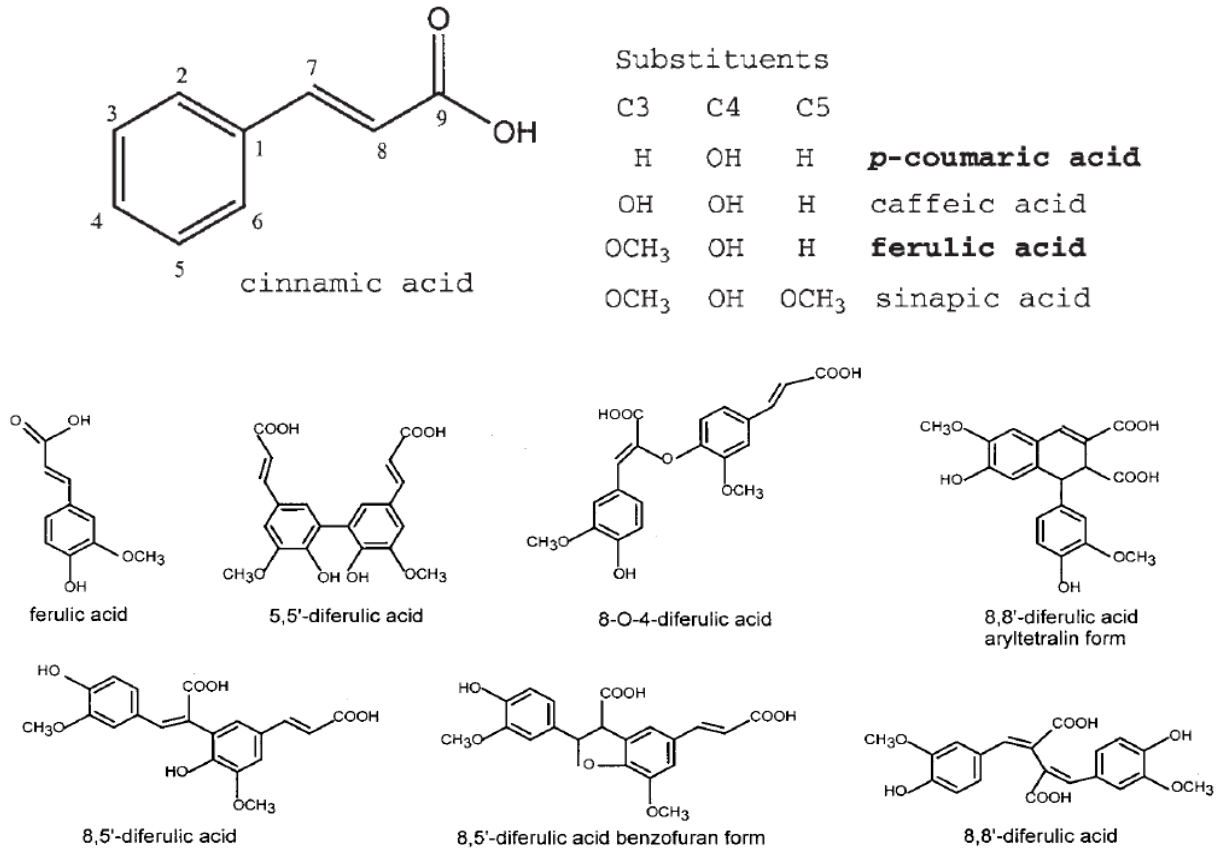

Fig. 3. Structures of ferulic acids from plant cell wall [4], [6]

FA has also a potential use as feedstock for the biocatalytic conversion into other valuable molecules such as styrenes, polymers, epoxides alkylbenzenes, vanillic acid derivatives, protocatechuic acid-related catechols, guaiacol, catechol and vanillin (the last is one of the most universally used aromatic molecules in the food, pharmaceutical and cosmetic industries).

There has recently been considerable growing interest in FAEs and their potential application in obtaining FA from agro-industrial waste materials such as those produced by the milling, brewing and sugar industries (wheat bran, maize bran, maize fiber, brewer's (or barley) spent grain, sugar beet pulp, coastal bermuda grass, oat hulls, jojobameal, wheat straw, coffee pulp and apple marc [2], residue of rice bran oil); FA can be lately transformed by microorganisms to natural vanillic acid and vanillin, the main flavour component of vanilla [1].

Classification of FAEs

Feruloyl esterases can be isolated from a wide range of microorganisms when they are grown on complex substrates such as xylan, pectin, wheat bran or sugar beet pulp. More than 30 FAEs have been purified and characterized from several microorganisms including fungi and bacteria, showing significant variations in physical characteristics such as molecular weight, isoelectric points and optimum hydrolytic reaction conditions [7].

Feruloyl esterases have been initially classified as either **type A** or **type B**, based on their ability to release diferulic acids from esterified substrates and their specificity for different aromatic substrates [8]. Few years back, a more elaborate classification (according to their primary amino acid sequence identity, specificity for the hydrolysis of hydroxycinnamic acid methyl esters, ability to release 5,5′-diferulic acid from model and complex substrates and inducible plant cell wall materials), has been proposed to consist of four subclasses: type A, B, C, and D ([5], [9]).

FAEs appear to be a very diverse set of enzymes, with little unifying sequence and physical characteristics to link them. The nomenclature of FAEs follows both the source of the enzyme and the type of the esterases (e.g. the type-A FAE produced by F. oxysporum is termed FoFaeA). It is extremely common for esterases to act on a broad range of substrates. [2]

Multiple sequence alignments of known feruloyl esterase amino acid sequences suggest that they can be subdivided by sequence similarities with lipase, acetylxylan esterase, chlorogenate esterase/tannase, and xylanase. These four subclasses, FAE types A, B, C, and D, are also distinguished by their substrate specificity. [6]. An overview of classification criteria of FAEs is shown in table 1.

Type A FAEs show preference for the phenolic moiety of the substrate containing methoxy substituents, especially at meta-position(s), as occurs in **ferulic and sinapinic acids**, while **type B** FAEs shows complementally activity to type A esterases, showing preference to substrates containing one or two hydroxyl substitutions like **in p-coumaric or caffeic acid**. Furthermore, type **A and D** FAEs in contrast to type **B and C** are also able to release low quantities of diFA. Type **C and D** FAEs exhibit broad **specificity** against synthetic **hydroxycinnamic acids** (ferulic, p-coumaric, caffeic and sinapic acid) showing difference only in the ability to release 5-5' diFA. Nature has evolved several types of FAEs that differ in affinity for **5-O- and 2-O-feruloylated a- L-arabinofuranosyl residues**. **Type A** esterases are active only on substrates containing FA ester linked to the **O-5** and not on substrates containing FA ester linked to the **O-2** linkages of **L-arabinofuranose**. In contrast, **type B** FAEs are active on substrates containing FA ester linked to both **O-5** and **O-2** of Larabinofuranose with different preferences depending on the esterase studied. The inability

of **type A** FAEs to hydrolyze the **O-2** linkage between FA and L-arabinofuranose residues could be a new criterion for the classification in this subclass of esterases. **Type C and D** FAEs are able to hydrolyse both linkages. [2]

Table 1: Overview of classification criteria of FAE

FAE type	Hydrolysis of methyl esters of hydroxycinnamic acids				Release of diferulic acid	Affinity for 5-O and 2-O feruloylated L-arabinofuranose	
	MFA	MSA	MCA	M*p*CA		5-O	2-O
A	*	*		*	Yes (5,5')	Yes	No
B	*		*	*	No	Yes	Yes
C	*	*	*	*	No	Yes	Yes
D	*	*	*	*	Yes (5,5')	Yes	Yes

Type A is active on methyl ferulate (MFA), methyl *p*-coumarate (M*p*CA), and methyl sinapate (MSA). These FAEs have sequences related to those of lipases and are able to hydrolyze synthetic ferulate dehydrodimers. Examples of this group of enzymes include *Aspergillus niger* FAE-A (AnFaeA).

Type B FAEs are specific against MFA, M*p*CA, methyl caffeate (MCA), but not MSA. These enzymes do not release diferulic acid and show sequence similarities to carboxylic esterase family 1-acetyl xylan esterase. *Penicillium funiculosum* FAE-B and *Neurospora crassa* FAE-I belong to this group.

