CLONING, EXPRESSION AND CHARACTERIZATION OF TANNASE FROM *ASPERGILLUS* SPECIES

BY

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Submitted in fulfilment of the requirements for the degree

MAGISTER SCIENTIAE

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January 2002

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Acknowledgements

I wish to express my gratitude to the following people and institutions:

- **Dr Esta van Heerden.** For her guidance, support, friendship and perseverance through hard times.

- **Dr J. Albertyn.** For his support, encouragement and wisdom as well as inspiring me to reach greater heights.

- **Professor D. Litthauer.** For his time, encouragement and guidance and dedicated humour throughout my studies.

- **Cornelia Casaleggio.** For her patience, endless support and friendship, as well as her wisdom and knowledge.

- **My fellow students and friends of the Molecular Biology Laboratory.** For their valuable advise, interest and friendship.

- **My parents (Cora and Hennie)** and **sister (Karen)**, for support and friendship and love.

- **Ilse – Marie**, for believing in me, for encouragement and love.

- **The National Research Foundation**, for financial support.

- **TO GOD, CREATOR OF ALL.**

“All that is gold does not glitter,
Not all those who wander are lost;
The old that is strong does not whither,
Deep roots are not reached by the frost.

From the ashes a fire shall be woken,
A light from the shadows shall spring;
Renewed shall be blade that was broken:
The crownless again shall be king”.

- J.R.R. TOLKIEN
“ The Lord of the Rings “

List of Abbreviations

a.a	Amino acids
Amps.	Ampicillin
AP	Alkaline phosphatase
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp.	Base pairs
cDNA	Complimentary deoxyribonucleic acid.
Da	Dalton
DIG	Digoxigenin-11-dUTP
DNA	Deoxyribonucleic acid
EDTA	Ethylene diaminetetraacetic acid
ES	Enzyme substrate
IPTG	Isopropyl-1-thio-galactoside
MEA	Malt extract agar

MEB	Malt extract broth
mRNA	Messenger ribonucleic acid
NBT	Nitroblue tetrazolium salt
ORF	Open reading frame
oryTAH	*Aspergillus oryzae's* tannase gene
pI	Iso-electric point
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSC	Tri- sodium citrate
TAE	Tris(2-amino-2-(hydroxymethyl)-1,3-propandiol)-acetate electrophoresis buffer
Tannase	Tannin acyl hydrolase
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol
Tween 20	Polyoxyethylenesorbitan monooleate
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside
YNB	Yeast nitrogen base

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Chapter 1

Literature review

1. Introduction

Tannin Acyl Hydrolase (E.C. 3.1.1.20) is commonly referred to as tannase. Teighem accidentally discovered this unique enzyme in 1867 (Teighem, 1867). He reported the formation of gallic acid when two fungal species were exposed to an aqueous solution of tannins. The fungal species were later identified as *Penicillium glaucum* and *Aspergillus niger* (Lekha & Lonsane, 1997).

Tannase is responsible for the hydrolysis of ester and depside linkages in tannins to liberate gallic acid and glucose. This was a very interesting observation due to the usual complexation of proteins with tannic acid and naturally occurring tannins to form water insoluble complexes that inactivates enzymes (Haworth *et al.,* 1985). Tannins have since been shown to be the natural substrate for the tannase enzyme. The enzyme also attacks gallic acid methyl esters, but it possesses high specificity towards the acyl moiety of the substrate.

It has been known that certain moulds and fungi belonging to the species *Aspergillus* and *Penicillium* produce the enzyme (Rajakumar & Nandy, 1983). According to the work done by Yamada *et al.,* (1968) the enzyme was mainly found intracellularly although the culture broth also contained the enzyme. *Aspergillus niger, A. flavus* and *A. oryzae* were found to be the best tannase producers on tannic acid as a sole source of carbon. From these growth studies it became evident that the tannase enzyme was an inducible enzyme (Gupta *et al.,* 1997, Jean *et al.,* 1981 and Mattiason & Kaul, 1994).

1.1 Tannins as substrate for tannase

Tannins are naturally occurring polyphenolic compounds with varying molecular weights that occur naturally in the plant kingdom. These phenolic compounds differ from others by having the ability to precipitate proteins from solutions. In the plant kingdom these tannins are found in leaves, bark and wood. Tannins are considered to be the plant's secondary metabolic products because they play no direct role in the plants metabolism. After lignin, tannins are the second most abundant group of plant phenolics. The large amount of phenolic hydroxyl groups allows the tannins to form complexes with proteins and to a lesser extent with other macromolecules like cellulose and pectin (Mueller–Harvey *et al.,* 1987). Tannins can be divided into two major groups on the basis of their structure and properties.

1.1.1 Hydrolysable tannins

Hydrolysable tannins are polyphenolic plant constituents derived from mono – to pentagalloyllated β-D–glucopyranose (Figure 1.1).

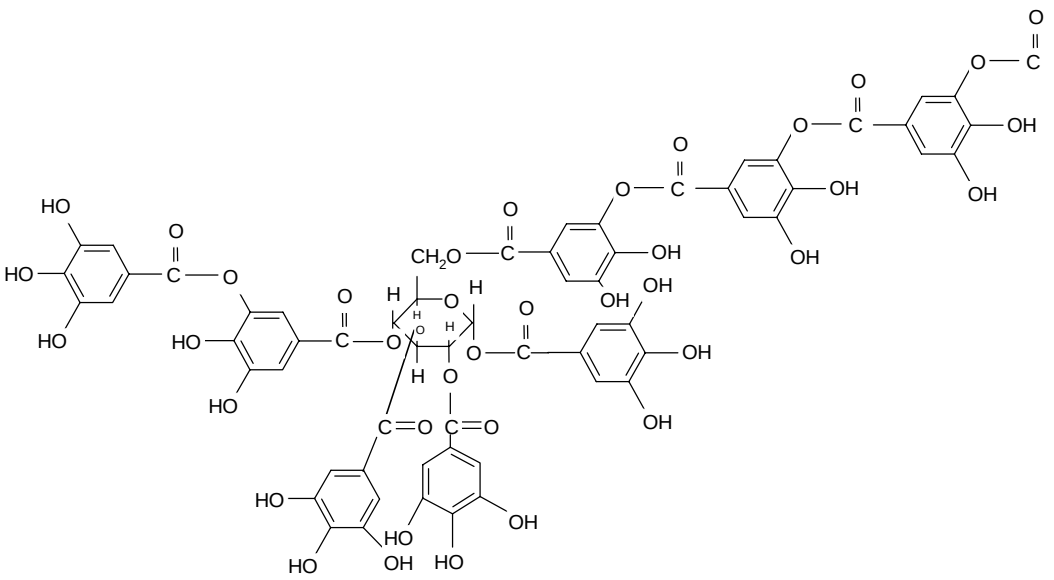


Figure 1.1. A hydrolysable tannin molecule with a glucose core.

These “simple esters” from the gallotannin subclass are extended by attachment of additional galloyl residues to the phenolic galloyl – OH groups to yield *meta*depsidic side–chains of variable length. The allagitannin subclass is characterized by oxidative linkages of spatially adjacent galloyl residues of the core unit with the formation of hexahydroxydiphenoyl bridges (Niehaus & Gross, 1997).

1.1.2 Condensed tannins

Condensed tannins are also known as proanthocyanidins, and consist of phenols of the flavon type flavonoids. They are also called flavolans because they are polymers of flavan-3-ols such as catechin or flavan–3,4–diols known as leucocyanidins. A very interesting difference between condensed tannins and hydrolysable tannins is the fact that condensed tannins do not contain any sugar moieties (Figure 1.2).

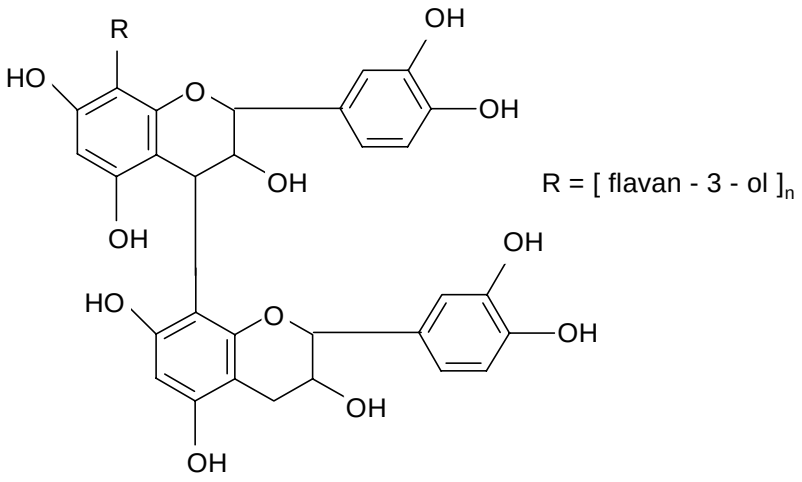


Figure 1.2. Condensed tannin (Procyanidin).

An intermediate group also exists that combines both characteristics of hydrolysable tannins and condensed tannins. This family of tannins is called the catechin tannins. The catechin tannins are most abundant in tea leaves (Graham, 1992). Table 1.1 summarizes the different types of natural occurring tannins that can serve as substrates for tannase.

Table 1.1. Different tannins as substrates for tannase (Haslam & Tanner, 1970).

Hydrolysable Tannins	**Catechin Tannins**	**Condensed Tannins**
1. **Gallotannins** – yield gallic acid and glucose on hydrolysis.	**Catechin and epicatechin gallates** – yield catechin, epicatechin and gallic acid on hydrolysis.	**Polymeric proanthocyanidins** – yields monomers of flavonoids e.g. flavan – 3, 4 – diols and flavan – 3 – ols.
2. **Ellagitannins** – yield ellagic acid and glucose on hydrolysis.		

1.2 Sources of tannase

Tannins are quite resistant to microbial attack and are known to inhibit the growth of some microorganisms. It is this anti-microbial effect of the tannins that slow down the rate of biodegradation of soil organic matter. Biodegradation of soil is usually a very complex process, the process usually involves degradation of organic matter by microorganisms to utilize the broken down constituents as carbon, energy or nitrogen sources.

The large amounts of polyphenolic compounds on the tannin substrate structure can form complexes with the extra and intracellular enzymes from the biodegradative organisms. This complexation leads to inhibition of the biodegradative enzymes (Scalbert, 1991), which in turn leads to a loss in the microbial growth and eventually an increase in the bioconversion time taken for the decomposition of soil organic matter. However Deschamps and co-workers found a number of bacteria, fungi and yeasts that are resistant to tannins and grow on them as a carbon source (Deschamps *et al.,* 1983).

1.2.1 Microbial tannase and tannin degradation

Of all the microorganisms able to produce tannase, *Aspergillus* sp. were commercially the most efficient producers of this enzyme. Tannase is produced as a membrane bound or intracellular enzyme. Not all tannase is equally active against the different tannin substrates. Fungal tannases have a better activity in degrading hydrolysable tannins, whereas yeast tannases degrade tannic acid better and has a lower affinity for naturally occurring tannins (Deschamps *et al.,* 1983). On the other end of the spectrum, bacterial tannase can degrade and hydrolyse natural tannin and tannic acid very efficiently (Deschamps *et al.,* 1983, Lewis & Starkey, 1969). In the beginning of the twentieth century the fungus, *Chryphonectria parasitica*, was found to cause chestnut blight in American chestnut trees, the rate of mycelial growth was shown to play an important role in the pathogenesis of the fungus during blight formation. The growth suggested that the fungus was able to utilize the tannins that are abundant in the chestnut bark. This behaviour would suggest that the fungi were able to use the tannins in the bark as an organic carbon source during pathogenesis. The type of tannin in the bark also played a major role in the susceptibility of the chestnut trees to blight. Hamamelitannin was found in high concentrations in blight susceptible chestnut trees of America and Europe (Elkins, 1981). However in the Japanese and Chinese blight resistant chestnuts no levels of hamamelitannin was found (Farias *et al.,* 1992). Farias and co-workers (1994) hypothesized that upon hydrolyses of the hamamelitannin gallic acid was liberated. The accumulating gallic acid had the potential of being toxic to the chestnut tissue, thus aiding the fungus in infecting the trees.

Some bacterial cultures have developed the ability to express extracellular tannase to degrade tannins, thus releasing gallic acid and glucose. Deschamps, (1983) showed that strains of *Bacillus pumilus, B. polymyxia, and Klebsiella planticola* were able to produce extracellular tannase with chestnut bark as the sole source of carbon. The most abundant group of bacteria able to degrade tannins is found in the gastrointestinal track of ruminants (Deschamps *et al.,* 1983).

Filamentous fungi also have the ability to degrade tannins as a sole source of carbon (Lewis & Starkey, 1969 and Hadi *et al.,* 1994). Researchers revealed that degradation of tannins increased with the addition of other metabolisable substances. Ganga *et al.,* (1977) found that *A. niger* and *Penicillium* spp. grew better on a medium containing glucose and tannin (Bhat *et al.,* 1997, 1998), which meant that the addition of carbon and nitrogen sources favoured the production of tannase for the subsequent cleavage of the tannin molecules to liberate a supply of carbon for growth.

Tannin degradation by yeasts has not been studied to its full potential. Aoki *et al.,* 1976 isolated and reported the enzymatic degradation of gallotannins by yeast species belonging to *Candida* that was able to produce tannase. The tannase from this yeast was able to hydrolyse the ester and depside linkages from tannic acid to liberate gallic acid and glucose. Table 1.2 is a table representing the different isolated microorganisms that are able to express tannase.

Table 1.2. Microorganisms capable of producing tannase (Adapted from Bhat *et al.*, 1998) (♠ Poor producer ∗ Moderate producer Good producer ♦ Best producer).

Bacteria	
∗ *Achromobacter* sp.	Lewis & Starkey, 1969
Bacillus pumilis	Deschamps *et al.*, 1983
Bacillus polymyxa	Deschamps *et al.*, 1983
Corynebacterium sp.	Deschamps *et al.*, 1983
Klebsiella planticola	Deschamps *et al.*, 1983
∗ *Pseudomonas solanacearum*	Deschamps & Lebeault, 1984
∗ *Selenomonas ruminatium*	Skene & Brooker 1995
Fungi	
♦*Aspergillus oryzae*	Bradoo *et al.*, 1996
∗*Aspergillus flavus*	Yamada *et al.*, 1968
♦*Aspergillus niger*	Bradoo *et al.*, 1996
♦*Aspergillus japonicus*	Bradoo *et al.*, 1996
∗ *Aspergillus aureus*	Bajpai & Patil, 1996
♦*Aspergillus awamori*	Bradoo *et al.*, 1996
∗ *Aspergillus fischeri*	Bajpai & Patil, 1996
∗ *Aspergillus rugulosus*	Bradoo *et al.*, 1996
∗ *Aspergillus terreus*	Bajpai & Patil, 1996
∗ *Penicillium chrysogenum*	Bradoo *et al.*, 1996
∗ *Penicillium notatum*	Ganga *et al.*, 1977
∗ *Penicillium islandicum*	Ganga *et al.*, 1977
∗ *Penicillium digitatum*	Bradoo *et al.*, 1996
∗ *Penicillium acrellanum*	Bradoo *et al.*, 1996
∗ *Penicillium carylophilum*	Bradoo *et al.*, 1996
∗ *Penicillium charlesii*	Bradoo *et al.*, 1996
∗ *Penicillium citrinium*	Bradoo *et al.*, 1996
Cryphonectria parasitica	Farias *et al.*, 1992
Fusarium solani	Bradoo *et al.*, 1996
∗ *Fusarium oxysporium*	Bradoo *et al.*, 1996
Rhizopus oryzae	Hadi *et al.*, 1994

Table 1.2. Continued:

Trichoderma viride	Bradoo *et al.*, 1996
∗ *Trichoderma hamatum*	Bradoo *et al.*, 1996
∗ *Trichoderma harzianum*	Bradoo *et al.*, 1996
∗ *Helicostylum* sp.	Bradoo *et al.*, 1996
∗ *Cunnighamella* sp.	Bradoo *et al.*, 1996
∗ *Syncephalastrum racemosum*	Bradoo *et al.*, 1996
♠*Neurospora crassa*	Bradoo *et al.*, 1996
Yeasts	
Candida sp.	Aoki *et al.*, 1976
Pichia spp.	Deschamps & Lebeault, 1984
∗ *Debaryomyces hansenii*	Deschamps & Lebeault, 1984

1.2.2 Plant tannase and tannin formation and degradation

Many tannin–rich plant materials have been isolated that contain tannase activity, for example Myrobolan fruits (*Terminalia chebula),* divi-divi pods (*Caesalpinia coriaria*) and from English oak (*Quercus robur*), Penduculate oak (*Quercus rubra*) and from the leaves of the Karee (*Rhus typhina*) tree (Niehaus & Gross 1997, Madhavakrishna *et al.,* 1960).

Cell free extracts from *Quercus robur, Quercus rubra and Rhus typhina* revealed the pronounced hydrolysis of the substrate β-glucogallin (1–O–galloyl-β-D glucopyranose) in *in vitro* assays. The esterase purified from the leaves of the Penduculate oak was shown to be an analogue to fungal tannase (Niehaus & Gross, 1997). It can be postulated that plant and microbial organisms have adapted a mechanism to overcome the degradative resistance of tannins and in return utilize them in their metabolism.

Why does tannase exist in the bark and leaves of plants and trees? Madhavakrishna *et al.,* (1960) suggested that upon growth, plants synthesize large amounts of gallic acid, chebulinic acid and hexahydroxyphenic acid, and as the plants produce fruit, the fruit ripens and it was envisioned that these acids might become esterified with glucose with the help of tannase to form complex tannins. Upon abscission of the fruit the esterase activity in the tannase may contribute to the hydrolysis of the preformed tannins.

Madhavakrishna *et al.,* (1960) also hypothesized that the condensed tannins are formed as intermediates or precursors that would later be transformed into complex tannin molecules. The tannin content in the plant material may also serve as a defence mechanism by which the plant may be able to protect itself against microbial invasion. They also suggested that tannase does not only protect the plant against microbial invasion, but also against attacks from herbivores. When the plant's leaves are under attack from herbivores the cells lose compartmentation, which brings the tannase into contact with the tannin substrate in the leaves. The substrate is then hydrolysed into harmful low molecular weight phenolic degradative compounds, which can be precursors for toxic substances in higher plants.

1.3 The physicochemical properties of tannase

1.3.1 pH optimum and pH stability

The optimum pH for tannase isolated from *A. niger* was shown to be between 5.0 and 6.0, with instability occurring at a pH above pH 6.0 (Iibuchi *et al.,* 1968). Barthomeuf *et al.,* (1994) confirmed that the tannase from *A. niger* contained both esterase and depsidase activity with the esterase and tannase activities peaking at a pH of 5.0. The stability was also good over a wide pH range between a pH of 3.5 and 8.0. Tannase isolated from the organism *Chryphonectria parasitica* had an optimum pH of 5.5 (Iibuchi *et al.,* 1968).

The plant tannase isolated from Penduculate oak was shown to be active over a wide pH range with an optimum of approximately 5.0 (Niehaus & Gross 1997). Good stability was maintained even if the enzyme was incubated for 24 hours at a pH of 5.0 (Madhavakrishna & Bose, 1962).

The tannase from *Candida* sp. K-1 showed an optimum activity at a pH value of 6.0. The investigation also revealed that the enzyme was stable over a wide pH range, from a pH of 3.5 to 7.5 (Aoki *et al.,* 1976).

The fungal tannase from *A. flavus* has also been characterised extensively and the authors showed that the enzyme could be preserved at a pH range of 5.0 and 5.5. A rapid decrease in activity occurred outside this pH range. An interesting observation was that on surface cultures the mycelial tannase activity peaked at a pH of 3 – 7 but in culture media the tannase activity was active between a pH of 4 – 7, here the activity increased with an increase in pH (Pourrat *et al.,* 1982, Yamada *et al.,* 1968).

Iibuchi *et al.,* 1968 purified a tannase enzyme from *A. oryzae.* The tannase was shown to be stable at a pH range of 3 – 7.5 for 12h, but at narrower pH range of 4.5 – 6.0 the stability was maintained for 25 hours. The authors concluded that the optimum pH for tannase from *A. oryzae* was pH of 5.5 (Iibuchi *et al.,* 1968).

Tannase from *Penicillium chrysogenum* showed broad pH dependence with optimum enzyme activity at a pH of 5.0 – 6.0, with the enzyme apparently stable at 16°C in a pH range of 4.0 to 6.5 (Rajakumar & Nandy, 1983).

1.3.2 Iso–electric focusing of tannase

Not many pI values has been reported for tannase. The iso-electric points reported to date are from the organism *Chryphonectria parasitica* with a pI value of 4.6 – 5.1 (Aoki *et al.,* 1976), and for *A. oryzae* tannase a pI value of near to pH 4.0 (Iibuchi *et al.,* 1968).

1.3.3 Optimum temperature and stability

The optimum temperature and stability values for tannase isolated from various organisms are shown in Table 1.3.

Table 1.3. The optimum temperature and stability of tannase.

Organism	**Optimum temperature**	**Temperature stability**	**Reference**
Fungal tannase			
A. flavus	50 – 60ºC	≤ 70°C	(Yamada *et al.,* 1968, Pourrat *et al.,* 1982)
A. oryzae	30-40°C	55°	(Beverini & Metche, 1990) (Iibuchi *et al.,* 1968)
A. niger	35°C	≤ 50°C	(Haslam & Tanner, 1970)
Penicillium chrysogenum	30-40°C	45°C	(Rajakumar & Nandy, 1983)
Chryphonectria parasitica	30°C	25 - 40°C	(Farias, *et al.,* 1992, Iibuchi *et al.* 1968)
Plant tannase			
Penduculate oak	35 and 40ºC	≤ 50ºC	(Niehaus & Gross, 1997)
Yeast tannase			
Candida sp. K-1	50ºC	≤ 50ºC	(Aoki *et al.,* 1976)

The optimum temperature for tannase ranged between 30-50°C, with a temperature stability ranging from as low as 0°C to as high as 80°C in the case of *A. oryzae.*

1.3.4 Molecular mass and carbohydrate content

The molecular weight of tannase was shown to vary from 186 000 Da to 300 000 Da as shown in Table 1.4. According to Hatamoto *et al.,* (1996) tannase from *A. oryzae* was shown to consist out of two subunits of 30 000 and 33 000 Da. They concluded that native tannase consisted out of four pairs of the two subunits, forming a hetero–octamer with a molecular mass of about 300 000 Da. Tannase from *Candida* sp. K – 1 also consisted out of two subunits of 120 000 Da each that could be separated after treatment with SDS and 2–mercaptoethanol (Aoki *et al.,* 1976).

Table 1.4. Molecular weight and carbohydrate content of tannase.

Organism	Molecular weight (Da)	Carbohydrate content (%)	Reference
A. flavus	192 000	25.4%	Yamada *et al.,* 1968, Adachi *et al.,* 1971
A. niger	186 000	43%	Barthomeuf *et al.,* 1994, Parthasarathy & Bose, 1976.
A. oryzae	300 000	22.7%	Hatamoto *et al.,* 1996, Abdel-Naby *et al.,* 1999
Candida sp. K-1	250 000	61.9%	Aoki *et al.,* 1976
Chryphonectria parasitica	240 000	64%	Aoki *et al.,* 1976
Penduculate oak	300 000	NA	Niehaus *et al.,* 1997

Niehaus *et al.,* (1997) reported that the tannase from the Penduculate oak exhibited 2 protein bands that contained esterase activity. After denaturation on SDS – PAGE they observed only one polypeptide band of molecular mass of 75 000 Da, which led the authors to the conclusion that the native enzyme consisted preferentially as a tetramer of apparently four identical subunits in slightly acidic medium, while dissociating partially or completely into still active dimers under more alkaline conditions (Madhavakrishna & Bose, 1962).

All fungal tannases reported are glycoproteins, primarily consisting of neutral sugars like mannose, galactose and hexosamines (Aoki *et al.,* 1976, Piater, 1999) (Table 1.4.). The polypeptide moiety for tannase was shown to be very small varying

between organisms, for example the tannase from *A. flavus* consisted of 12.5% nitrogen in contrast to tannase from *Candida* sp. K-1 consisting of 38% protein.
The biological significance of such high carbohydrate content is of yet unknown, however it is strongly suggested that the carbohydrate moiety protects the carboxyl groups of the protein peptide bonds against hydrogen bond formation due to the large amount of phenolic hydroxyl groups present in the substrate for tannase (Lekha & Lonsane, 1994).

1.4 The specificity of tannase

1.4.1 The mode of hydrolytic action

It is known that tannase hydrolyses the ester bonds of tannic acid although tannic acid is known to denature proteins. According to research done by Iibuchi *et al.*, 1972, tannase was shown to hydrolyse tannic acid (Figure 1.3. (I)) completely to gallic acid and glucose through 2,3,4,6, -tetragalloyl glucose (Figure 1.3. (III)) and two kinds of monogalloyl glucose (Figure 1.3. (IV)). This is supported by the facts that the same products were detected in the hydrolysate of 1,2,3,4,6, -pentagalloyl glucose, and that depsidic gallic acid of methyl–*m*–digallate was liberated first. Where R_1 and R_2 are gallate and digallate respectively.

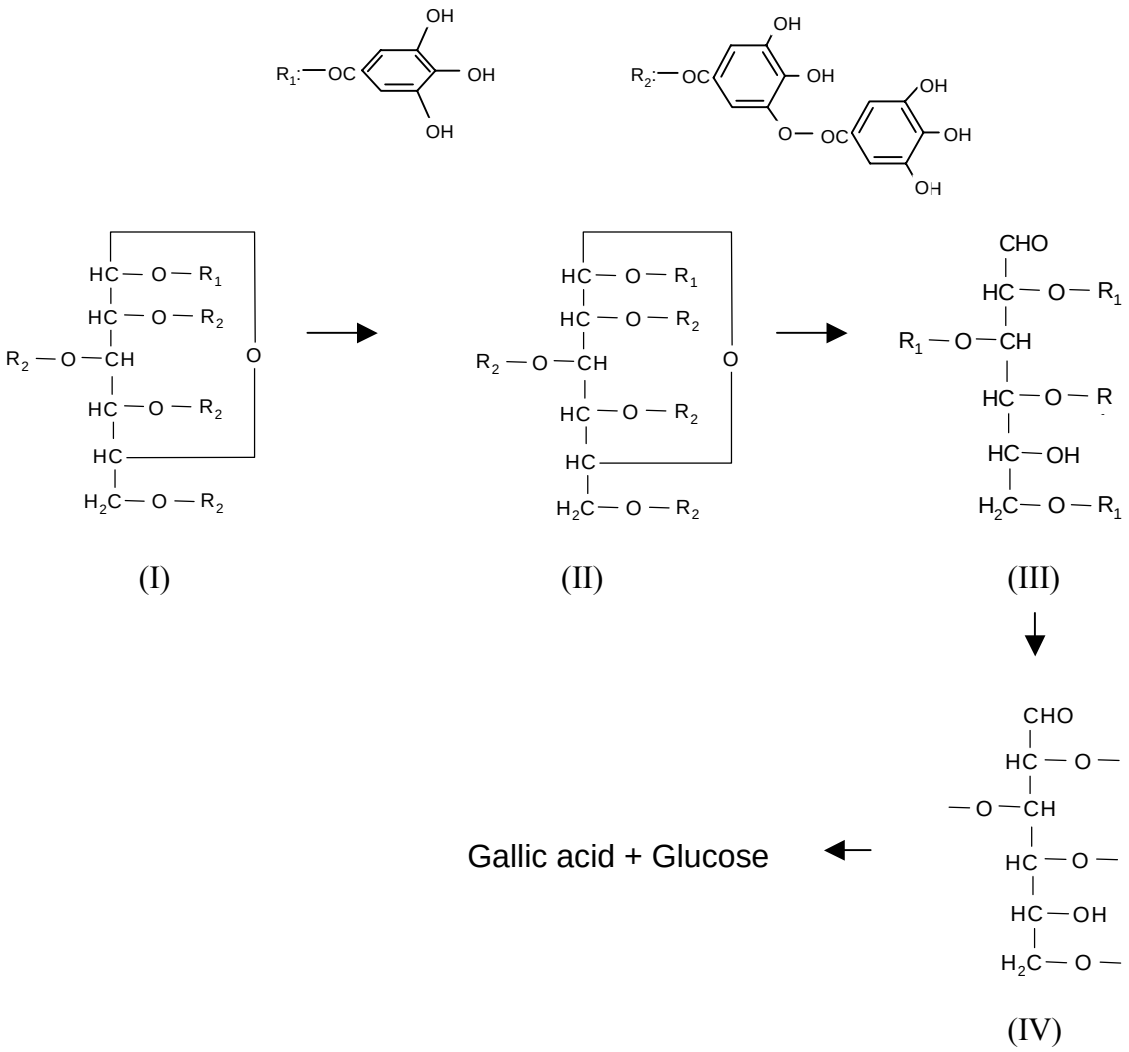


Figure 1.3. Hydrolysis pathway of tannic acid by tannase.

In affect this meant that the enzyme would react with any phenolic hydroxyl group, but for a true enzyme substrate complex to form the substrate had to be an ester compound of gallic acid (Iibuchi *et al.,* 1972).