Type C and D act on all four hydroxycinnamic acid methyl esters. **Type C** enzymes do not release diferulic acids from model and complex substrates, whereas **type D** enzymes are able to hydrolyse dimers.

Type C and D shows sequence similarities to chlorogenate esterase (tannase) and xylanase, respectively. **Type C** includes *A. niger* FAE-B (AnFaeB) and *Talaromyces stipitatus* FAE-C. **Type D** enzymes include *Piromyces equi* EstA and *Celluvibrio japonicus* EstD. [6]

Aim of the thesis

In the recent years, many articles have been published about subjects such as purification and characterization of different types of FAEs from various microorganisms, especially after the mapping of certain genomes There is a growing interest in using these enzymes at a larger, industrial, scale, due to their broad range of potential, not only in agriculture and food processing, but also in paper and bio-fuel industry. One of the areas where FAEs have been proved to be particularly valuable is within the production of bioethanol. The fungus *Fusarium oxysporum* is, for this purpose, highly interesting, as it in its genome not only has the genes encoding for FAEs, but also the genes responsible for the subsequent fermentation of the sugars made accessible by the FAEs.

In order to clone and express a feruloyl esterase from *Fusarium oxysporum* in *Pichia pastoris,* the following steps were planed to be taken:

1) Identification of the hypothetical protein DNA sequence to clone from the recently released genomic DNA of *Fusarium oxysporum* (http://www.broad.mit.edu/annotation/genome/fusarium_group/MultiHome.html)

2) Design of primers for directional cloning of the specific gene into the pPICZα expression plasmid, containing the tightly regulated AOX1 promoter and the *Saccharomyces cerevisiae* α-factor secretion signal, located immediately upstream of the multiple cloning site. Primers have also been designed for the exclusion of the intron region positioned in the target gene using fusion PCR technique.

3) DNA sequencing of the recombinant pPICZα plasmid.

4) Transformation of *P. pastoris* using the electroporation method.

5) Identification of recombinant colonies using fluorescent screening assay.

6) Expression experiments in order to choose the best producer of the recombinant feruloyl esterase.

7) Purification and biochemical characterization including SDS/IEF-PAGE and substrate specificity experiments against various model substrates for the classification of this esterase.

Materials and methods

Chemicals and reagents

Vent high-fidelity DNA polymerase was purchased from New England Biolabs (Ipswich, Massachusetts, USA). NucleoSpin® Extract II and NucleoSpin® Plasmid Kits were purchased from Macherey Nagel (Düren, North Rhine-Westphalia, Germany).

Zero Blunt® PCR Cloning Kit, pPICZα vectors and EasySelect™ *Pichia* Expression Kit were purchased from Invitrogen (Carlsbad, California, USA) while restriction enzymes were purchased from Takara (Ōtsu, Shiga, Japan). All chemicals were of analytical grade.

Strains, vectors and media

For the cloning of the feruloyl esterase gene from *F. oxysporum*, *E. coli* One Shot® Top10 (Invitrogen) and Zero Blunt® PCR Cloning Kit (Invitrogen) were used as the host–vector system. *P. pastoris* host strain X-33 and pPICZαC (Invitrogen) were used for protein expression. The wildtype (WT) strain of *F. oxysporum* isolated from cumin (by prof. Christakopoulos and his research group in 1989), was maintained on potato dextrose agar at 4℃.

E. coli was grown at 37℃ in LB medium containing 50 μg kanamycin ml^{-1} for selection of clones transformed with the Zero Blunt® PCR vector. *P. pastoris* was routinely grown in shaking flasks at 30℃ in a rich medium containing 1% (*w/v*) yeast extract, 2% (*w/v*) peptone, 0.1 M potassium phosphate buffer, pH 6.0, 1.34% (*w/v*) yeast nitrogen base, 4×10^{-5}% biotin and 1% (*v/v*) glycerol (BMGY) before induction or 0.5% (*v/v*) methanol (BMMY) for induction. For maintaining cultures and plates, 1% (w/v) yeast extract, 2% (*w/v*) peptone and 2% (*w/v*) dextrose (YPD) medium was used, and for selection of transformants, YPD plates containing 1 M sorbitol (YPDS) and 100 μg ml^{-1} Zeocin™ at final concentration were used.

Cell cultivation

From a stock culture of *Fusarium oxysporum* cells were transferred into slants prepared with solid agar growth medium (39 g/l of Potato Dextrose Agar (PDA) and 1 g/l of yeast extract) and incubated four days. After this a new transfer was done, this time to flasks with liquid medium (1 g/l KH_2PO_4, 0.3 g/l $CaCl_2$, 0.3 g/l $MgSO_4 \bullet 7H_2O$, 15.6 g/l $NaH_2PO_4 \bullet 2H_2O$, 1.52 g/l $Na_2HPO_4 \bullet 2H_2O$(or 1.2 g/l Na_2HPO_4) and 10 g/l $(NH_4)_2HPO_4$). These flasks were incubated at 30°C and 30 RPM, initially for three days, and then later extended another day, as the growth of *F. oxysporum* had not been as great as desired.

In addition, a second transfer to liquid medium was made in parallel to the first, just in case the first would turn out to be contaminated. An examination in light microscope however revealed that this was not to be the case. Meanwhile a transfer was also made from a stock culture of *Pichia pastoris* to solid YPD agar medium (1% yeast extract, 2% peptone, 2% dextrose, 2% nutrient agar) on plates, incubated at 30°C, to be used further on in the experiment.

After the flasks with liquid media had been incubated, their content was transferred into a number (4 per flask) of 25 ml sterilin tubes, placed in ice. These tubes were centrifuged in pairs at 180 RPM for 7 minutes. The supernatant was discarded while the pelleted biomass was gathered in weighted beakers and the mass of the wet biomass could be determined to 13.4 g. The beakers were stored for 5 minutes at -80ºC before being placed in freeze dryer to remove the water. After having been freeze dried the content from all beakers was gathered in a single weighted sterilin tube and the weight of the dry biomass was 0.96 g.

DNA isolation

Cellular DNA from *Fusarium oxysporum* was isolated by a modified version of the method of Lee, Milgroom & Taylor for isolation of total genomic DNA from fungi.

Freeze-dried cells staying in a sterilin tube were powdered using a spatula. Two eppendorf tubes were filled with around 100 mg of biomass each.
750 µl of lysis buffer (50 mM Tris-HCl, pH 7.5, 50 mM EDTA, 3% SDS and 1% 2-mercaptoethanol) was added into each tube, then mixed and followed by incubation at 65°C for one hour.

After incubation the tubes were mixed once more and 700 µl of a mixture of chloroform/isoamylic alcohol, 24:1 (*v/v*) was added. The solution was now mixed (inverted) to form an emulsion that was centrifuged at 10,000 RPM for 10 minutes at room temperature. The upper (aqueous) phase was then carefully removed and transferred to a new tube in which 700 µl of chloroform was added, the content was mixed and after that centrifuged again in the same way for another 10 minutes. The upper (aqueous) phase was then once more carefully removed and transferred to a new tube in which this time 20µl 3M NaOAc was added, followed by 1ml of isopropanol. After that the tubes had been mixed (inverted) they were centrifuged for 30 seconds to pellet the DNA and the supernatant was discarded. Following another centrifugation the tubes were then left open to become dry.

300µl of EB buffer (as a substitute for Tris–EDTA buffer) was added and the tubes were incubated at 65°C for 15 minutes. After the incubation 10 µl of NaOAc and 1 ml of ethanol were added, the tubes were centrifuged for 2 minutes and the supernatant was

discarded. Next 700 µl of ethanol and 300 µl of water were added, effectively resulting in the addition of 1ml of 70% ethanol. After having been gently mixed, the tubes were now centrifuged again and the supernatant discarded. Once more the tubes were staying with open lid to get dry.

Finally 100µl of EB buffer was added and the tubes were marked and placed for storage at –20°C.

Cloning of the gene from FOXG_17021.2

The gene to be studied, the sequence from the ORF *FOXG_17021.2*, was amplified by PCR from genomic DNA using primers having a design based on the available information of the sequenced *Fusarium oxysporum* genome (*F. oxysporum* Sequencing Project, Broad Institute of Harvard and MIT; http://www.broad.mit.edu). The two primers were designed in the following way: 5′-GCATCGATGGCGCTGCCCTCCAATGGAAC-3′ including a site for *Cla*I (underlined) and 5′-CGTCTAGATACACAGGGATCTTGAAAGCATTG-3′ including a site for *Xba*I (underlined).