For a true enzyme-substrate complex to form the following criteria had to be met:

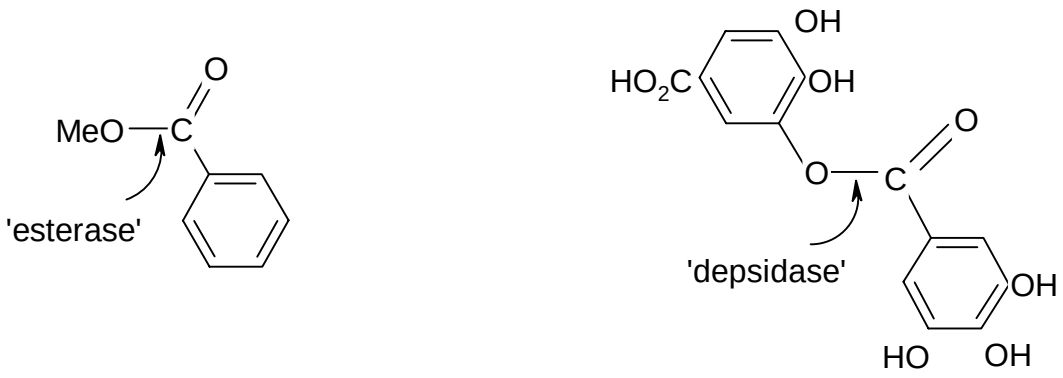
- There should be no restriction on the structure of an alcohol composing a substrate ester, although the acid should be gallic acid.
- Any phenolic hydroxyl might react with the binding site of the enzyme and prevent the enzyme from forming a true ES-complex.
- An ester bond or carboxyl does not link to the enzyme by itself, because an ester or carboxylic compound is not hydrolysed by or inhibits the enzyme unless it has phenolic hydroxyls (Iibuchi *et al.,* 1972).

Adachi *et al.,* (1971) proposed that tannase contained one essential serine molecule. From their data they detected that when tannase was incubated with ^{32}P – labelled phosphate, 1 mole of phosphate was incorporated into 1 mole of tannase to give complete enzyme inhibition. This suggested that tannase contained a single essential serine amino acid in its catalytic centre.

1.4.2 Kinetic parameters of tannase catalytic activity

Barthomeuf *et al.,* (1994) showed that the tannase enzyme from *A. niger* contained esterase activity that catalyses the hydrolysis of the galloyl esters that are attached to glucose moieties. Depsidase activity hydrolyses the depside linkages between two galloyl residues (Haslam & Tanner, 1966). Gallotannins are exclusively poly–O–galloyl–D glucose with varying complexity according to the plant source. In gallotannins a certain proportion of the galloyl groups are bound in the form of *m*–depsides. It is suggested that the depsidically linked gallolyl groups are not randomly distributed but that they form one polygalloyl chain of variable length linked to a carbohydrate nucleus at one specific position. Tannase was shown to contain two separate activities containing esterase and depsidase activities with specificity for methyl gallate (Figure 1.4, I) and m–digallic acid (Figure 1.4, II) ester linkages.

The tannase enzyme isolated from *A. niger* was subjected to a series of experiments in which it was possible to vary the ratio of esterase/depsidase activities of the enzyme; i.e. the activity against methyl gallate (Figure 1.4, I)/*m*–digallic acid (Figure 1.4, II) ester linkages (Haslam & Stangroom, 1966).



I Methyl gallate II *m* – digallic acid

Figure 1.4. A model showing the esterase and depsidase activities of tannase from *A. niger.*

The authors showed that when *A. niger* was grown on a depside-free media, in this case methyl gallate, a tannase was yielded with an increase in the esterase/depsidase ratio. This was in contrast with tannase yielded upon the growth of the organism on gallotannin media. They reported that each of these enzymes were capable of hydrolysing both esters and depsides of gallic acid (Figure 1.4, I and II) and that each enzyme had a relative specificity, one for esters and the other for depsides (Haslam & Stangroom, 1966).

The only two K_m values found in literature are from *A. flavus* and from *P. chrysogenum.* The values available for the two species are from different substrates, and not for the natural substrate on which the organism grows. Tannase from *P. chrysogenum* had a K_m value of 0.48 X 10^{-4} M for tannic acid as substrate (Rajakumar & Nandy, 1983). In the case of *A. flavus* the K_m values were 0.5 X 10^{-4} M for tannic acid as substrate, and 1.4 X 10^{-4} M for glucose–1–gallate (Yamada *et al.,* 1968).

1.5 Molecular aspects of tannase

Hatamoto *et al.*, (1996) cloned and sequenced the tannase gene from *A. oryzae* and reported that the gene sequence did not have any introns. They found that the gene code for a 588 amino acid sequence with an 18 amino acid signal sequence, and a molecular weight of approximately 64 000 Da. They hypothesized that their tannase consisted out of two subunits with molecular weights of 30 000 and 33 000 Da linked by a disulfide bond. The tannase gene was transcribed as a single polypeptide chain after which the 18 amino acid signal sequence was cleaved off and the polypeptide chain was cleaved into two subunits. It was reported that the single polypeptide chain was cleaved by a KEX-II like protease in two separate polypeptide chains. They concluded that native tannase consisted of four pairs of the two subunits, forming a hetero-octamer with a molecular weight of about 300 000 Da (Figure 1.5).

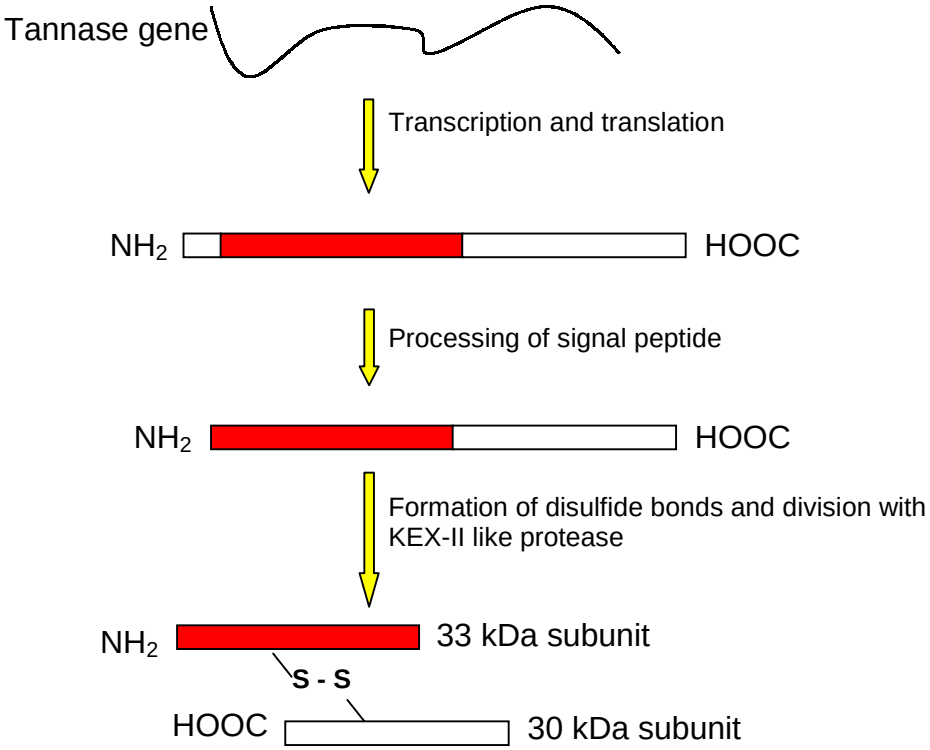


Figure 1.5. Proposed posttranslational modification of tannase precursor (Hatamoto et *al.*, 1996).

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1.6 Industrial uses of tannase

1.6.1 Cold tea products

In producing the above-mentioned beverages the most important factor is to produce a product with a high 'cold water solubility', which is a very large problem in the manufacturing of instant tea, as tea–cream is formed when the tea is stored at or below temperatures of 4°C (Powell *et al.,* 1993). This haze formation is due to the coacervation of tea flavonoids, consisting mainly of epicatechin, epicatechin gallate, epigallocatechin and epigallocatechin gallate. Tea polyphenols also form hydrogen bonds with caffeine, which leads to the cream formation. Consumers would prefer clear products, thus the compounds forming the haze must be removed in order to leave a product that is free of turbidity and chemicals used as clarifiers. Methods used to prepare cold water–soluble teas, thus preventing the haze formation, frequently affect the flavour quality of the beverage, tannase on the other hand has the catalytic activity to remove gallic acid moieties from tannins and the polyphenols from tea extract, resulting in cold water-soluble products. The reaction that follows is a deesterification between galloyl groups and various compounds in unconverted tealeaves (Figure 1.6).

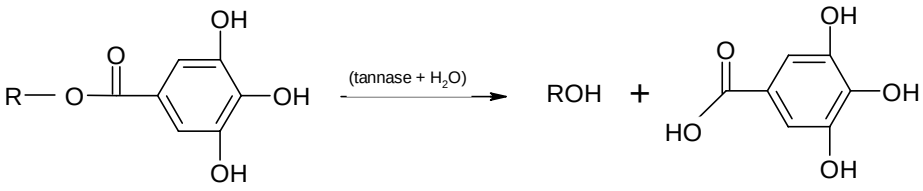


Figure 1.6. Deesterification of tea polyphenols by tannase. Where ROH is epicatechin or epigallocatechin.

The treatment of tea with tannase enhances the natural levels of epicatechin and gallic acid, which in turn favours the formation of epitheaflavic acid, which is responsible for the bright reddish colour of tea. This means that the treatment of tea products with tannase yields tea with a good cold-water solubility and colour.

1.6.2 Beer and wine production

A tannase from a certain strain of *A. flavus* has been shown to dramatically reduce the haze formation in beer after storage. This implicates tannase in the hydrolysis of wort phenolics which complex with the other chemicals in the beer mixture and results in the haze formation. Giovanelli, (1989) showed that upon treatment of the stored beer with tannase the potential of haze formation was dramatically reduced.

In the early days wine was treated chemically to remove the unfavoured phenolics. Now tannase is being employed to hydrolyse chlorogenic acid to caffeic acid and quinic acid, which influences the taste of the wine favourably (Chae *et al.,* 1983). Even fruit juices are being treated with a mixture of lactase and tannase to stabilize and clarify the product (Canterelli *et al.,* 1989).

1.6.3 Pharmaceutical industry

Gallic acid has been synthesized chemically, but this chemical synthesis has been known to be very expensive and not always very selective. Gallic acid is one of the products liberated upon hydrolysis of tannic acid with tannase (Iibuchi *et al.,* 1972). It is used as a synthetic intermediate for the production of pyrogallols and gallic acid esters. Today gallic acid is mainly used for the synthesis of trimethoprim, as well as for the production and synthesis of propyl gallate, which is used as an anti–oxidant in fats and oils (Weetal, 1985). Now by employing biotechnological means to synthesize gallic acid huge expenses can be saved with better and more selective yields (Deschamps & Lebeault, 1984).

1.7 Concluding remarks

Tannase has been shown to be a very versatile enzyme. The enzyme finds application in the food, beverage, industrial and pharmaceutical industry, however due to insufficient knowledge about the enzyme, the large-scale application of tannase is currently still limited. Tannase have been isolated from a wide range of organisms, from bacteria, fungi and yeasts (Lewis and Starkey, 1969, Deschamps *et al.,* 1983, Deschamps & Lebeault, 1984, Bradoo *et al.,* 1996, Yamada *et al.,* 1968, Bajpai & Patil 1997, Ganga *et al.,* 1977, Farias *et al.,* 1992, Hadi *et al.,* 1994). The tannase from all these organisms have been superficially characterized, however from literature it became evident that at gene level almost no knowledge exists on the tannase gene structure from the various organisms (Adachi *et al.,* 1971, Haslam & Tanner, 1966, Barthomeuf *et al.,* 1994, Haslam & Stangroom, 1966, Rajakumar & Nandy, 1983, Yamada *et al.,* 1968, Iibuchi *et al.,* 1968, Aoki *et al.,* 1976, Pourrat *et al.,* 1982, and Parthasarathy & Bose, 1976). The only available gene sequence for tannase was the cDNA sequence for tannase from *A. oryzae* (Hatamoto *et al.,* 1996). Elucidation of the gene sequence for tannase from various other organisms would help in understanding the structure function and relationship of tannase for its natural substrate, and the post transcriptional modification of the protein.

Chapter 2

Introduction to the present study

Teighem was the first researcher to report the formation of gallic acid when *P. glaucum* and *A. niger* were grown on tannic acid. Tannase, the responsible enzyme, hydrolyses the ester and depside bonds in hydrolysable tannins such as tannic acid to liberate gallic acid and glucose (Lekha & Lonsane, 1994). The enzyme is used in food and beverage processing, however the practical use of this enzyme is at present limited due to insufficient knowledge about its properties, optimal production and large-scale application.

Because tannins are present in plant material and thus in animal feed, the tannins can exert negative effects on the passage of nutrients through the gut wall of ruminants (Lekha & Lonsane, 1997). This is due to the complexation of tannins with the digestive enzymes as well as with the proteins on the outer cellular layer of the gut wall. By implication this complexation hinders the absorption of nutrients.

The high tannin content in plant material is associated with the resistance of plants to microbial invasion. The reason for this is due to the fact that for germination of spores and penetration of fungal hyphae the microorganism has to make use of extracellular enzymes. These extracellular enzymes are inactivated by the complexation that occurs with the tannins in the plant material (Lekha & Lonsane, 1997). However certain species of fungi, bacteria and yeasts have developed the ability to express tannase (Lewis and Starkey, 1969, Deschamps *et al.,* 1983, Deschamps & Lebeault, 1984, Skene & Brooker 1995, Bradoo *et al.,* 1996, Yamada *et al.,* 1968, Bajpai & Patil 1997, Ganga *et al.,* 1977, Farias *et al.,* 1992, Hadi *et al.,* 1994). In microorganisms tannase helps in invading plant material for infection as well as for the decomposition of plant organic matter.

Tannase has been shown to be an inducible enzyme, therefore tannase is only expressed in the presence of its substrate or a substrate analogue, such as tannic acid or its end product e.g. gallic acid (Haslam & Tanner, 1970). During submerged fermentation Rajkumar & Nandy, (1983) reported that tannase from most fungi and especially from *A. niger* was completely intracellular during the initial 48 hours of growth. Beverini (1990) reported that the enzyme was probably located between the cell wall and the plasmalamella but not membrane bound. Depending on the type of fermentation the tannase produced in solid-state fermentation could be completely extracellular (Lekha & Lonsane, 1993).

For many years work has been done on characterizing tannase enzymes from different organisms, but what was interesting was that up to date there was only one DNA sequence deposited for the tannase gene in the available databases. The only available DNA sequence for the tannase gene was the complimentary DNA sequence for tannase from *A. oryzae* (GenBank, accession number D63338) (Hatamoto *et al.,* 1996).

The outline of the present study became:

1. To investigate other *Aspergillus* spp. for tannase activity, to sequence the genes of positive isolates.
2. To use the knowledge available to express a tannase gene from one of the *Aspergillus* isolates in *Saccharomyces cerevisiae*, to characterize and compare the recombinant tannase with the published properties of the other tannases.

Chapter 3

Materials and Methods

3.1 Fungal and bacterial strains and plasmids used

Fungal, bacterial strains and plasmids used in this study are listed in Table 3.1.