After the ordered primers had arrived a calculated amount of water (137 µl and 109 µl respectively) was added to achieve a stock solution of 100 pmol/µl of each primer. These were left to stay 1 hour in order for the primers to properly dissolve before 25µl of each stock solution was transferred into an eppendorf tube together with 25 µl of water to prepare 50µl of a 50 pmol/µl solution of each primer.

A high-fidelity Vent DNA polymerase producing blunt ends was used for the DNA amplification, which was carried out with an initial denaturation of 5 minutes at 94°C, followed by 30 cycles of denaturation (25 s at 94°C), annealing (25 s at 56°C) and extension (120 s at 72°C) finally followed by 5 minutes of further extension at 72°C.

In order to determine the DNA sequence, the PCR product was cloned into the pCR-Blunt® vector according to the method described by the Zero Blunt® PCR Cloning Kit. After an agarose gel electrophoresis had been run, the fragment was extracted from the gel according to the protocol of the kit NucleoSpin® Extract II (Macherey Nagel) and then ligated into the pCR®-Blunt vector by mixing 5µl fragment, 2µl water, 1µl 10x ligation buffer, 1µl pCR®-Blunt vector and 1µl T4 DNA ligase and incubating it at 16ºC for 90 minutes. Next an eppendorf tube containing 100µl of *E. coli* One Shot® TOP10 cells was taken out from -80ºC freezer and placed in ice. After having stayed around 5 minutes in ice, 5µl of ligation reaction was added to the tube while gently stirring with the tip of the pipette. The tube was then incubated in the ice for 30 minutes before receiving a heat shock of 42ºC for 90 seconds and then placed back again in the ice. Next 200µl of LB medium (10 g/l tryptone, 5g/l yeast extract and 10 g/l NaCl, pH 7.4) was added and the tube was incubated at 37ºC for 1 hour.

After that 200 µl of the content from the tube was spread on one LB Km plate (LB medium with 15 g/l nutrient agar and 50 mg/l) while the remaining part was spread on another LB Km plate. Plates were incubated at 37ºC over night, using kanamycin resistance to screen for transformants.

Next, selected colonies (transformants resistant to kanamycin) from the plates were transferred into sterilin tubes containing 5 ml of LB medium with 50 µg/ml of kanamycin added. The tubes were incubated at 37ºC and 180 RPM over night. After that the plasmids were isolated from the tubes according to the protocol of the kit NucleoSpin® Plasmid. To verify the presence of the fragment of interest, a portion of the isolated plasmids from each tube was digested with restriction enzymes by preparing a mixture of 5 µl plasmid, 2 µl 10x M buffer, 1 µl *Cla*I, 1µl *Xba*I and 11 µl water, incubated at 37ºC for 2 hours and then analysed by agarose gel electrophoresis.

The samples indicating the strongest presence of the fragment were chosen to be sent for sequencing and two marked eppendorf tubes, each filled with 15 µl of sample, was prepared for each chosen sample (one for the forward primer and the other for the backward primer). While waiting for the result of the sequencing to be returned, more *E. coli* One Shot® TOP10 cells were transformed with plasmids in order to have a bigger amount available.

To be able to achieve secreted expression of the gene, the *E. coli*/*P. pastoris* vector, pPICZαC, was used. This vector contains the tightly regulated *AOX1* promoter and the *S. cerevisiae* α-factor secretion signal located immediately upstream of the multiple cloning site.

After that the result from the sequencing had turned out positive, one of the plasmid samples was chosen for expression. The sample (containing the pCRBlunt® vector carrying the gene) was digested by preparing a mixture of 40 µl plasmid, 6 µl 10x M buffer, 2 µl *Cla*I, 2µl *Xba*I and 6 µl water, incubated at 37ºC for several hours before adding another 2 µl of *Cla*I and 2µl of *Xba*I and incubating at 37ºC over night.

Following that, the fragment was extracted by running an agarose gel electrophoresis, cutting the fragment out from the gel and then once more follow the protocol of the kit NucleoSpin® Extract II (Macherey Nagel). Another agarose gel electrophoresis was run afterwards to verify the presence of fragment in the purified sample.

To clone the gene into the pPICZαC vector three different ligation mixes were prepared, each one with a different ratio between amount of vector and amount of insert. The composition of each mixture is given in table 2.

Table 2: Composition of different ligation mixtures used to clone the gene into the pPICZαC vector.

Ligation mixture	Water [µl]	10x Ligation Buffer [µl]	pPICZαC vector [µl]	Insert [µl]	T4 DNA ligase [µl]	V_{tot} [µl]
1:3	13	2	1	3	1	20
1:5	11	2	1	5	1	20
1:7	9	2	1	7	1	20

The tubes prepared with the different ligation mixtures were incubated at 16ºC for several hours before being stored at –20ºC until the day of transformation. To amplify the resulting pPICZαC vector with *foxg-17021.2* gene, plasmids from each of the three ligation mixtures were transformed into *E. coli* One Shot® TOP10 cells in the same way as before, except that in this case low salt LB medium was used (10 g/l tryptone, 5g/l yeast extract and 5 g/l NaCl, pH 7.5) and the cells were plated on low salt LB plates (Low salt LB medium with 15 g/l nutrient agar and 25 mg/l kanamycin).

The next day, in the same way as before selected colonies (this time transformants resistant to) from the plates were transferred into sterilin tubes containing 5 ml of low salt LB medium with 25 µg/ml of Zeocin™ added. The tubes were then incubated as before at 37ºC and 180 RPM over night. After that, just as before, the plasmids were isolated, a portion of the isolated plasmids was digested with restriction enzymes and then analysed by agarose gel electrophoresis to confirm the presence of the fragment and the successful cloning of the gene into the vector pPICZαC, now ready to be transformed into *P. pastoris*.

Transformation of P. pastoris and screening of recombinant transformants

In order to be transformed into *P. pastoris* the circular plasmid pPICZαC/*foxg-17021.2* was first going to be linearized with *Sac*I. This was done by preparing a digestion mix consisting of 35 µl plasmid, 5 µl *Sac*I, 10 µl 10x L buffer and 50 µl water, incubated at 37ºC for several hours and then washed (to remove by *Sac*I) by adding 600 µl of wash buffer A4 from the kit NucleoSpin® Plasmid, transfer everything into a NucleoSpin® Plasmid Column placed in a collecting tube, centrifuge for 1 min, discard flow through and then follow the last two steps of the protocol of the kit.

To prepare for the transformation a sterilin tube containing 5ml of YPD medium (10 g/l yeast extract, 20 g/l peptone and 20 g/l dextrose) was inoculated with cells from a stock culture of *P. pastoris* and incubated at 30ºC and 200 RPM over night. After that 50 µl was transferred from the sterilin tube with cells into each one of three 250 ml flasks containing 100 ml of YPD medium. The flasks were incubated at 30ºC and 200 RPM over night.

Next, one flask was used to measure OD_{600} while the content of the other two was pored over into a sterilized centrifugation tube. The tube was centrifuged at 1500 x g for 5 minutes and the supernatant discarded. Next the pellet was resuspended with 250 ml ice-cold (0ºC) sterile water, centrifuged in the same way again and the supernatant once more discarded. Again the pellet was resuspended with another 250 ml ice-cold (0ºC) sterile water, centrifuged in the same way and the supernatant discarded. After this the pellet was resuspended with 20 ml of ice-cold (0ºC) sterile 1M sorbitol, centrifuged in the same way and the supernatant discarded. Following this the pellet was resuspended with 1 ml of ice-cold (0ºC) sterile 1M sorbitol to a final volume of approximately 1.5 ml.

The transformation of *P. pastoris* was done by electroporation, according to the EasySelect™ *Pichia* Expression Kit. Normally 80 µl of cells is mixed with 30µl of linearized plasmid, but in this case, since the linear plasmid had been eluted into 50 µl of water instead of 30 µl, 80 µl of cells was added directly to the tube containing the linear plasmid and the content was transferred into an electroporation cuvette. After having been incubated 5 minutes on ice, the cuvette received a pulse of 2000 V during 5ms generated by the electroporation machine. Immediately after the electroporation 1 ml of ice-cold (0ºC) sterile 1M sorbitol was added to the cuvette and the content was transferred into a 15 ml Falcon tube. The tube was incubated at 30ºC for 2 hours.

After that 300 µl was transferred from the tube to each one of three YPDS plates (YPD medium with 1M sorbitol (182.2g/l), 20 g/l agar and 100 mg/l Zeocin™) and the rest was transferred to a fourth YPDS plate. The plates were incubated at 30ºC for three days, using Zeocin™ resistance to screen for transformants. When three days had past the plates were taken out from the incubator and placed in fridge.

Production of the enzyme

To start preparing for expression, cells were transferred from each one of the three colonies visible over onto marked areas of a MD plate (13.4 g/l yeast nitrogen base (YNB), 0.4 mg/l biotin, 20 g/l dextrose and 15 g/l agar) using the tip of a pipette. The plate was incubated at 30°C over night. Next, using a loop, three baffled 250 ml flasks (sterilized, with cotton in neck and marked 1,2 and 3) containing 50 ml of BMGY medium (10 g/l yeast extract, 20 g/l peptone, 0.1M potassium phosphate buffer, pH=6, 13.4 g/l yeast nitrogen base (YNB), 0.4 mg/l biotin, 10 ml/l glycerol) were inoculated with cells from the corresponding sector (1,2 and 3) of the MD plate. The flasks were incubated at 30°C and ~200 RPM over night.

After that a smaller amount (~5-10 ml) was transferred from each one of the three flasks into marked sterilin tubes in order to measure OD_{600}. Another amount of the same size

was transferred to a fourth sterilin tube, to be centrifuged and used to set OD_{600}=0 for medium without cells. 1:4 dillutions were prepared of the four samples (1,2,3, only medium) by adding 250 µl of sample together with 750 µl of water into a spectrophotometer cuvette. Table 3 gives the results from the measurements of OD_{600} and the subsequent calculations of preculture volumes using equation 1.

$$V_{preculture} = \frac{100}{OD_{600}} \quad (1)$$

Table 3: Measurements of OD_{600} and calculated preculture volumes for each one of the three samples.

Sample	1:4	OD_{600}	$V_{preculture}$ [ml]
1	1.193	4.772	21.0
2	1.165	4.660	21.4
3	1.230	4.920	20.3

Based on these calculations around 20 ml (the preculture) was transferred from each one of the three flasks into (marked and sterile) sterilin tubes. The tubes were centrifuged for five minutes and the supernatant was discarded. Next the cells were to be resuspended in BMMY (10 g/l yeast extract, 20 g/l peptone, 0.1M potassium phosphate buffer, pH=6, 13.4 g/l yeast nitrogen base (YNB), 0.4 mg/l biotin, 5 ml/l methanol) medium to induce expression of the enzyme. 20 ml of BMMY medium was transferred into each one of the sterilin tubes, while another 80 ml of BMMY medium was transferred into each one of three (sterilized, with cotton and marked) baffled 250 ml flasks. The cells were then resuspended by inverting and vortexing the sterilin several times before the content was transferred into the baffled 250 ml flasks, now containing a total volume of 100 ml each. The flasks were incubated at 30℃ and 200 RPM over night. The following three days 500 µl of methanol was added every day to each one of the flasks to maintain the induction of enzyme expression. The fourth day a small amount (~5 ml) of cell culture was transferred from each one of the flasks into (used and marked) sterilin tubes before the adding of methanol. This small amount was to be used for an assay of esterase activity.

Enzyme and protein assays

Esterase activity was assayed using as substrate and the visual detection of green colour (due to the release of *p*-nitrophenol) as a sign of positive outcome. To begin with, some 2 ml were transferred from the each one of the three-sterilin tubes with cell culture into (marked) 2 ml eppendorf tubes. These were centrifuged for about two minutes to pellet the cells. Next a small amount of *p*-nitrophenyl acetate was added to each one of three (marked) 1.5 ml eppendorf tubes, followed by around 900 µl of phosphate buffer, pH=6 and 100 µl of supernatant from the tubes with centrifuged cell culture. The tubes were then incubated at 40℃ for a while until the possible detection of greenish colour.

Results and discussions

Amplification of FOXG_17403.2

Recently, in the *Fusarium oxisporum* genome, ten translated ORFs were identified, based on primary sequence similarities, as potential FAEs, which is consistent with the expectation that F. oxysporum is capable of producing more than one type of FAE. One of these ORFs, FOXG_17403.2, encodes putative FAE with 46–47% identity with NcFaeD from *N. crassa* and CjFaeD from *Cellvibrio japonicus*, showing type A or D FAE activity [7]. This sequence was the one initially chosen for study.

The amplification through PCR of the sequence from the ORF FOXG_17403.2 turned out to be much harder than expected and came to require a rather big number of repeated attempts. Not until the temperature for hybridisation (annealing) had been varied several times, another DNA sample had been used as template, another polymerase had been used and even another set of primers from another company, was it possible to achieve satisfying results. Figure 4 shows the result of the first attempt to amplify the gene, yielding only weak, hardly visible bands at the expected product size when running an agar gel electrophoresis with the product from the amplification.

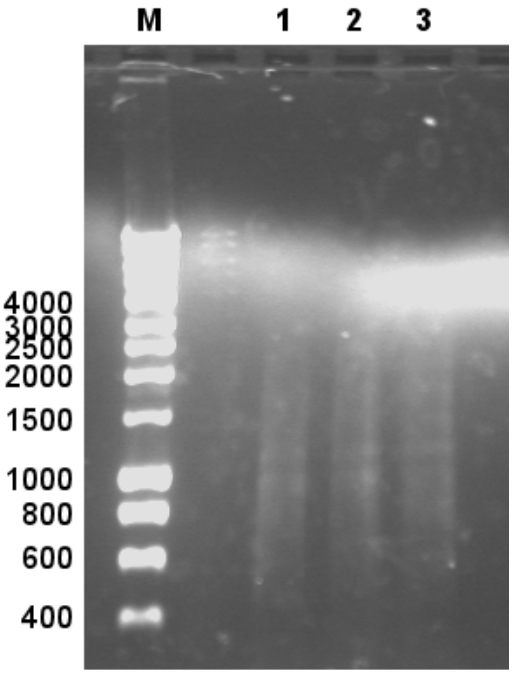

Fig. 4: Agarose gel electrophoresis of the product resulting from the first attempt to amplify the sequence from FOXG_17403.2. In lanes 1-3, a weak band at the size of 800-900 bp can be noticed, in particular for the first two.

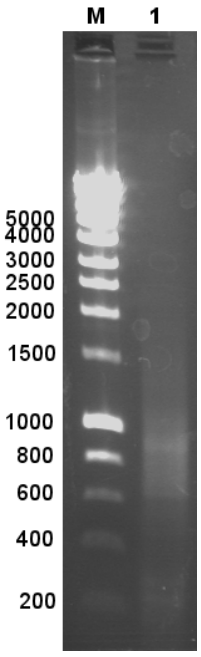

Fig. 5: Agarose gel electrophoresis showing the amplified product visible in lane 1.

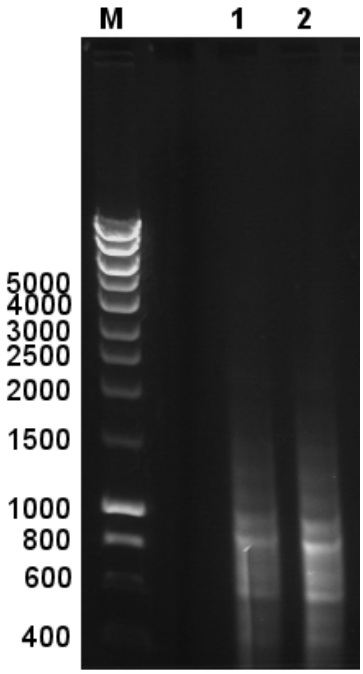

Fig. 6: Agarose gel electrophoresis used for gel extraction. The product band can be seen slightly above another band of similar intensity, of which both were cut and processed separately, in lanes 1 and 2.

Many unsuccessful attempts then followed until finally the amplified product could be seen again, as shown in figure 5. The product from this amplification was used for gel extraction and the resulting gel, from which a slice containing the product band was cut, can be seen in figure 6:

Cloning of the gene from FOXG_17403.2 into pCR®-Blunt vector

Samples of plasmids, purified from ten different transformed colonies, were each one digested with restriction enzymes (*Pst*I, *Xba*I) and analysed by agarose gel electrophoresis. As can be seen in figure 7, all ten samples have fragments at the size of the vector, but only the first sample contains a fragment of the expected gene size.