Table 3.1. Fungal and bacterial strains and plasmids

Strains or plasmids	**Relevant characteristics**	**Source or reference**
Strains		
Aspergillus alliaceus		Culture collection, UFS.
Aspergillus fumigatus		Culture collection, UFS.
Aspergillus niger		Culture collection, UFS.
Aspergillus oryzae		Culture collection, UFS.
Saccharomyces cerevisiae Σ1278b background	L5529, *MAT*α, *ura*3-52, *his3::hisG*.	Gimeno & Fink, 1994.
Saccharomyces cerevisiae W303 – 1A		Gimeno & Fink, 1994.
E. coli SURE® 2 strain	$CaCl_2$ competent	Stratagene
Plasmids		
pGEM® – T Easy Vector (Figure 3.1, A.)	Contains the following sequence reference points: a) T7 RNA Polymerase transcription initiation site, b) SP6 RNA Polymerase transcription initiation site, c) T7 RNA Polymerase promoter, d) SP6 RNA Polymerase promoter, e) multiple cloning site, f) *Lac* Z start codon, operon sequence and operator, g) β-lactamase coding region, h) phage f1 region.	Promega
pRS426 ATCC Number: 77107 (Figure 3.1, B.)	Multi–copy YE-type (episomal) shuttle vector. Contains Amp^R and *URA*3 markers. It also contains an f1 replicon and *Lac* Z start codon, operon sequence and operator.	Christianson *et al*., 1992

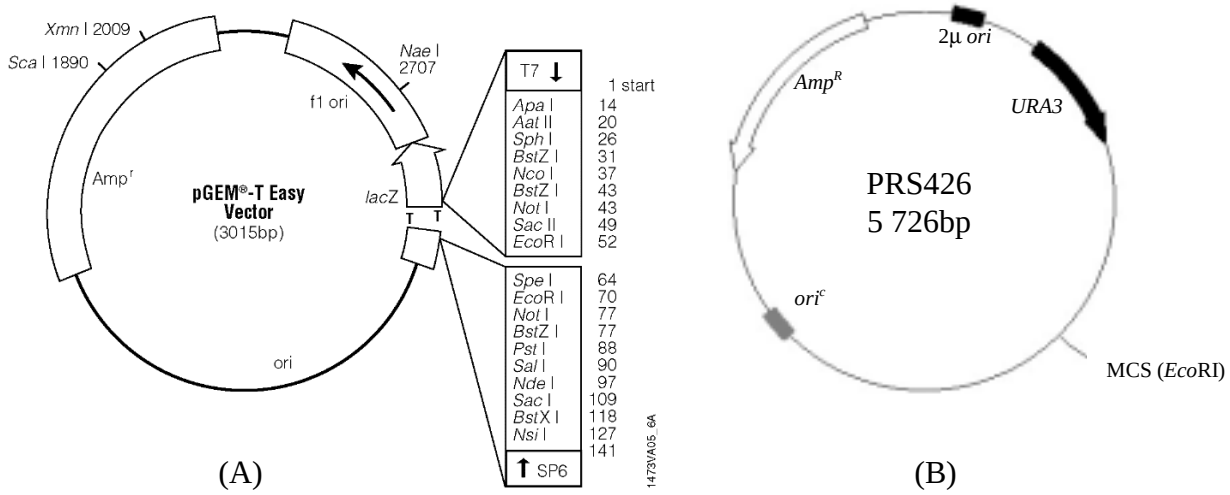


Figure 3.1. Schematic representation of the plasmid (A) pGEM®–T Easy and (B) pRS426.

3.2 Enzymes, Chemicals and Kits

The restriction enzymes used in this study (*Eco*RI, *Bam*HI, *Xba*I, *Hind*III, *Pst*I and *Acc*65I), Taq DNA Polymerase, Expand Long template Taq DNA Polymerase, modifying enzymes (Klenow and Alkaline phosphatase) and the DIG labeling and detection kit were obtained from Roche Molecular Chemicals. Ligations were performed using T4 DNA Ligase and pGEM®–T easy from Promega.

All chemicals were of analytical or molecular biology grade and were used without further purification. All chemicals were obtained from Merck or Sigma unless stated otherwise. The chemicals were sodium chloride ($NaCl_2$), Sodium dodecyl sulfate (SDS), two-amino-2- (hydroxymethyl)-1,3-propanediol (Tris), ethylenediaminetetraacetic acid (EDTA), Tri-Sodium Citrate ($Na_3C_6H_3O_7.2H_2O$) and Maleic Acid, 2-Mercaptoethanol, Tween 20®, Uracil and Ampicillin. Positively charged nylon membranes (Magnacharge) were from Osmonics INC. The agarose used for the electrophoresis of DNA was of molecular biology grade.

3.3 Cultivation and enzyme induction

All fungal strains (*Aspergillus alliaceus, Aspergillus fumigatus, Aspergillus niger* and *Aspergillus oryzae)* were cultivated on malt extract agar (MEA) or in malt extract broth (MEB). One litre of MEA contained malt extract (15 g) (Biolab, Merck) and agar (12 g) (Biolab, Merck). The malt extract broth contained the same ingredients, but no agar was added. The fungal pre-inoculum was prepared by inoculating fungal spores grown on MEA plates in MEB (80 ml) in a 250 ml Erlenmeyer flask. The pre-inoculum was grown at 30°C on a rotary shaker at 160 oscillations per minute, for 24 hours. The pre-inoculum (80 ml) was then transferred to malt extract broth (250 ml) in a one litre Erlenmeyer flask and cultivated at 30°C on a rotary shaker (160 oscillations per minute) for 5-7 days.

For tannase production the pre-inoculum was inoculated in Czapek Dox's minimal medium (0.035 M $NaNO_3$, 0.0067 M KCl, 0.002 M $MgSO_4$, 0.000036 M $Fe(II)SO_4$ and 0.0057 M K_2HPO_4). All the above mentioned chemicals were dissolved in 0.01 M phosphate buffer (pH 6.0) and autoclaved. For induction of the tannase 1% tannic acid was added to the medium. The tannic acid was filter sterilized with a 0.22 μm filter (Osmonics INC.).

A tannase plate assay was used to screen the fungal cultures for tannase production. Screening was performed by point inoculation of fungal spores on Czapek Dox's minimal medium, with tannic acid as the sole carbon source. The plates contained 3% agar and 0.5% Quinine Hydrochloride and were incubated at 30°C for one to two weeks (Bradoo *et al.*, 1996).

Esherichia coli was grown on LB media containing tryptone (10 g/L), NaCl (5 g/L), yeast extract (5 g/L) and 0.001 M NaOH.

Saccharomyces cerevisiae was cultivated on yeast nitrogen base with amino acids (YNB), glucose and uracil. One liter contained YNB (6.7 g/L) with amino acids, glucose (20 g/L) and uracil (0.014 g/L) at pH 6.2.

3.4 Recombinant DNA techniques

3.4.1 Fungal Genomic DNA isolation

Genomic DNA was isolated from *A. alliaceus, A. fumigatus, A. niger* and *A. oryzae*. A 5-7 day old culture (section 3.3) was harvested by means of filtration through Whatman® No. 3 filter paper. The harvested mycelia was washed with distilled water and again filtered to remove residual media. The mycelia were then ground with a -20°C pre-cooled mortar and pestle with liquid nitrogen until a fine powder remained. Ground mycelia (2.5 g) was resuspended in extraction buffer (12.5 ml) consisting of 200 mM Tris-HCl (pH 8.5), 250 mM NaCl, 25 mM EDTA and 0.5% SDS, after which phenol (pH 7.9) (8.75 ml) preheated to 60°C was added followed by the addition of chloroform/isoamylalcohol [24:1 (v/v)] (3.75 ml). The suspension was carefully inverted a few times. After centrifugation (18900 x g) in a Beckman J2 - 21 centrifuge for 60 minutes at 4°C the top liquid phase was removed containing the DNA. To remove excess RNA from the liquid phase 500 µl (5 mg/ml) RNase H was added and incubated for 15–20 minutes at 37°C. One part phenol was added to the mixture after incubation with the RNase and the mixture was again centrifuged (18900 x g for 20 minutes) at 4°C. The liquid phase was removed and the DNA was precipitated with 0.54 volumes of isopropanol. The mixture was centrifuged (18900 x g for 15 minutes) at 4°C and the resulting pellet was washed with 70% (v/v) ethanol. The sample was centrifuged (18900 x g for 2 minutes) at 4°C after which the ethanol was aspirated and the pellet was dried under vacuum. The pellet containing the isolated DNA was then dissolved in 1 ml TE buffer [10 mM Tris (pH 7.8) and 1 mM EDTA] and stored at -20°C for further manipulation.

3.4.2 Primers for amplifying and sequencing of the tannase gene from A. niger

The only available sequence for tannase from *Aspergillus* sp. was the cDNA sequence of *A. oryzae*. The sequence was obtained from GenBank (accession number D63338). From this sequence four upstream and four downstream primers were designed for the amplification of the tannase gene from *A. niger* (Table 3.2).

Table 3.2. Designed forward and reverse primers for the amplification of the tannase gene from *A. niger*.

Primer name:	**Forward primers**
OTP 1 F	5' – ATg CgC CAA CAC TCg CgC – 3'
TTH 1 F	5' – CgA CTA CgA gAA CCg TTT CTA CgT TgC Tgg – 3'
TAH 1 F	5' – gTg Agg AgT ATg ACg gTg CgA TTA CTg gTg – 3'
TAN 1 F	5' – gAA CTA gCA CTT CgC TTg gTT TCg gCT TC – 3'
	Reverse primers
OTP 2 R	5' – CTA gTA TAC Agg gAC CTT gAA ggC Tgg g – 3'
TTH 2 R	5' – gCA gCA CAg TAg TAA ggC TCA CCg ATg ATA gAg – 3'
TAH 2 R	5' – TCg Agg TTC Agg AgC TgA ATg AAC TTg gTg – 3'
TAN 2 R	5' – CgA CCC AgT CgA TCA TAA TCT CCA TgT TgT TC – 3'

A PCR was performed on the isolated genomic DNA from *A. niger* to amplify the tannase gene. The PCR was performed with the primer pairs OTP 1 F, OTP 2 R or TTH 1 F, TTH 2 R or TAH 1 F, TAH 2 R or with TAN 1 F and TAN 2 R as well as combinations of the forward and reverse primers. The PCR mixture contained 1µl of isolated genomic DNA (250 ng) from *A. niger*, 1µl of each primer (100 pmol/µl), 2µl 10 mM deoxyribonucleosidetriphoshate, 5 µl 10X PCR buffer containing no $MgCl_2$, 8 µl 25 mM $MgCl_2$ and 1 µl of 5 U/µl *Taq* DNA Polymerase. The final $MgCl_2$ concentration in the total reaction volume of 50 µl was 4 mM. The PCR reaction was conducted with the initial denaturation of the template DNA at 95°C for 5 minutes followed by 5 cycles of

denaturation at 94°C for 60 seconds, annealing at 58°C for 30 seconds and elongation at 72°C for 45 seconds. These 5 cycles was then followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds and elongation at 72°C for 90 seconds followed by an extended final elongation step at 72°C for 15 minutes. Sequence specific primers were designed from the obtained sequence for the tannase gene from *A. niger* to aid in sequencing and cloning of the gene (Table 3.3).

Table 3.3. Sequence specific primers for sequencing of the tannase gene from *A. niger*.

Primer name	
INP 1	5' – CAT Tgg AAA Cag TgC AgA gAT Cgg ACA g – 3' (Inverse PCR primer)
INP 2	5' – CgT AgC TgT Agg AgA Agg CgT CgT Ag – 3' (Inverse PCR primer)
FNP 1	5' – CgA CCC AgT CgA TCA TAA TCT CCA TgT TgT TC – 3'
FNP 2	5' – CgA CCC AgT CgA TCA TAA TCT CCA TgT TgT TC – 3'

3.4.3 Cloning and sequencing of the tannase gene fragments from A. niger

All PCR products and fragments were electrophoresed in a 1% (w/v) agarose gel containing 2.5 mg/µl ethidium bromide. The agarose gels were prepared and electrophoresed in TAE – buffer [0.1 M Tris, 0.05 M Na_2EDTA (pH 8.0) and 0.1 mM glacial acetic acid]. The gel electrophoresis was conducted for 1 hour at 86V. DNA bands were visualized under a low radiation UV light. The desired DNA fragments were excised from the gel and the DNA was eluted from the gel slice by using the NucleoSpin® DNA purification Kit obtained from Macherey-Nagel, according to the manufacturer's specifications.

The isolated PCR products were cloned into the pGEM®-T Easy vector system II (Promega) according to the manufacturer's specifications. The ligated plasmids were transformed into $CaCl_2$ competent cells (*E. coli* SURE 2) (Tang *et al.*, 1994, Inoue *et al.*, 1990). The transformed bacteria were selectively grown on LB agar plates containing 50 µg/ml ampicillin, 10 µg/ml isopropyl-1-thio-galactoside (IPTG) and 40 µg/ml 5-bromo-

4-chloro-3-indolyl-β-D-galactoside (X-gal) at 37°C for 16 hours. Colonies containing plasmids with inserts were identified by blue/white colony selection. Isolated colonies were grown in 5 ml LB containing 50 μg/ml ampicillin at 37°C for 16 hours after which the plasmid DNA was isolated from the bacterial cells by using the alkaline lysis plasmid isolation procedure (Maniatis *et al.* 1989). The isolated plasmid DNA was then analysed by restriction analysis and sequencing.

pGEM-T® Easy vectors containing the desired inserted DNA fragments as well as PCR products were sequenced with either primers, Sp6, T7 or sequence specific primers. Sequencing was performed using the ABI Prism BigDye™ Terminator V3.0 Cycle Sequencing Kit from Applied Biosystems. The sequencing reactions were analyzed on an ABI PRISM™ 377 Automatic DNA sequencer. The DNA sequences were analyzed by using the ABI PRISM™ 377 Automatic DNA sequencer software provided by Perkin Elmer.

3.4.4 Inverse-PCR amplification of the flanking regions of the tannase gene of A. niger

Genomic DNA (5 μg) from *A. niger* was digested with *Hind*III according to the manufacturer's specifications. The digested DNA was electrophoresed on 1% (w/v) agarose gel containing TAE buffer. Appropriate fragments were excised from the gel and the DNA was eluted using the NucleoSpin column Kit. For circularisation 0.1 μg of the isolated restriction fragment was ligated with T4 DNA ligase for 16 hours at 12°C. The inverse PCR was performed in reactions containing 0.1 μg of circularised DNA and 50 pmol of each inverse primer INP 1 and FNP 2 (Table 3.3) (Ochman *et al.*, 1988).

The inverse PCR reaction was conducted with the initial denaturation of the template DNA at 95°C for 5 minutes followed by 30 cycles of denaturation at 94°C for 60 seconds, annealing at 55°C for 30 seconds and elongation at 72°C for 6 minutes followed by an extended final elongation step at 72°C for 15 minutes. The resultant PCR products

were fractionated on a 1% (w/v) agarose gel in TAE buffer. The resultant positive PCR product was excised from the gel isolated and sequenced with the sequence specific primers INP 1 and FNP 2 (Table 3.3).

3.4.5 PCR amplification of the tannase gene of A. oryzae

A PCR was performed on the isolated genomic DNA from *A. oryzae* to amplify the tannase gene. The PCR was performed with primer pair OTP 1 F and OTP 2 R (Table 3.2). The PCR mixture contained 1 µl (250 ng) of isolated genomic DNA from *A. oryzae*, 1 µl of each primer (100 pmol/µl), 2 µl 10 mM deoxyribonucleosidetriphoshate, 5 µl 10X PCR buffer containing no $MgCl_2$, 8 µl 25 mM $MgCl_2$ and 1 µl of 5 U/µl *Taq* DNA Polymerase. The final $MgCl_2$ concentration in a total reaction volume of 50 µl was 4 mM. The PCR reaction was conducted with the initial denaturation of the template DNA at 95°C for 5 min followed by 5 cycles of denaturation at 94°C for 60 seconds, annealing at 58°C for 30 seconds and elongation at 72°C for 45 seconds. These 5 cycles was then followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds and elongation at 72°C for 90 seconds followed by an extended final elongation step at 72°C for 15 minutes.

3.5 Southern hybridisations

A PCR was performed on the genomic DNA isolated from *A. oryzae* with the primers OTP 1 F and OTP 2 R (Table 3.2). The PCR fragment contained the complete coding region of the tannase gene from *A. oryzae*. This fragment designated *ory*TAH was labeled as a DIG-probe for hybridisation studies on the genomes of the remaining fungal species. The PCR product was random prime labeled with Klenow enzyme according to the manufacturer's specifications of the DIG DNA labeling and Detection Kit. The probe was dissolved in 10 ml hybridisation buffer [0.6 M NaCl, 0.06M tri- sodium citrate, 0.1% (w/v) N-Lauroyl Sarkosyn, 0.02% (w/v) SDS and blocking solution (1%(w/v) blocking

reagent in maleic acid buffer (0.1M maleic acid and 0.15M NaCl; pH 7.5)] to a final concentration of 25 ng/ml.

Isolated genomic DNA (3 μg) from *A. alliaceus*, *A. fumigatus* and *A. niger* was digested with *Bam*HI, *Xba*I, *Hind*III or *Pst*I and 3 μg of genomic DNA from *A. oryzae* was digested with *Eco*RI to serve as a control. Restriction digestion was allowed to proceed for 16 hours at 37°C according to the manufacturer's specifications. The digested genomic DNA was electrophoresed on a 1% TAE agarose gel (w/v) for 2 hours at 86V. The separated DNA in the Agarose gel was subsequently nicked in a GS Gene Linker™ UV Chamber (Bio-Rad). The DNA was then transferred to a positively charged nylon membrane (Magnacharge, Osmonics INC.) according to the method as described by Southern, (1975).

After transfer of the digested DNA to the nylon membrane the DNA was cross-linked to the membrane by use of a GS Gene Linker™ UV Chamber (Bio-Rad). The membrane was placed in a roller tube and 15 ml of standard hybridisation buffer was added. The roller tube was placed in a hybridiser (Techne Hybridiser HB1) and pre-hybridised at 67ºC for 45 min. To denature the labeled *ory*TAH probe, the probe was placed in a boiling water bath for 10 minutes after which it was rapidly cooled in an ice-water bath. After removal of the pre-hybridisation solution, 5 ml of hybridisation solution containing the denatured probe was added. Hybridisation was performed at 67ºC for 18 hours, after which the probe was removed and frozen at -20°C for later use. The membrane was washed twice in 2 X SSC, 0.1% SDS wash buffer for 5min. After this initial washing step the membrane was washed twice in 0.1 X SSC, 0.1% SDS wash buffer at 67ºC for 15 minutes. The membrane was then incubated in DIG washing buffer no. 1 (100 mM maleic acid, 150 mM NaCl; pH 7.5 and 0.3% (v/v) Tween® 20) for one minute at 25°C to remove any residual SDS. The membrane was subsequently blocked in blocking solution [10% (w/v) blocking reagent in maleic acid buffer (1 M maleic acid and 1.5 M NaCl; pH 7.5] for 60 minutes, after which the Anti-DIG AP conjugate was added [1 μl Anti-DIG AP conjugate (200 μl polyclonal sheep anti-digoxigenin, Fab fragments, conjugated to alkaline phosphatase, 750 units/ml) dissolved in 10 ml blocking solution]. Binding of the

antibody was allowed to proceed for 30 minutes at 25°C. After 30 minutes the antibody was removed and the membrane was washed two times for 15 minutes in DIG washing buffer no. 1 at 25°C to remove any unbound antibodies. Detection was achieved by dissolving 80 µl of NBT/BCIP (1.25 ml nitroblue tetrazolium salt, 75 mg/ml in dimethylformamide, 70% (v/v) and 0.9 ml 5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt, 50 mg/ml, in dimethylformamide) in 10 ml detection buffer (100 mM Tris-HCl, 100 mM NaCl; pH 9.5). The membrane was incubated in the substrate for 16 hours in the dark or until bands of sufficient intensity was visible.

3.6 Colony hybridisations

After analysing the data obtained from the developed southern membranes, the restriction digestion of the genomic DNA from the four fungal species were repeated. After electrophoresis of the digested DNA on a 1% (w/v) agarose gel containing TAE buffer, the appropriate DNA fragments were isolated from the gel. The gel isolated digested genomic DNA was ligated into 0.5 µg/µl pGEM®-T Easy plasmid cut with the same restriction enzyme as the isolated genomic DNA (*Hind*III or *Pst*I) and dephosphorylated with 1 U/µl alkaline phosphatase (Roche) to hinder self-ligation. The ligated plasmids containing the isolated genomic DNA fragments were transformed into $CaCl_2$ competent *E. coli* SURE 2 cells and selectively propagated for 16 hours at 37°C on LB agar containing 50 µg/ml ampicillin, IPTG and X-gal.

The colonies were blotted directly from the petri-dish onto a positively charged nylon membrane (Magnacharge, Osmonics INC.). After transfer of the colonies to the membrane it was placed on top of filter papers saturated with 10% SDS solution. The membrane was incubated in the SDS for 3 minutes after which it was transferred and incubated for 5 minutes on filter paper saturated with denaturing solution (0.5 N NaOH, 1.5 M NaCl). After denaturing the membrane was neutralized in neutralization solution (1.5 M NaCl, 0.5 M Tris-HCl pH 7.4) for 5 minutes, and equilibrated in 2X SSC solution. The nylon membrane was subsequently cross-linked (GS Gene Linker™ UV Chamber

[Bio-Rad]) and the probe hybridisation and detection procedure was carried out as explained in section 3.5. Chemo-luminescent detection was also performed on the hybridised nylon membranes according to the DIG DNA labelling and detection kit manual.

3.7 Tannase expression in *A. alliaceus, A. fumigatus, A. niger and A. oryzae*

3.7.1 Extraction of tannase produced by the four fungal cultures in liquid media

For the induction and expression of the tannase enzyme in the four fungal species, the cultures were grown in Czapek Dox's minimal liquid medium as described previously. After one to two weeks of growth, depending on the fungus used, the biomass was harvested by means of filtration of the liquid culture through Whatman® No. 3 filter paper and the biomass was washed with distilled H_2O to remove any residual media. The isolated biomass was then frozen in liquid nitrogen and ground to a fine powder in a cold mortar and pestle. The ground biomass was resuspended in 0.1 M sodium phosphate buffer pH 5.75. The suspended biomass was then ultrasonicated on ice with a Branson Sonifier Cell Disrupter Model B-30. Sonification was conducted for three pulsed cycles with one-minute intervals between cycles. Mycelial cell debris was removed by centrifugation at 18 900 x g in a Beckman J2 - 21 centrifuge for 30 minutes at 4°C. The supernatant was isolated and subjected to the tannase assay for measurement of tannase activity.

3.7.2 The tannase enzyme assay

The assay was based on the monitoring of the hydrolysis of the ester bond spectrophotometrically at 272nm at 30°C in a Beckman DU650 spectrophotometer. The substrate used for enzyme activity measurements was (-)-epigallocatechin-3-ol-gallate. The 0.0014 mM substrate was prepared by mixing 0.7 mg of (-)-epigallocatechin-3-ol-

gallate in 15 ml sodium phosphate buffer at a pH of 5.75. To 500 µl of the substrate, 10 µl of extracted tannase was added to start the reaction. The decrease in absorbance was measured over a time period of 15 minutes. To compensate for any spontaneous oxidation or hydrolysis of the substrate, a blank rate was also measured by monitoring the change in absorbance of the substrate without the enzyme, over the same time interval. A change in absorbance of 0.01 absorbance units per minute under these conditions was defined as one unit of tannase activity.

3.8 Expression of the recombinant tannase enzyme in *S. cerevisiae*

3.8.1 Amplification of the PDC1 promoter from S. cerevisiae

The glucose induced *PDC1* promoter region was PCR amplified from genomic DNA of *S. cerevisiae* W303-1A using the primer pair PDC1-1F (5' – Tgg gAT CCg AAA gAA gAT CAA gCg AgT CCA – 3') and PDC1-1R (5' – ggA ATT CgA TTT gAC TgT gTT ATT TTg Cg – 3'). The primers that were used for the amplification of the *PDC1* promoter contained a *Bam*HI restriction site at the forward primer 5' side and an *Eco*RI restriction site at the reverse primer 5' side. This allow the introduction of a *Bam*HI and *Eco*RI restriction site at the 5' and 3'ends of the PCR product sequence to facilitate cloning (Hohmann, 1991).

The PCR was carried out under standard conditions, with an initial denaturation of the template DNA at 95°C for 5 min followed by 25 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds and elongation at 72°C for 45 seconds followed by an extended final elongation step at 72°C for 3 minutes.

3.8.2 Construction of the expression system for the tannase gene from A. oryzae in S. cerevisiae

The pRS426 multi-copy shuttle vector (0.5 μg) was digested with the restriction enzymes *Bam*HI and *Eco*RI at 37°C for 1 hour. The amplified *PDC1* promoter was ligated into *Eco*RI/*Bam*HI digested pRS426. After ligation the pRS426 plasmid containing the *PDC1* promoter in its multiple cloning site, was transformed into $CaCl_2$ competent *E. coli* SURE 2 cells (section 3.4.3). Transformants (6 colonies) containing the pRS426 plasmid with the inserted *PDC1* promoter were identified by plasmid isolation and restriction analysis with *Bam*HI and *Eco*RI.

The cloned tannase gene from *A. oryzae* in the vector pGEM-T® Easy (section 3.4.5) was subjected to restriction digestion with *Eco*RI liberating the inserted tannase gene from the pGEM-T® easy plasmid. The digested pGEM-T® Easy plasmids were loaded on a 1% (w/v) agarose gel and the DNA was electrophoresed at 86V for 1 hour. The tannase gene fragment was isolated from the agarose gel by using the NucleoSpin DNA extraction and purification kit.

The constructed pRS426 plasmid containing the *PDC1* promoter was subjected to restriction digestion with *Eco*RI and dephosphorylated by using alkaline phosphatase (Roche) (according to manufacturer's specifications) to hinder self-ligation of the plasmid. The *Eco*RI digested *A. oryzae* tannase gene fragment was then ligated into the dephosphorylated pRS426 plasmid. Ligation proceeded for 16 hours at 14°C. The ligated plasmids were then transformed into $CaCl_2$ competent *E. coli* SURE 2 cells and positive transformants containing the plasmid with the inserted ligated tannase gene were identified by plasmid isolation and restriction analysis with *Eco*RI. The expression multi copy shuttle vector was designated pRS426-*PDC1*-*ory*TAH.

For the tannase gene to be expressed the gene had to be ligated in the right orientation, with its start coding directly downstream to the *PDC1* promoter. To determine the orientation of the inserted tannase gene, restriction analysis was performed on the plasmid with restriction enzymes *Kpn*I, *Eco*RI and *Hind*III.

3.9 Transformation of the pRS426-*PDC1-ory*TAH plasmid into the expression host *S. cerevisiae*

Transformation of the yeast *S. cerevisiae* Σ1278b was performed according to the method described by Chen *et al.*, (1992). The transformants were selectively grown on 2% glucose YNB media containing amino acids without uracil. Cells able to grow on the media were isolated as positive transformants containing the transformed plasmid pRS426-*PDC1-ory*TAH.

3.10 Expression of the tannase gene from *A. oryzae* in *S. cerevisiae*

The transformed yeast cells were selectively grown on 2% glucose, YNB agar plates containing all amino acids without uracil. For expression studies, cells were grown in 500 ml YNB with amino acids and uracil substituted with 2% glucose, at 28°C on a rotary shaker set at 160 oscillations per minute for 21 hours. The cultures were harvested in late exponential phase by means of centrifugation at 15 000 x g in a Beckman J2 - 21 centrifuge for 20 minutes at 4°C. A tannase assay was performed to verify tannase activity in the isolated culture broth as described previously. To concentrate the expressed tannase enzyme the culture broth was filtered through an Amicon® ultra filtration membrane with a molecular mass cut-off of 30 000 Da. The 500 ml isolated culture broth was concentrated to 2 ml containing the recombinant expressed tannase enzyme and stored at 4°C.

3.11 Properties of the recombinant tannase expressed by *S. cerevisiae*

3.11.1 Optimum pH of the recombinant extracellularly expressed tannase

The optimum pH of the recombinant tannase enzyme was determined over a pH range of pH 3-8. The pH assessment was carried out in a Tris-Glycine buffer calibrated at the various pH values. Enzyme assays were performed (section 3.7.2) in duplicate at each pH value. The average activity was used to calculate the recombinant enzymes optimum pH.

3.11.2 Kinetics

A concentration range of 0.01-0.2 mM (-)-epigallocatechin-3-ol-gallate was used with the standard assay procedure as described in section 3.7.2. The kinetic constants were calculated by fitting the data directly to the Michaelis–Menten equation using non-linear regression with the GraphPad Prism® software version 3.0 (GraphPad software incorporated Copyright 1994 – 1999).

Chapter 4

Results and Discussion

4.1 Screening for tannase production by the fungal cultures

4.1.1 Screening for tannase production on solid media

A. alliaceus, A. fumigatus, A. niger and *A. oryzae* were screened for the production of tannase on solid media as described in section 3.3. Tannic acid was added to the media as the sole carbon source. After three days clear zones appeared around the tannase producing colonies. Zones formed due to hydrolysis of tannic acid to gallic acid and glucose (Bradoo *et al.,* 1996), leading to a decrease in opacity of the media. All the fungal cultures expressed tannase activity as illustrated in Figure 4.1.