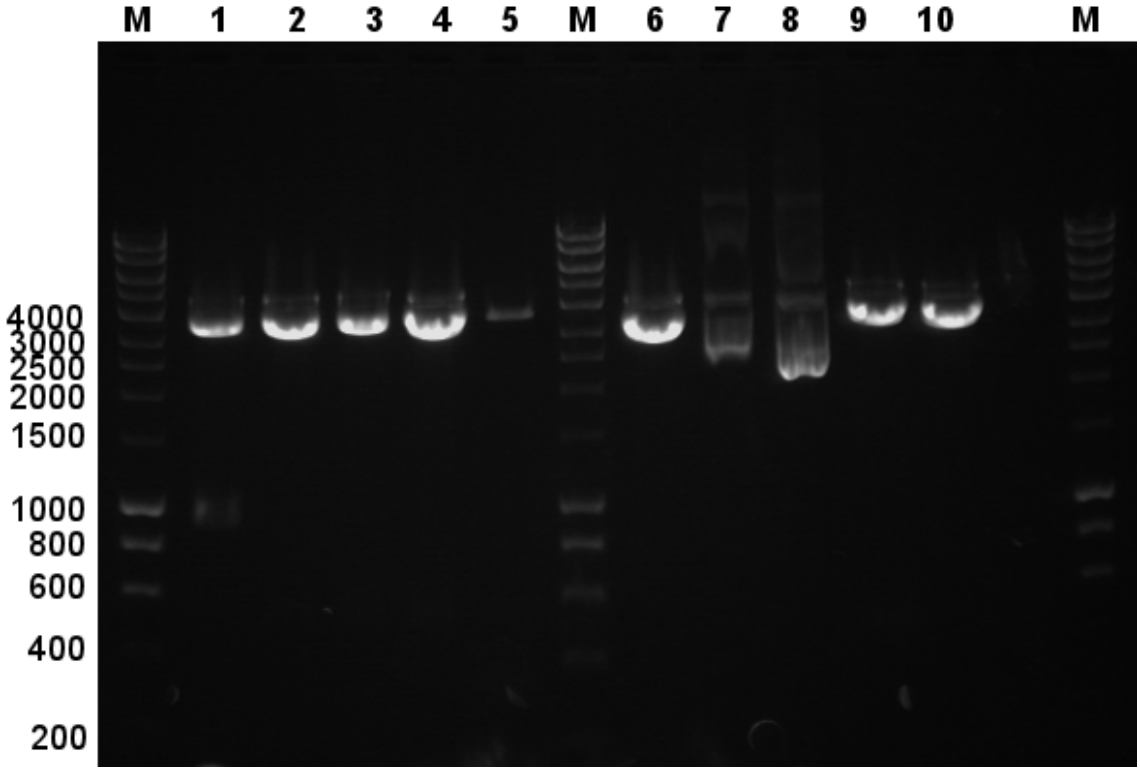

Fig. 7: Agarose gel electrophoresis showing the result from the first cloning of the sequence from FOXG_17403.2 into pCR®-Blunt vector. Fragments at the size of the vector can be seen in all 10 samples, but only in sample 1 can be seen a fragment at the size of the gene.

To verify that the fragment of expected size really is the gene, a crosstest was performed, by running a PCR with a different combination of primers, using the regular set, together with another one, designed to eliminate the intron in the middle of the gene. If the fragment really is the gene then the result from the crosstest should be the detection of a fragment at about half the size of the actual fragment. However, as can be seen in figure 8, even after two such tests repeated, very little (at best) of such fragment size could be seen.

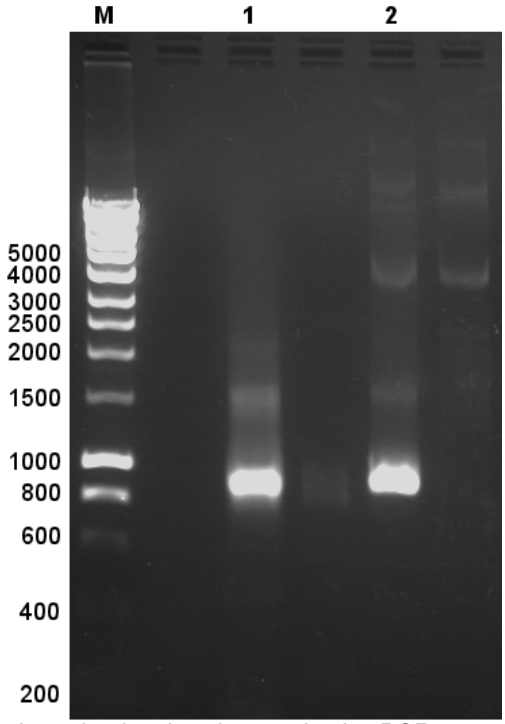

Fig. 8: Agarose gel electrophoresis showing the result of a PCR crosstest, using combinations of different primers to test the presence of the gene. The strong band in lane 1 and 2, at around the size of the whole gene, in contrast to the hardly visible band at the expected fragment size for this combination (about half the size of the gene) demonstrates that the fragment is most likely not the gene of interest.

Instead, the main band appears to be at the size of the whole gene, something that suggests that the fragment obtained is not the gene in question.

Amplification of FOXG_17403.2, using new primers

Since the fragment obtained did not turn out to be the gene, a new strategy was tried, to amplify the gene using a new set of primers ordered from a different company. Unfortunately this seemed to have little effect, as it still turned out hard to amplify the gene. Not until after several attempts and the use of another DNA sample as template and another polymerase could a fragment of the expected size be seen, first on a gel run with product from a PCR with $T_{annealing}$=56℃, as seen in figure 9, then on another gel this time with $T_{annealing}$=58℃, as seen in figure 10. The products from both these amplifications were used for gel extraction. Figure 11 shows the resulting gel that a slice was cut from.

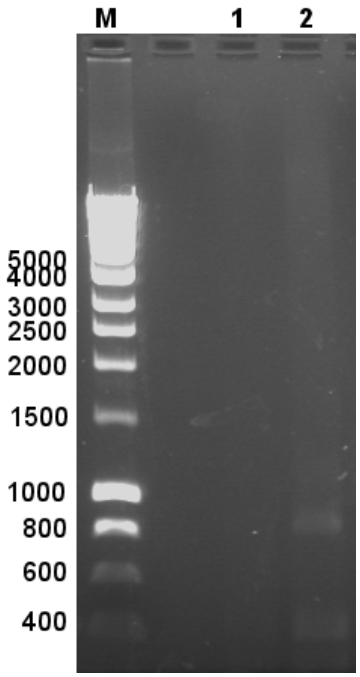

Fig. 9: Agarose gel electrophoresis showing the result of a PCR run with the new primers ordered, $T_{annealing}$=56℃ and two different samples, one with concentrated DNA as template the other with diluted (1/10) DNA. A band of the expected size can be seen in the sample with concentrated DNA (lane 2) but not in the one with diluted DNA (lane 1).

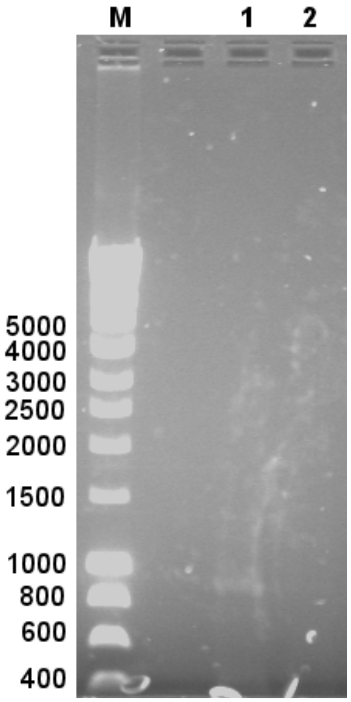

Fig. 10: Agarose gel electrophoresis showing the result of another PCR, this time with two samples, one with $T_{annealing}$=58℃ (lane 1) and the other with $T_{annealing}$=60℃ (lane 2). A weak band can be seen in lane 1.