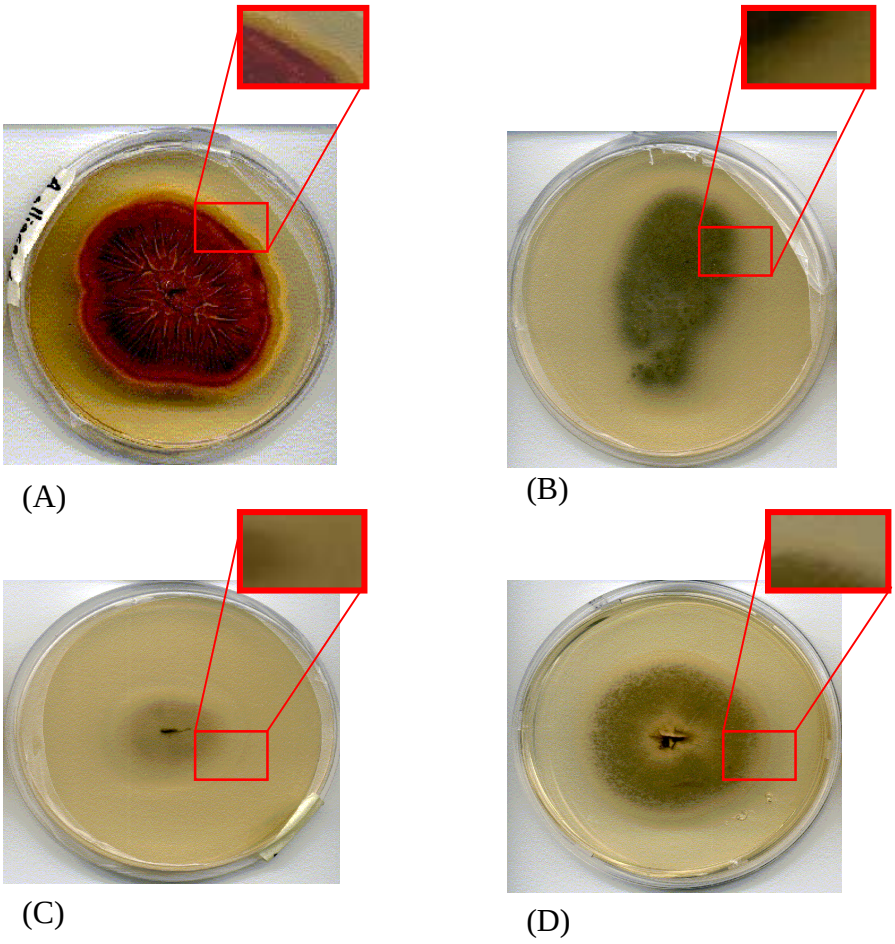


Figure 4.1. Agar plates showing the zones of clearance after expression of the tannase activity by the fungal cultures. (A) *A. alliaceus.* (B) *A. fumigatus.* (C) *A. niger.* (D) *A. oryzae.*

4.1.2 Fungal tannase production in liquid media

A. alliaceus, A. fumigatus, A. niger and *A. oryzae* were grown on media as described in section 3.3. The intracellular tannase was extracted and enzyme assays were performed on the tannase from each fungal strain. The enzyme activity was estimated as described previously (section 3.7.2). Table 4.1 represents the tannase activity obtained from the intracellular isolated tannase from each fungal culture.

Table 4.1. Total activity of the tannase isolated (intracellularly) from the four fungal species during growth on Czapek Dox's minimal medium containing 1% tannic acid as sole carbon source.

Specie	Total tannase activity (Units/g wet biomass)
A. alliaceus	0.17
A. fumigatus	1.04
A. niger	1.22
A. oryzae	1.03

From the screening of the fungal isolates on solid media and liquid media, it was evident that the tannase gene was resident in the genome of the chosen four fungal isolates and that the tannase gene was inducible in the presence of tannic acid as sole carbon source, with this positive evidence the search for the gene in the genomic DNA of the fungal species continued. The higher titres in production of tannase by *A. niger* correlated well with the values described by Lekha & Lonsane (1997) during solid-state fermentation. The high tannase activity produced by *A. fumigatus* and *A. oryzae* correlated well with findings by Bradoo *et al.,* (1996).

4.2 Fungal genomic DNA isolation

Fungal genomic DNA was isolated from the four fungal cultures as described in section 3.4.1. Figure 4.2 depicts the pure genomic DNA isolated from *A. alliaceus, A. fumigatus, A. niger* and *A. oryzae*.

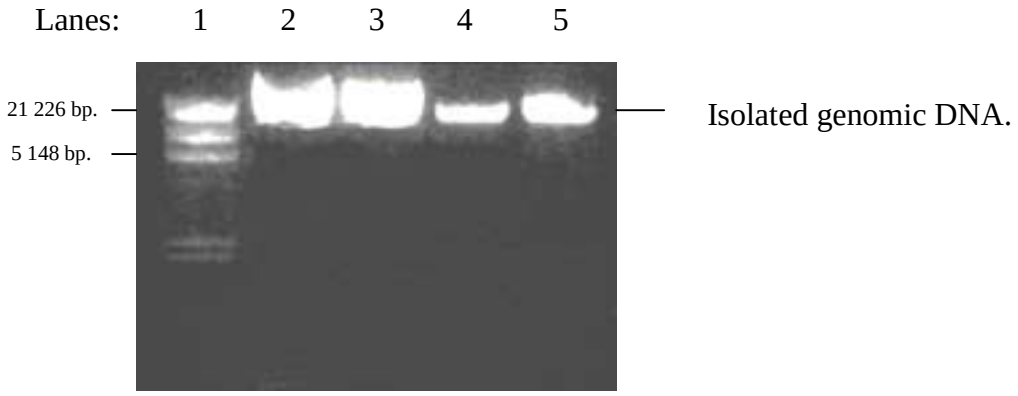


Figure 4.2. An ethidium bromide stained 1% agarose gel showing the isolated fungal genomic DNA. Lane 1, lambda III marker. Lane 2, genomic DNA isolated from *A. alliaceus.* Lane 3, genomic DNA isolated from *A. fumigatus.* Lane 4, genomic DNA isolated from *A. niger* and Lane 5, genomic DNA isolated from *A. oryzae.*

4.3 PCR amplification of the tannase gene from *A. oryzae*

The tannase gene of *A. oryzae* has a coding region of 1 767 nucleotides coding for a protein of 588 amino acids in length as well as an 18 amino acid signal sequence. This gene was cloned by designing two primers (OTP-1-F and OTP-2-R, Table 3.2) to amplify the full-length gene from the *A. oryzae* genomic DNA (Figure 4.3). The primers were designed from the complementary DNA (cDNA) sequence for tannase from *A. oryzae* (GenBank accession nr. D63338). The cDNA sequence for tannase has been compared to the genomic DNA sequence for this gene, and the results indicated that the gene contained no introns (Hatamoto *et al.*, 1996).

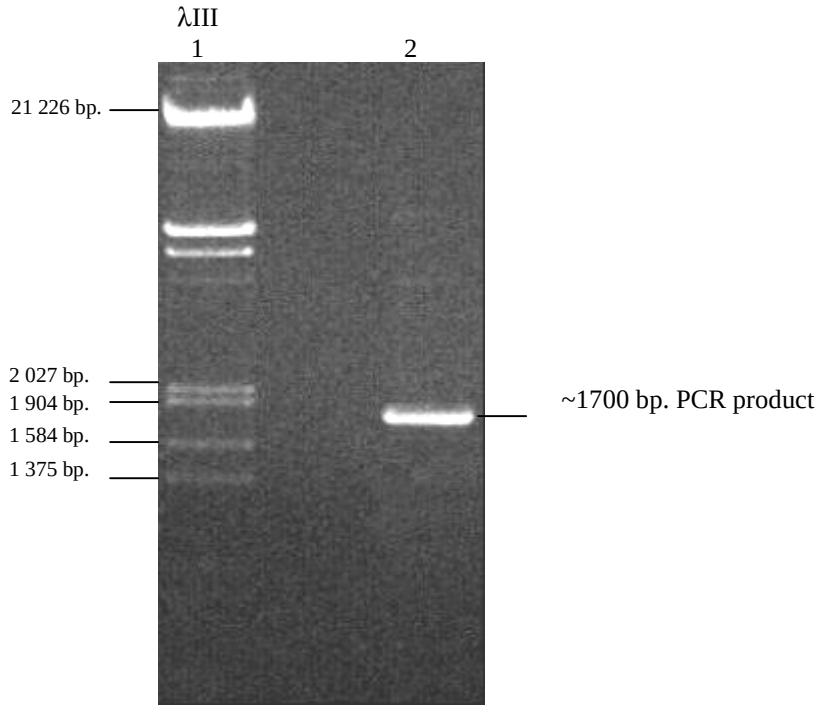


Figure 4.3. An ethidium bromide stained 1% agarose gel showing the 1 767 bp. PCR product. Lane 1, λIII marker DNA. Lane 2, represents the putative amplified tannase gene from the genome of *A. oryzae*.

Amplification of the genomic DNA yielded a 1.7 kb. fragment which correlated with the expected 1.7 kb. tannase gene sequence from *A. oryzae* (Hatamoto *et al.*, 1996). To verify that the PCR product was the tannase gene from *A. oryzae*, a restriction map was compiled according to the known sequence of this gene, showing the expected fragments upon restriction digest with *Hind*III, *Ava*I or *Asp*718 (Figure 4.4, A). From the known sequence for tannase a restriction analysis with the above enzymes would liberate fragments of the following sizes, [*Hind*III- two fragments 932 bp. and 835 bp., correlating with the restriction profile (Figure 4.4, C), *Ava*I- two fragments with the sizes 1 459 bp. and 308 bp. (Figure 4.4, B), and finally *Asp*718 three fragments with the sizes 1 470 bp., 149 and 148 bp. in size (Figure 4.4, D)]. All the expected fragments were obtained. The 1.7 kb. PCR product was cloned into the vector pGEM-T Easy and sequence analysis was performed using the primer T7, this primer had a binding site on the 5' end of the pGEM-T-Easy vector facilitating the sequencing of a

cloned product in the vector. A 336 bp. sequence obtained was aligned with the known sequence of *A. oryzae*. A 100% sequence identity at nucleotide level was obtained (Figure 4.5). Results obtained from both restriction (Figure 4.4.) and sequence analysis (Figure 4.5.) indicated that the amplified PCR product was indeed the tannase gene fragment from *A. oryzae*. The tannase gene fragment from *A. oryzae* was designated *oryTAH*.

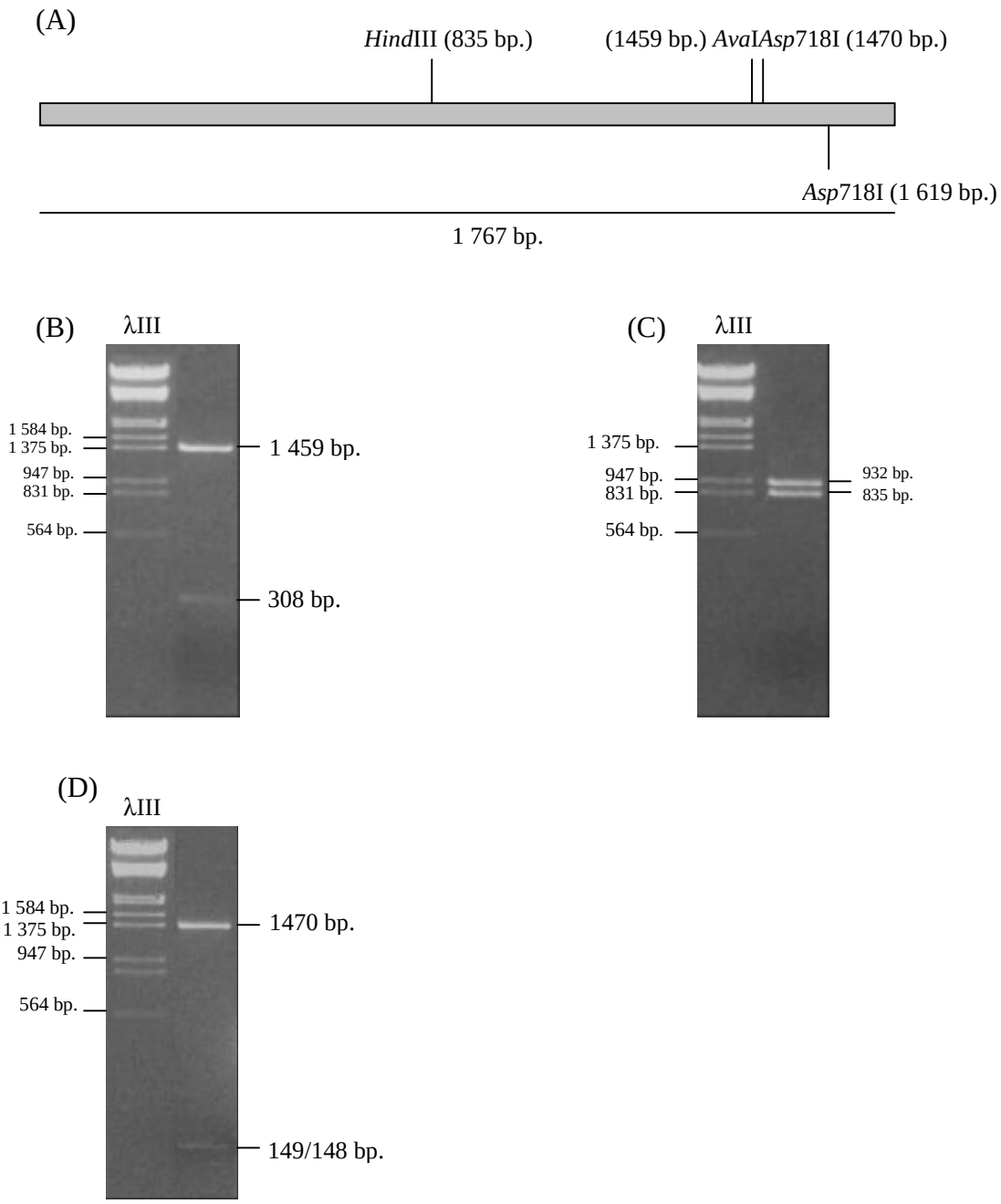


Figure 4.4. Ehidium bromide stained 1% agarose gels, showing the restriction analysis of the 1.767 kb. putative tannase PCR fragment.

(A) A schematic representation of the restriction map of the 1 767 bp. tannase gene fragment from *A. oryzae* with the restriction enzymes *Ava*I, *Hind*III and *Asp*718.

(B) An ethidium bromide stained 1% agarose gel showing the digested 1.7 kb. PCR product after digestion with *Ava*I. Lane 1, λIII marker DNA. Lane 2, shows the liberated fragments, 1 459 and 308 bp. in size.

(C) An ethidium bromide stained 1% agarose gel showing the digested 1.7 kb. PCR product after digestion with *Hind*III. Lane 1, λIII marker DNA. Lane 2, shows the liberated 2 fragments, 932 and 835 bp. in size.

(D) An ethidium bromide stained 1% agarose gel showing the digested 1.7 kb. PCR product after digestion with *Asp*718. Lane 1, λIII marker DNA. Lane 2, shows the liberated 3 fragments, 1 470, 149 and 148 bp. in size.

```
t7orytah  1   GTATACAGGGACCTTGAAGGCTGGGAACTCGTAGGTCCAGCTGTCA 46
oryzae    1   GTATACAGGGACCTTGAAGGCTGGGAACTCGTAGGTCCAGCTGTCA 46

t7orytah  47  ATCGACTTCTCGTCGTTGACACAGTCGAAGCTGGAGTTGCCGCGCC 92
oryzae    47  ATCGACTTCTCGTCGTTGACACAGTCGAAGCTGGAGTTGCCGCGCC 92

t7orytah  93  AGAGAGGACGCTTGGGCCACTGGCAAAGCATCTGGGTCTCGCCGGC 138
oryzae    93  AGAGAGGACGCTTGGGCCACTGGCAAAGCATCTGGGTCTCGCCGGC 138

t7orytah  139 GTAGGTACCCGAAGAAACAGTGGCATTGAGACGGGACGGCTTGTTG 184
oryzae    139 GTAGGTACCCGAAGAAACAGTGGCATTGAGACGGGACGGCTTGTTG 184

t7orytah  185 CCGTTCTCGACCCAGTCGATCATAATCTCCATGTTGTTCTCAGGGT 230
oryzae    185 CCGTTCTCGACCCAGTCGATCATAATCTCCATGTTGTTCTCAGGGT 230

t7orytah  231 AAGGTCCGGGCTGGAGAGAGTTGGTTCCGCAGTGGGCGGCACCGGG 276
oryzae    231 AAGGTCCGGGCTGGAGAGAGTTGGTTCCGCAGTGGGCGGCACCGGG 276

t7orytah  277 GATTAGGTAGAACTGGTACCAGTCCTCGAGAGCCTCCAGGGCCTCC 322
oryzae    277 GATTAGGTAGAACTGGTACCAGTCCTCGAGAGCCTCCAGGGCCTCC 322

t7orytah  323 TCTTCCGTCTTGTC 336
oryzae    323 TCTTCCGTCTTGTC 336
```

Figure 4.5. A partial sequence alignment between the known cDNA sequence for tannase from *A. oryzae* (oryzae) and the amplified 1 767 bp. PCR product from the genome of *A. oryzae* (t7orytah).