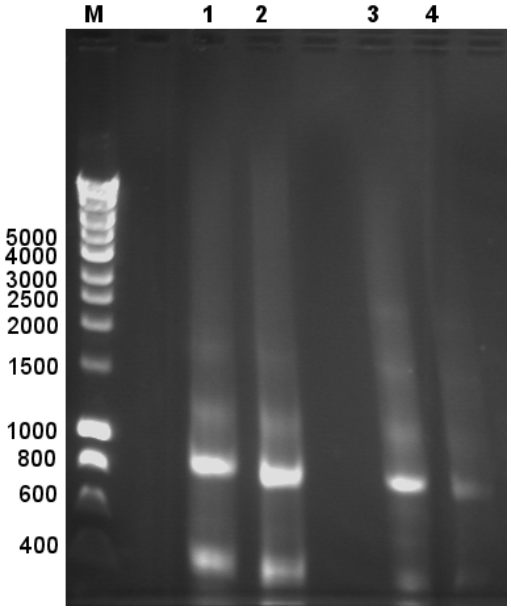

Fig. 11: Agarose gel electrophoresis used for gel extraction, lane 1 and 2 originating from the 56℃ product while the other two (lane 3 and 4) originating from the 58℃ product. Both pairs of bands were cut out with a scalpel and placed in eppendorf tubes, 56℃ product being purified while 58℃ product was stored in freezer.

Cloning of the gene from FOXG_17403.2 into pCR®-Blunt vector

Just as before plasmids samples from different colonies (this time five), were digested with restriction enzymes (now *Sac*I and *Xba*I) and analysed by agarose gel electrophoresis. As can be seen in figure 12, all five samples have fragments at the size of the vector, while the samples (1), 3,4 and 5 contain a fragment of the expected gene size.

Sample four was chosen to proceed with as it showed the strongest and clearest band at the right size. A crosstest was done, as previously, this time indicating a positive result, bands at the sizes ~300 bp and ~400 bp respectively. So, the sample was sent away for sequencing. The result that came back from the sequencing however, turned out negative. Finally it was decided to stop working with this sequence altogether and instead make an attempt to clone another similar ORF, namely FOXG_17021.2

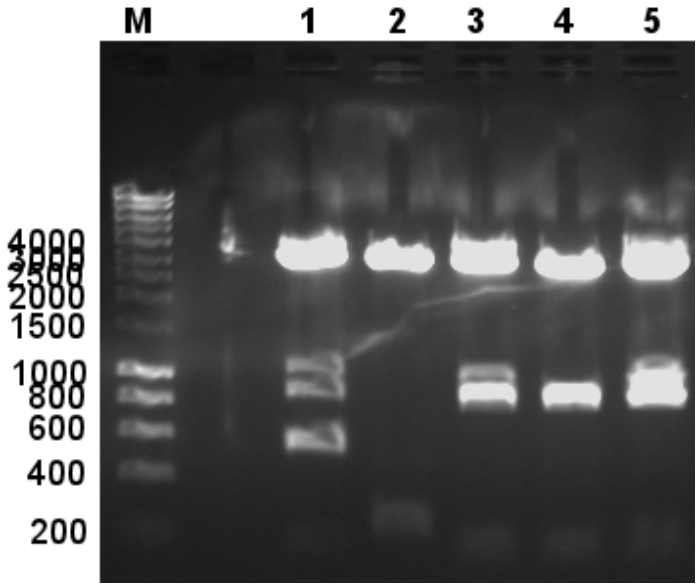

Fig. 12. Agarose gel electrophoresis showing the result from the second cloning of FOXG_17403.2 into pCR®-Blunt vector.

It is hard to know the exact reason how come this sequence turned out so hard to obtain, one possible explanation could be minor differences between the genome of the *F. oxysporum* strain used in the laboratoty (F3) and the one used to map the genome in the USA. Such differences are usually relatively small, but perhaps in the case of this particular ORF, they happen to be of significant importance for the primers' ability to match to the sequence?

Amplification of FOXG_17021.2

Since the attempt to clone FOXG_17403.2 was unsuccessful, another similar sequence from the *F. oxysporum* genome was chosen, namely the sequence of the ORF FOXG_17021.2.

In order to have an idea what to expect when cloning this ORF, a BLAST search of the translated protein sequence was made. The result is shown in figures A and B in the appendix.

At a first glance it seems clear that this enzyme most likely belongs to the tannase and feruloyl esterase family. Judging from the top scoring hits it's very tempting to say that it is a tannase, but it's important to keep in mind that tannases and feruloyl esterases, type C share a great deal of similarities in terms of sequence, so it would be rather risky to make such a conclusion, especially since these two classes of enzymes have different properties and act upon different substrates. Not until a proper investigation has been made concerning the activity and substrate specificity of the enzyme is it possible to conclude what type of enzyme it really is.

The PCR amplification of the sequence from the ORF FOXG_17021.2 turned out to be a whole lot easier than how it had been with FOXG_17403.2. As can be seen in figure 13, already the first PCR run resulted in clear bands at the expected size of the fragment. In this case three reactions had been run, all with different concentrations of DNA as template, one being a 1/10 dillution, the next a 1/6 dillution and the last a 1/3 dillution. Between these, the 1/10 product was chosen for gel extraction. The successful extraction of the fragment from the 1/10 product can be seen in figure 14, showing the positive result of the subsequent cleanup after gel extraction.

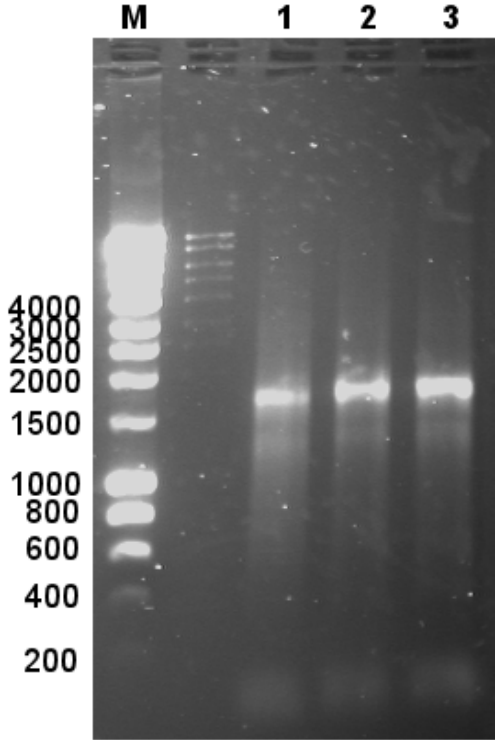

Fig. 13: Agarose gel electrophoresis showing the result (positive) of the first amplification of FOXG_17021.2. Three reactions had been run, all with different DNA concentration as template. From left to right:1/10 dilution of DNA (lane 1), 1/6 dilution (lane 2) and 1/3 dilution (lane 3). Among these the 1/10 dilution was used to prepare for extraction.

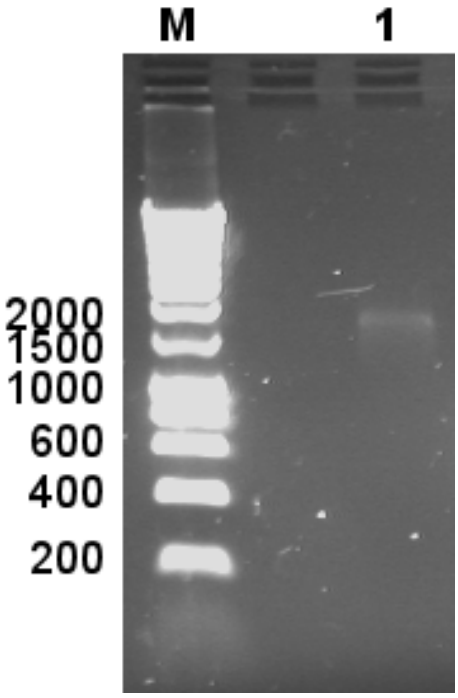

Fig. 14: Agarose gel electrophoresis of the extracted fragment after gel cleanup. A weak band can be seen in lane 1, indicating a successful cleanup.

Cloning of the gene from FOXG_17021.2 into pCR®-Blunt vector

In the same way as previously plasmids samples originating from different colonies (this time four), were digested with restriction enzymes (in this case *Cla*I and *Xba*I) and analysed by agarose gel electrophoresis. The result is shown in figure 15. It can be seen (in the lower row) that all four samples have fragments at the size of the vector, while the samples 1,2 and 4 contains a fragment of the expected gene size as well. Sample 1 and sample 4 shows the clearest bands and were therefore chosen to be sent for sequencing.