4.4 Construction of a mini-genomic DNA library

Southern blot analysis was performed to confirm that the tannase gene from *A. alliaceus, A. fumigatus* and *A. niger* were present in these fungi and that these tannase genes were homologous to the tannase gene from *A. oryzae*. The genomic DNA from the four different fungal isolates were isolated as described in section 3.4.1 and digested with the restriction enzyme *Eco*RI (Figure 4.6, A) and probed with the random primed labeled *oryTAH* PCR product. Southern blot analysis (Fig. 4.6, B) showed that the tannase labeled probe hybridised to certain fragments of digested DNA of the four fungal species. A single hybridisation band was visible on the digested genomic DNA of *A. oryzae* to which the *oryTAH* probe hybridised (Figure 4.6, B, lane 4). In contrast to this intense hybridisation, the intensity of hybridisation was very low for the genomic DNA of *A. alliaceus, A. fumigatus* and *A. niger* (Figure 4.6, B, lane 1, 2, 3). The hybridisation pattern suggested that the tannase gene sequence in *A. alliaceus* contained an *Eco*RI restriction site in the coding sequence of this gene, showing 2 hybridisation bands with the *oryTAH* gene probe (Fig. 4.6, B, lane 1). The same reasoning applied for *A. fumigatus* (Figure 4.6, B, lane 2). Only one hybridisation band was visible in *A. niger's* profile, suggesting no *Eco*RI restriction site in the tannase gene sequence from this fungal specie (Figure 4.6, B, lane 3).

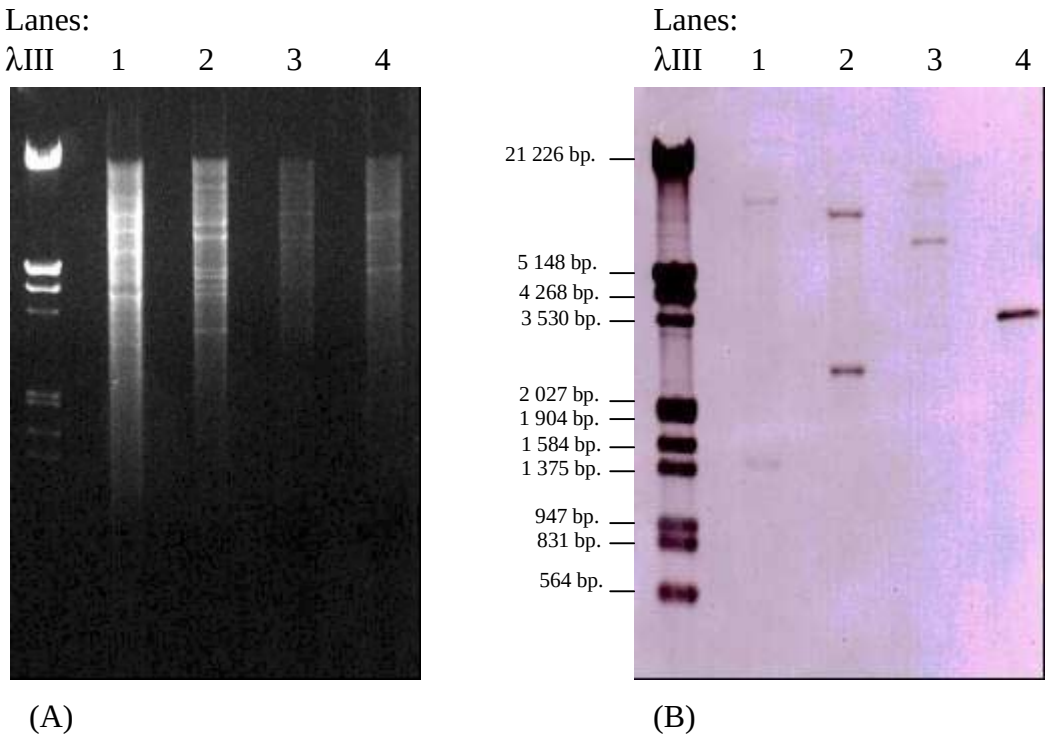
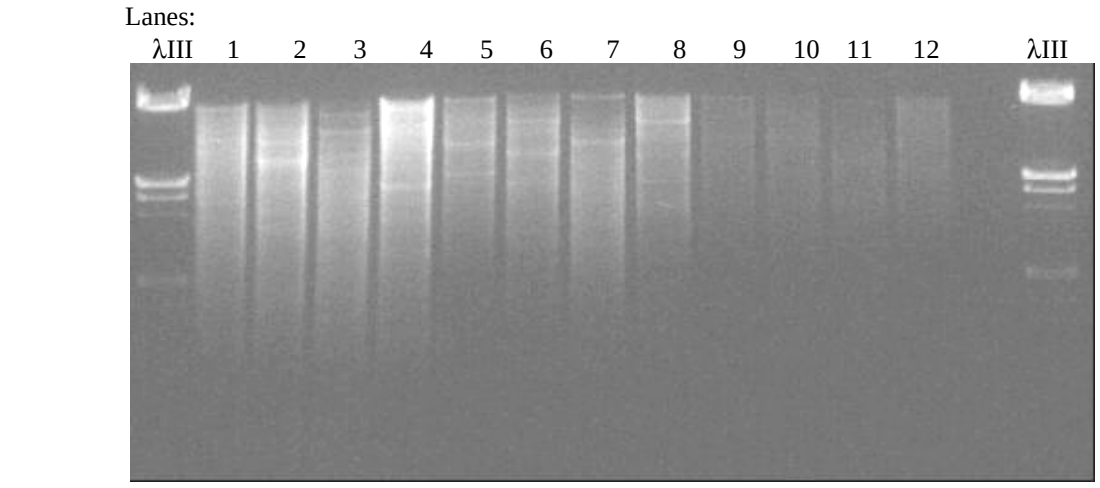


Figure 4.6. Southern hybridisation of the *Eco*RI digested fungal genomic DNA.

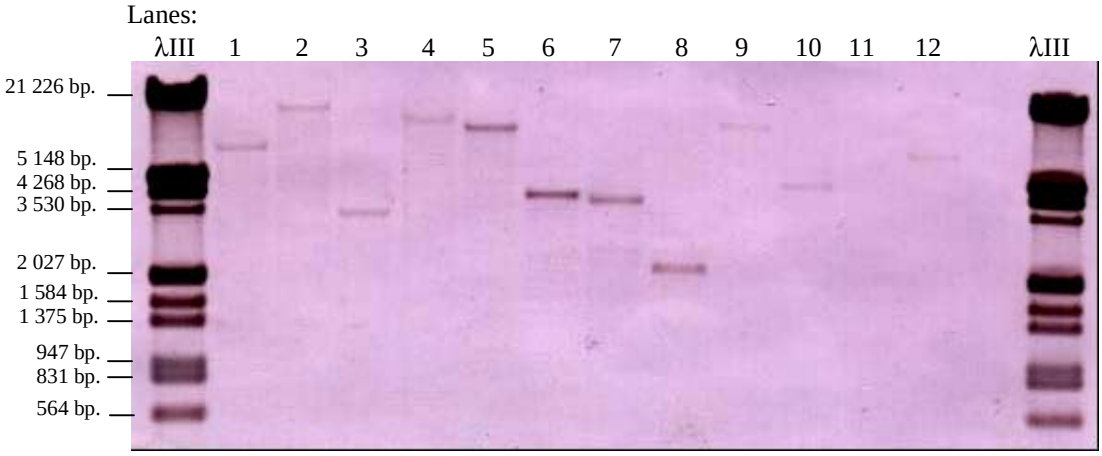
(A) An ethidium bromide stained 1% agarose gel showing the *Eco*RI digested fungal genomic DNA. Lane 1, digested genomic DNA from *A. alliaceus.* Lane 2, digested genomic DNA from *A. fumigatus.* Lane 3, digested genomic DNA from *A. niger.* Lane 4, digested genomic DNA from *A. oryzae.*

(B) A probed and developed Southern transfer membrane showing the hybridisation bands on the fungal *Eco*RI digested genomic DNA. Lane 1, *A. alliaceus.* Lane 2, *A. fumigatus.* Lane 3, *A. niger.* Lane 4, *A. oryzae.*

Southern blot analysis was performed on genomic DNA digested with different restriction enzymes to determine the size and hybridisation profile of the resultant fragments (Figure 4.7). This was necessary to find a restriction enzyme that does not have a restriction site in the coding region of the tannase genes from the three fungal species (*A. alliaceus, A. fumigatus* and *A. niger*). Due to the probable presence of an *Eco*RI restriction site in the tannase gene from *A. alliaceus* and *A fumigatus* four additional restriction enzymes assessed in digestion of the genomic DNA from the other *Aspergillus* spp. Genomic DNA from *A alliaceus*, *A fumigatus* and again from *A. niger* was digested with *Bam*HI, *Xba*I, *Hind*III or *Pst*I (Figure 4.7, A). After gel electrophoresis the digested genomic DNA was transferred and probed with the DIG labeled o*ryTAH* probe (Figure 4.7, B). Different sized hybridisation fragments were visible (Figure 4.7, B, lanes 1 - .12). This was due to the different restriction enzymes recognizing different restriction sites in the genomic DNA from the fungal cultures. The smaller less intense hybridisation fragments in Figure 4.7,B, lanes 7 and 8 can be explained by hypothesising that the restriction endonuclease most probably digested a small piece of the DNA upstream or downstream in the tannase gene from that organism, resulting in a fragment containing a piece of the tannase gene that was able to hybridise with the labeled probe. Only fragments of the same size or larger than the known tannase genes size were isolated. This was done to increase the probability for finding and cloning the entire coding region for tannase on one fragment. The digested DNA was electrophoresed exactly as in the experiment depicted in Figure 4.7, A, and the DNA was isolated from the gel area where the single band hybridised fragments were visible. Single band hybridised fragments were obtained with the digestion of *A. alliaceus* genomic DNA with *Pst*I (Figure 4.7, B, lane 3) and *Hind*III digestion of *A. fumigatus* and *A. niger's* genomic DNA (Figure 4.7, B, lane 6 and 10). The sizes of the fragments isolated were ~3.4 kb. in the case of *A. alliaceus* and ~4 kb. each in the case of *A. fumigatus* and *A. niger.* These isolated gel fragments were cloned into the plasmid pGEM® - T Easy and transformed into $CaCl_2$ competent *E. coli* cells. The reasoning being that the isolated, DNA would increase the probability of finding the correct hybridisation fragment containing the tannase gene.



(A)



(B)

Figure 4.7. Southern hybridisation of genomic DNA of *A. alliaceus, A. fumigatus* and *A. niger* digested with *Bam*HI, *Hind*III, *Pst*I and *Xba*I.

(A) An ethidium bromide stained 1% agarose gel showing the digested fungal genomic DNA. Lanes 1-4 contained digested genomic DNA from *A. alliaceus* digested with *Bam*HI, *Hind*III, *Pst*I and *Xba*I in each respective lane. Lanes 5-8 contained digested genomic DNA from *A. fumigatus,* digested with *Bam*HI, *Hind*III, *Pst*I and *Xba*I in each lane respectively. Lanes 9-12 contained digested genomic DNA from *A. niger* digested with *Bam*HI, *Hind*III, *Pst*I and *Xba*I in each lane respectively.

(B) The probed and NBT/BCIP colour developed southern transfer membrane, showing single band hybridised fragments in all the lanes.

4.4.1 Colony hybridisations

Colony hybridisations were performed to search for bacterial clones containing the cloned tannase genes, however problems were encountered which made it impossible to distinguish between positive and false positive clones.

4.5 PCR amplification of the tannase gene from *A. niger*

According to recent literature there are no reports of a cloned tannase gene for any other fungal or bacterial species other than the tannase gene from *A. oryzae*. It is this limited knowledge about tannase that motivated the cloning and search for other genes encoding tannase. Due to the problems encountered with the colony hybridisations and because *A. niger* displayed the highest tannase activity (Table 4.1) it was decided to focus only on the cloning of the tannase gene(s) from this fungus. The new approach to clone the gene was based on PCR.

The PCR was based on a set of PCR amplifications using primers designed on the known tannase DNA sequence from *A. oryzae* (Table 3.2). Figure 4.8 is a schematic representation of how the tannase gene from *A. niger* was cloned with a PCR approach. The PCR reactions were optimized at 250 ng of template DNA, and a $MgCl_2$ concentration of 4 mM. The PCR was performed with the primer pairs OTP 1 F (OTP 2 R), TTH 1F (TTH 2 R), TAH 1 F (TAH 2 R) and TAN 1 F (TAN 2 R) (Table 3.2.).

The PCR products that yielded bands with expected sized fragments were amplified using primers TTH 1 F, TTH 2R and TAN 1 F and TAN 2 R. The schematic representation in Figure 4.8, C, indicated that primers TTH 1 F and TTH 2 R should amplify a fragment of 600 bp. according to the known sequence of tannase from *A. oryzae*, from which the primer sequences were designed (Table 3.2). Figure 4.9, lane 3, showed the positive PCR product of ~600 bp. Primer pair TAN 1 F and TAN 2 R were expected to amplify a piece of the tannase gene consisting out of 684 bp. based on the known tannase gene sequence from *A. oryzae* (Figure 4.8, E). Upon conducting the experiment on the genomic DNA from *A. niger* an ~684 bp. PCR product was

amplified (Figure 4.9, lane 2). Unfortunately no PCR product was found when the primers TAH 1 F, TAH 2 R were used to amplify a 580 bp. central part of the tannase gene (Figure 4.8, D, Figure 4.9, lane 1). Attempts to amplify the coding region of the tannase gene from *A. niger* from the start to the stop codon with the primers OTP 1 F and OTP 2 R also failed (Figure 4.8, A and Figure 4.9, lane 8). Controls were also included in the PCR amplifications to ensure reproducible results with no false positive data due to possible contamination of the reagents (Figure 4.9, lane 4, 5, 6 and 7). The PCR products were cloned into pGEM®-T Easy and sequenced using the plasmids primers Sp6 and T7. The amplified products gave positive alignments with the known tannase gene sequence from *A. oryzae*. The data suggested that it was possible to clone the tannase gene from *A. niger* by means of amplifying the gene with PCR.