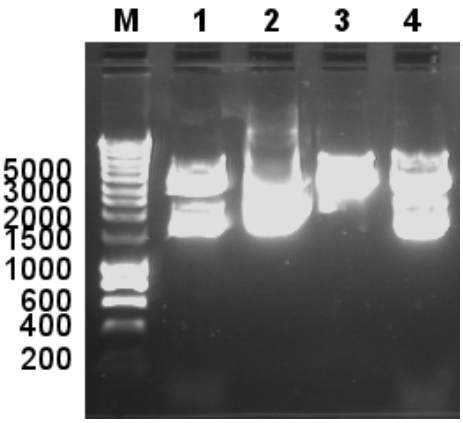

Fig. 15: Agarose gel electrophoresis showing the result of cloning FOXG_17021.2 into pCR®-Blunt vector. Bands at the size of the vector can be seen in all four samples while bands at the size of the fragment can be seen in samples 1,2 and 4.

Cloning of the gene from FOXG_17021.2 into pPICZα vector

As before plasmids samples coming from different colonies (this time ten), were digested with restriction enzymes (in this case *Cla*I and *Xba*I) and analysed by agarose gel electrophoresis. The first attempt however turned out unsuccessful so another ten colonies were chosen and plasmids were purified from each one of them. As can be seen in figure 16, the result was positive this time and it was possible to proceed with the transformation of *Pichia pastoris* and the subsequent expression of the enzyme.

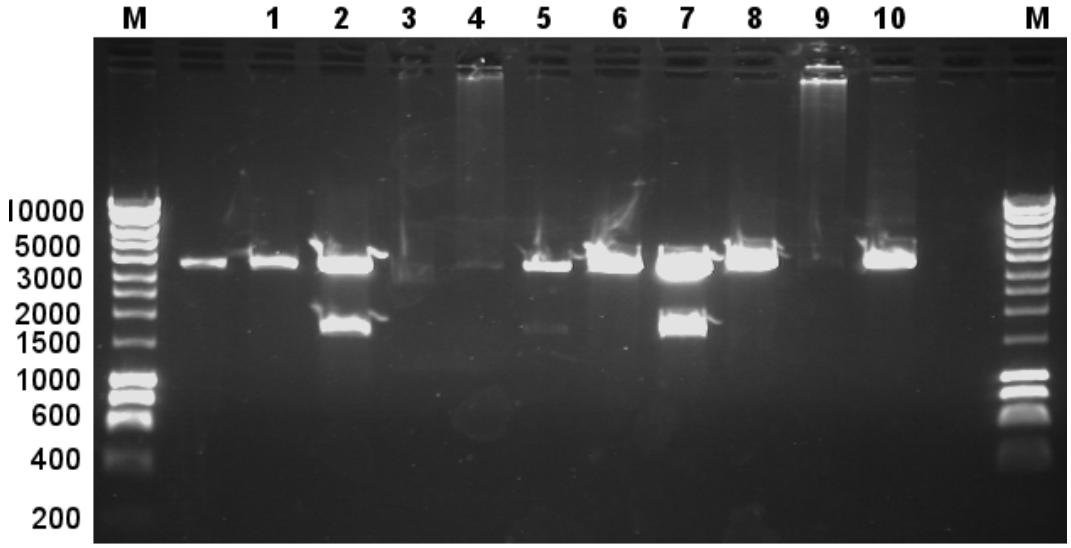

Fig.16: Agarose gel electrophoresis showing the result of the second attempt (successful) to clone FOXG_17021.2 into pPICZα vector. Positive result (band at the size of the fragment) can be seen for samples 2 and 7. Note that band seen in the lane immediately to the right of the markers in the left is merely the result of spillage from the well to the right of it (sample 1) and that the number of samples really is ten.

Transformation of P. pastoris and expression of the enzyme

The presence of colonies on YPDS plates with Zeocin™ suggests that cells of *P. pastoris* were successfully transformed.

After having been induced for expression during four days an esterase assay was carried out using *p*-nitrophenyl acetate as substrate, resulting in a green colour if positive (due to the release of *p*-nitrophenol). Figure 17 shows that among the three sample that were tested, all three yielded a positive result for esterase activity.

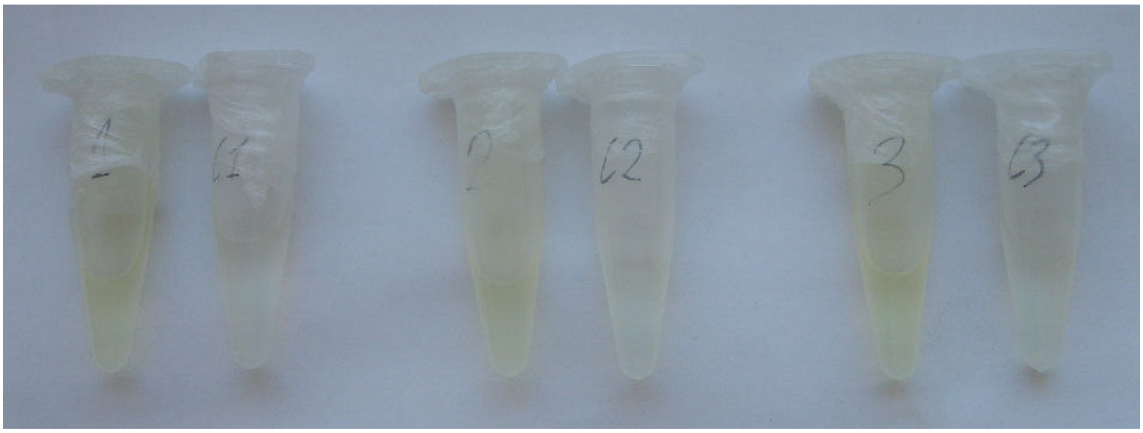

Fig.17: Eppendorf tubes (marked 1, 2 and 3) containing *p*-nitrophenyl acetate, phosphate buffer, pH=6 and a sample from the supernatant of the transformed *P. pastoris* cultures, induced to express the enzyme, pictured next to control samples (marked C1, C2 and C3) lacking the supernatant sample. Green colour (caused by the release of *p*-nitrophenol) indicating esterase activity in the tubes containing supernatant sample, demonstrating the presence of an enzyme with esterase activity.

The outcome of this assay clearly shows that the enzyme expressed from the ORF FOXG_17021.2 has esterase activity. It is however not possible in this moment to say if it is a feruloyl esterase. Strictly speaking this cannot be said for sure until it has been established if the enzyme is capable of releasing ferulic acid from plant cell wall. Unfortunately due to lack of time any further investigation of activity could not be covered with this report.

Conclusions

The isolation and cloning of the ORF FOXG_17403.2, due to some unknown reason, turned out much more difficult than expected and because of the time available being limited, the investigation was postponed.

Concerning the ORF FOXG_17021.2, this one was, on the other hand, successfully cloned and later on also expressed in *P. pastoris.* It could be shown that the ORF encodes an enzyme that possesses esterase activity. It could however not be concluded weather it is the question of a feruloyl esterase or not.

References

1. Benoit, I., Danchin, E. G. J.; Bleichrodt, R.-J.; De Vries, R. P., *Biotechnol. Lett.* (**2008**), 30, 387–396.

2. Topakas, E., Vafiadi, C., Christakopoulos, P., *Proc. Biochem.* (**2007**), 42, 497–509.

3. Mathew, S., Abraham, T.E., *Crit. Rev. Biotechnol.,* **(2004)**, 24(2–3), 59–83.

4. De Vries, R. P., Visser, J., *Microbiol Molec. Biol. Rev.,* (**2001**), 497–522.

5. Vafiadi, C., Topakas, E., Bakx, E. J., Schols, H. A., Christakopoulos**,** P., *Molecules* (**2007**), 12, 1367-1375.

6. Wong, D. W. S., *Applied Biochem. Biotechnol.*, (**2006),** 133, 87-112.

7. Moukouli, M., Topakas, E., Christakopoulos, P., *Appl. Microbiol. Biotechnol.* **(2008),** 79, 245–254.

8. Crepin, V. F., Faulds, C. B., Connerton, I. F., *Biochem. J.* **(2003),** 370, 417–427.

9. Crepin, V. F., Faulds, C. B., Connerton, I. F., *Appl. Microbiol. Biotechnol.* **(2004),** 63, 647–652.

Appendix

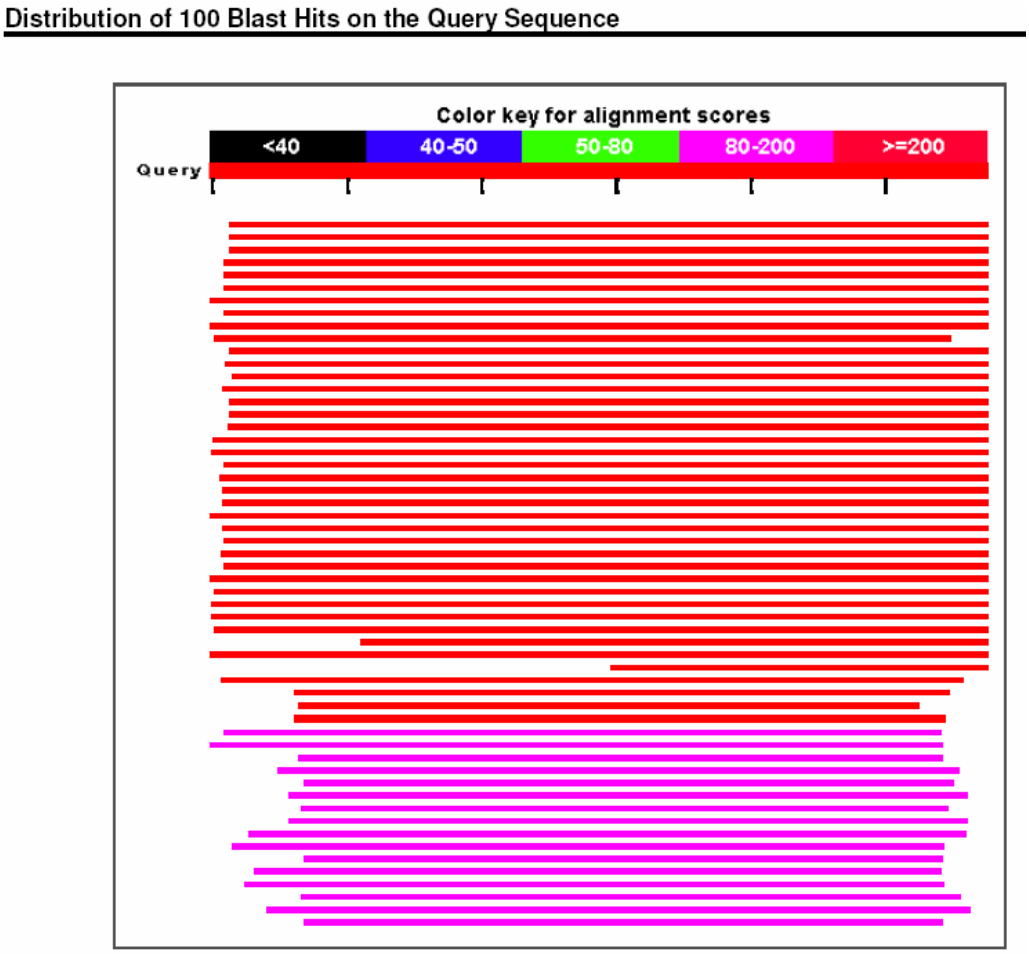

Fig. A: Graphical overview of the result from a BLAST search of the translated protein sequence from the ORF FOXG_17021.2

Sequences producing significant alignments:	Score (Bits)	E Value
ref|XP_001262462.1| tannase and feruloyl esterase family prot...	875	0.0
ref|XP_746534.1| tannase [Aspergillus fumigatus Af293] >gb|EA...	873	0.0
gb|EDP47357.1| tannase, putative [Aspergillus fumigatus A1163]	870	0.0
gb|ABJ51876.1| tannase [Aspergillus oryzae]	866	0.0
ref|XP_001826685.1| hypothetical protein [Aspergillus oryzae ...	860	0.0
ref|XP_001402486.1| hypothetical protein An10g00950 [Aspergil...	854	0.0
ref|XP_001216558.1| hypothetical protein ATEG_07937 [Aspergil...	853	0.0
ref|XP_001553529.1| hypothetical protein BC1G_08253 [Botryoti...	842	0.0
ref|XP_682472.1| hypothetical protein AN9203.2 [Aspergillus n...	842	0.0
gb|ABX89592.1| tannase [Aspergillus niger]	773	0.0
ref|XP_660301.1| hypothetical protein AN2697.2 [Aspergillus n...	773	0.0
ref|XP_456970.1| hypothetical protein DEHA0A15114g [Debaryomy...	749	0.0
ref|XP_001393409.1| hypothetical protein An09g00890 [Aspergil...	747	0.0
ref|XP_001547571.1| hypothetical protein BC1G_13815 [Botryoti...	704	0.0
ref|XP_001558054.1| hypothetical protein BC1G_03086 [Botryoti...	686	0.0
ref|XP_001590621.1| hypothetical protein SS1G_08361 [Scleroti...	684	0.0
ref|XP_001548537.1| hypothetical protein BC1G_12932 [Botryoti...	627	9e-178
ref|XP_001389868.1| hypothetical protein An01g14800 [Aspergil...	620	1e-175
ref|XP_001393089.1| hypothetical protein An08g09800 [Aspergil...	615	3e-174
ref|XP_001396513.1| hypothetical protein An13g03810 [Aspergil...	600	1e-169
ref|XP_001399173.1| hypothetical protein An02g00350 [Aspergil...	594	7e-168
ref|XP_001560056.1| hypothetical protein BC1G_01615 [Botryoti...	580	1e-163
ref|XP_001398312.1| hypothetical protein An17g00830 [Aspergil...	574	6e-162
ref|XP_748839.1| tannase [Aspergillus fumigatus Af293] >gb|EA...	572	4e-161
ref|XP_001820636.1| hypothetical protein [Aspergillus oryzae ...	570	8e-161
ref|XP_001390411.1| hypothetical protein An03g05300 [Aspergil...	563	1e-158
ref|XP_001211829.1| predicted protein [Aspergillus terreus NI...	561	5e-158
ref|XP_001401809.1| hypothetical protein An04g04430 [Aspergil...	560	9e-158
ref|XP_001261622.1| tannase and feruloyl esterase family prot...	559	3e-157
ref|XP_001394358.1| hypothetical protein An11g03590 [Aspergil...	544	8e-153
ref|XP_001594065.1| hypothetical protein SS1G_05493 [Scleroti...	536	2e-150
ref|XP_001388709.1| hypothetical protein An01g02740 [Aspergil...	493	2e-137
ref|XP_001558852.1| hypothetical protein BC1G_02486 [Botryoti...	488	4e-136
ref|XP_001827335.1| hypothetical protein [Aspergillus oryzae ...	399	3e-109
ref|XP_001912284.1| unnamed protein product [Podospora anseri...	339	3e-91
ref|XP_001551827.1| hypothetical protein BC1G_09533 [Botryoti...	263	2e-68
ref|XP_001805090.1| hypothetical protein SNOG_14920 [Phaeosph...	221	1e-55
ref|XP_001821143.1| hypothetical protein [Aspergillus oryzae ...	211	2e-52
ref|XP_001589546.1| hypothetical protein SS1G_09267 [Scleroti...	206	3e-51
ref|XP_001555592.1| hypothetical protein BC1G_05867 [Botryoti...	202	7e-50
ref|XP_001389566.1| hypothetical protein An01g11560 [Aspergil...	189	4e-46
ref|XP_001394129.1| hypothetical protein An11g01220 [Aspergil...	188	1e-45
ref|XP_001560161.1| hypothetical protein BC1G_00993 [Botryoti...	179	6e-43
ref|XP_001822863.1| hypothetical protein [Aspergillus oryzae ...	179	7e-43
ref|XP_001228631.1| hypothetical protein CHGG_10704 [Chaetomi...	178	1e-42
ref|XP_001521975.1| hypothetical protein MGCH7_ch7g93 [Magnap...	178	1e-42

Fig. B: Listing of the highest scoring results from a BLAST search of the translated protein sequence from the ORF FOXG_17021.2