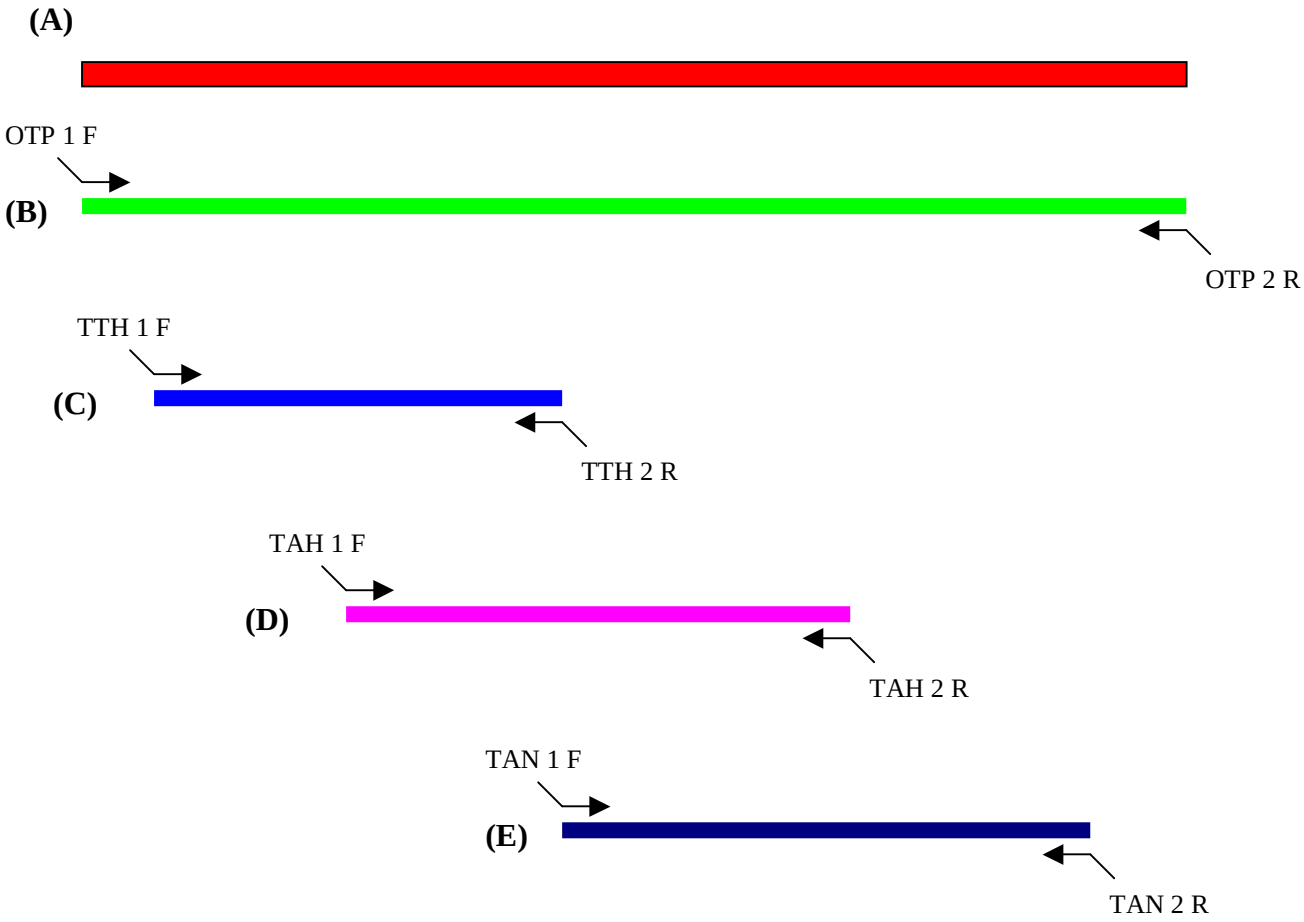


Figure 4.8. A schematic representation on the PCR amplification of the tannase gene from *A. niger* showing expected fragments.

(A) Represents the known 1 767 bp. ORF for tannase from *A. oryzae.*

(B) Represents the entire tannase gene that would be amplified if the primers OTP 1 F and OTP 2 R were able to anneal and amplify the tannase gene from *A. niger*.

(C) If the primers TTH 1 F and TTH 2 R were able to amplify the tannase gene from *A niger* a fragment of approximately 600 bp. was expected.

(D) If the primers TAH 1 F and TAH 2 R were able to amplify the tannase gene from *A. niger a* PCR fragment of approximately 580 bp. was expected.

(E) If the primers TAN 1 F and TAN 2 R were able to amplify the tannase gene from *A. niger a* PCR fragment of approximately 684 bp. was expected.

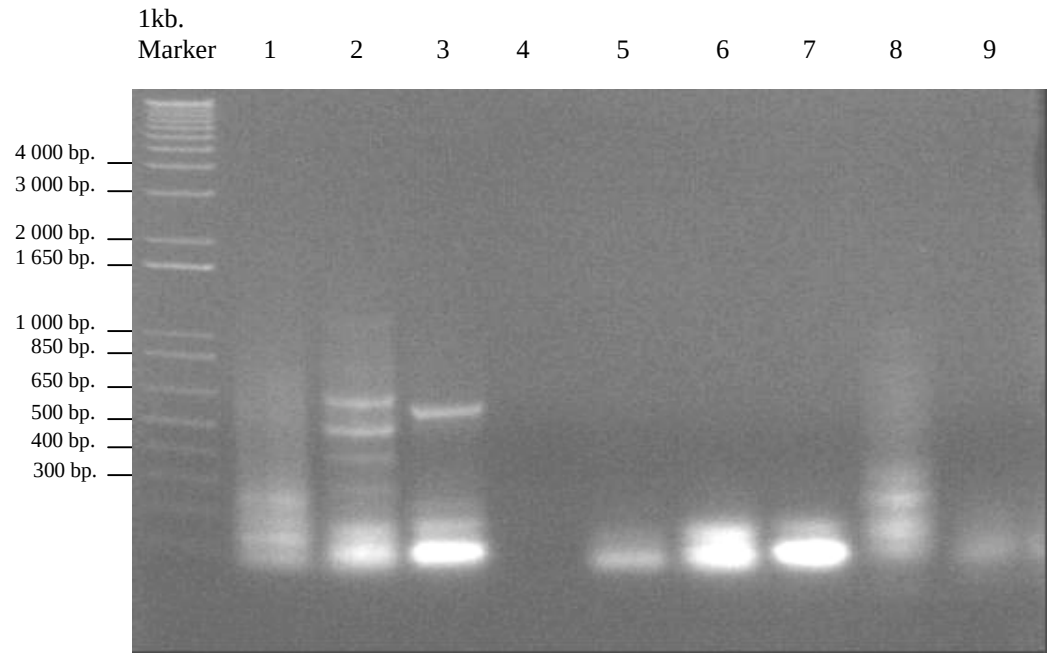


Figure 4.9. An ethidium bromide stained 1% agarose gel showing amplified PCR products with primers OTP, TTH, TAH and TAN.

Lane 1: PCR with primers TAH 1 F and TAH 2 R. No PCR products were visible.

Lane 2: PCR with primers TAN 1 F and TAN 2 R. Various PCR products were visible, with a PCR product having the expected ~684 bp. size.

Lane 3: PCR with primers TTH 1 F and TTH 2 R. Single PCR product was visible with the expected size of 600 bp.

Lane 4: A Control reaction with template DNA without primers.

Lane 5: A Control reaction without template DNA with primers TAH 1 F and TAH 2 R.

Lane 6: A Control reaction without template DNA with primers TAN 1 F and TAN 2 R.

Lane 7: A Control reaction without template DNA with primers TTH 1 F and TTH 2 R.

Lane 8: PCR with primer pair OTP 1 F and OTP 2 R with no product amplification.

Lane 9: Control without template DNA with primers OTP 1 F and OTP 2 R.

After the PCR amplification of the tannase gene from *A. niger* with the primer pairs TTH 1 F, TTH 2 R and TAN 1 F and TAN 2 R, a 1 284 bp. fragment of the tannase gene from *A. niger* was cloned and sequenced. From the schematic representation of the tannase gene from *A. oryzae* (Figure 4.10, A), 287 bp. upstream from the TTH 1 F primer annealing site still needed to be cloned as well as ~196 bp. downstream from the TAN 2 R primer annealing site. Thus to clone and sequence the whole tannase gene from *A. niger* more PCR amplifications were performed using combinations of the primers in table 3.2 (Figure 4.10). Combinations of the primers designed from the known tannase gene sequence from *A. oryzae* (Table 3.2) were used to amplify and clone the remaining parts of the tannase gene from *A. niger*. Figure 4.10, A, represents the schematic size of the known tannase gene sequence from *A. oryzae* (1 767 bp). The already cloned and sequenced part of the tannase gene sequence from *A. niger* is represented in Figure 4.10, B and C. Combinations between primers TAN 1 F and OTP 2 R (Table 3.2) were used to amplify an expected 878 bp. PCR product (Fig. 4.10, F), but unfortunately when the PCR was performed with the primers TAN 1 F and OTP 2 R on the genomic DNA from *A. niger*, no product was found. A combination between primers TTH 1 F and TAN 2 R was used to amplify a 1 286 bp. PCR product (Figure 4.10, G). Upon using the primers TTH 1 F and TAN 2 R a fragment of 1 286 bp. was amplified (Fig. 4.11, B) from the genomic DNA of *A. niger* correlating with the expected sized fragment. From Figure 4.10, D a PCR with the primers OTP 1 F and TTH 2 R were to amplify a 888 bp. PCR product, as was found in Figure 4.11, A. To amplify more than ¾ of the tannase gene from *A. niger* the primers OTP 1 F and TAN 2 R were used in combination to amplify a product of 1 572 bp. in size (Figure 4.10, E). Figure 4.11, C represents the expected 1 572 bp. tannase gene product. All the positive PCR products were cloned into pGEM-T Easy and sequenced, all of the PCR fragments gave positive alignments with the known DNA sequence for tannase from *A. oryzae*. PCR amplification with the primers OTP 1 F and TAN 2 R produced a fragment of 1 572 bp. representing more than 75% of the expected tannase gene from *A. niger*.

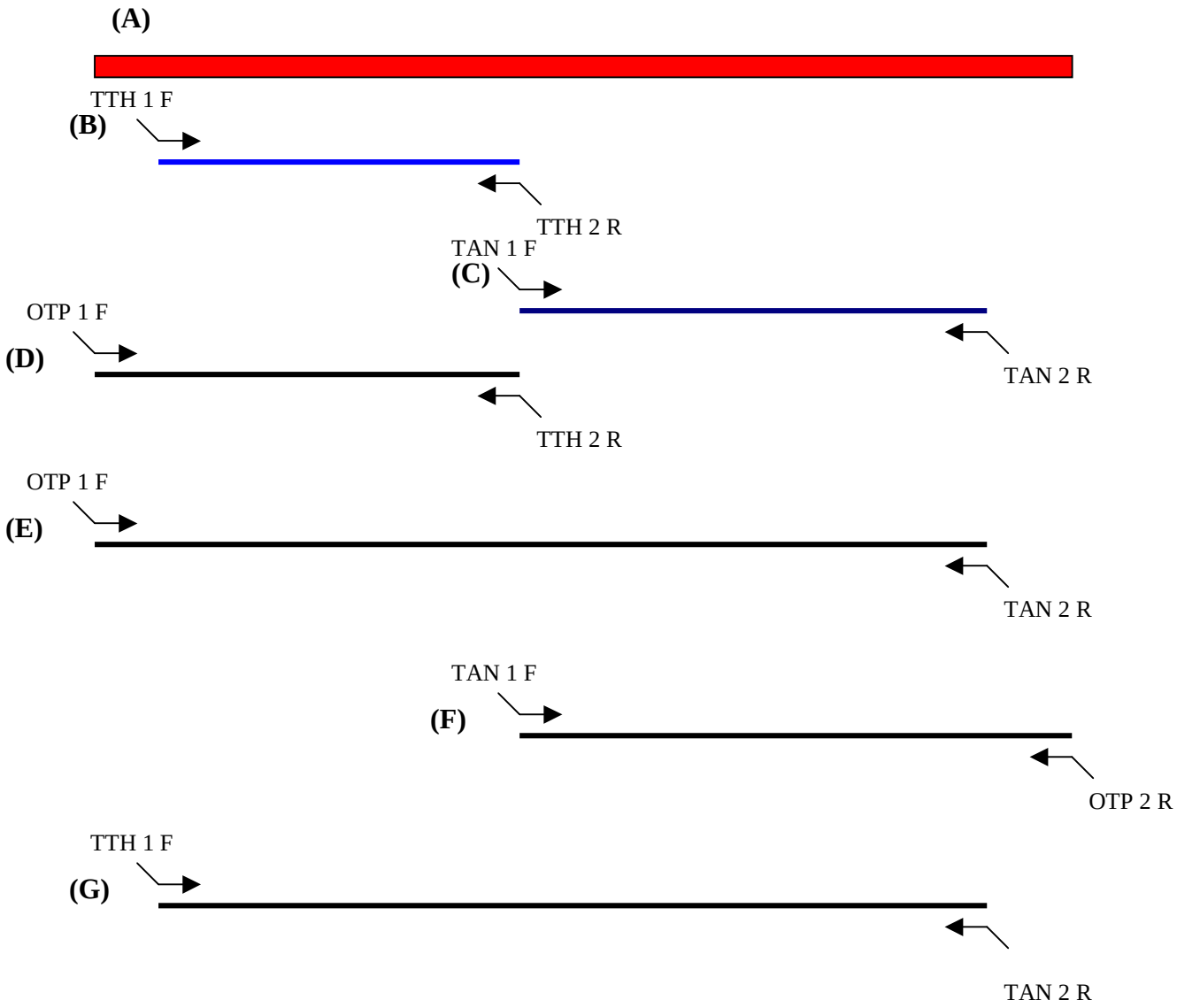


Figure 4.10. A schematic representation on the PCR amplification of the tannase gene from *A. niger* by using combinations of the designed primers from Table 3.2.

(A) Represents the known 1 767 bp. ORF for tannase from *A. oryzae.*

(B), **(C)** Represents the PCR fragments that were cloned and sequenced. The cloned fragments gave positive nucleotide alignments with the DNA sequence for tannase from *A. oryzae*. **(D)** A schematic representation of an expected 888 bp. PCR fragment if primer combination OTP 1 F and TTH 2 R were able to amplify the tannase gene fragment from *A. niger*. **(E)** A schematic representation of an expected 1 572 bp. PCR fragment if primer combination OTP 1 F and TAN 2 R were able to amplify the tannase gene fragment from *A. niger*. **(F)** A schematic representation of an expected 878 bp. PCR fragment if primer combination TAN 1 F and OTP 2 R were able to amplify the tannase gene fragment from the genomic template DNA from *A. niger*. **(G)** A schematic representation of an expected 1 286 bp. PCR fragment if primer combination TTH 1 F and TAN 2 R were able to amplify the tannase gene fragment from the genomic template DNA from *A. niger*.

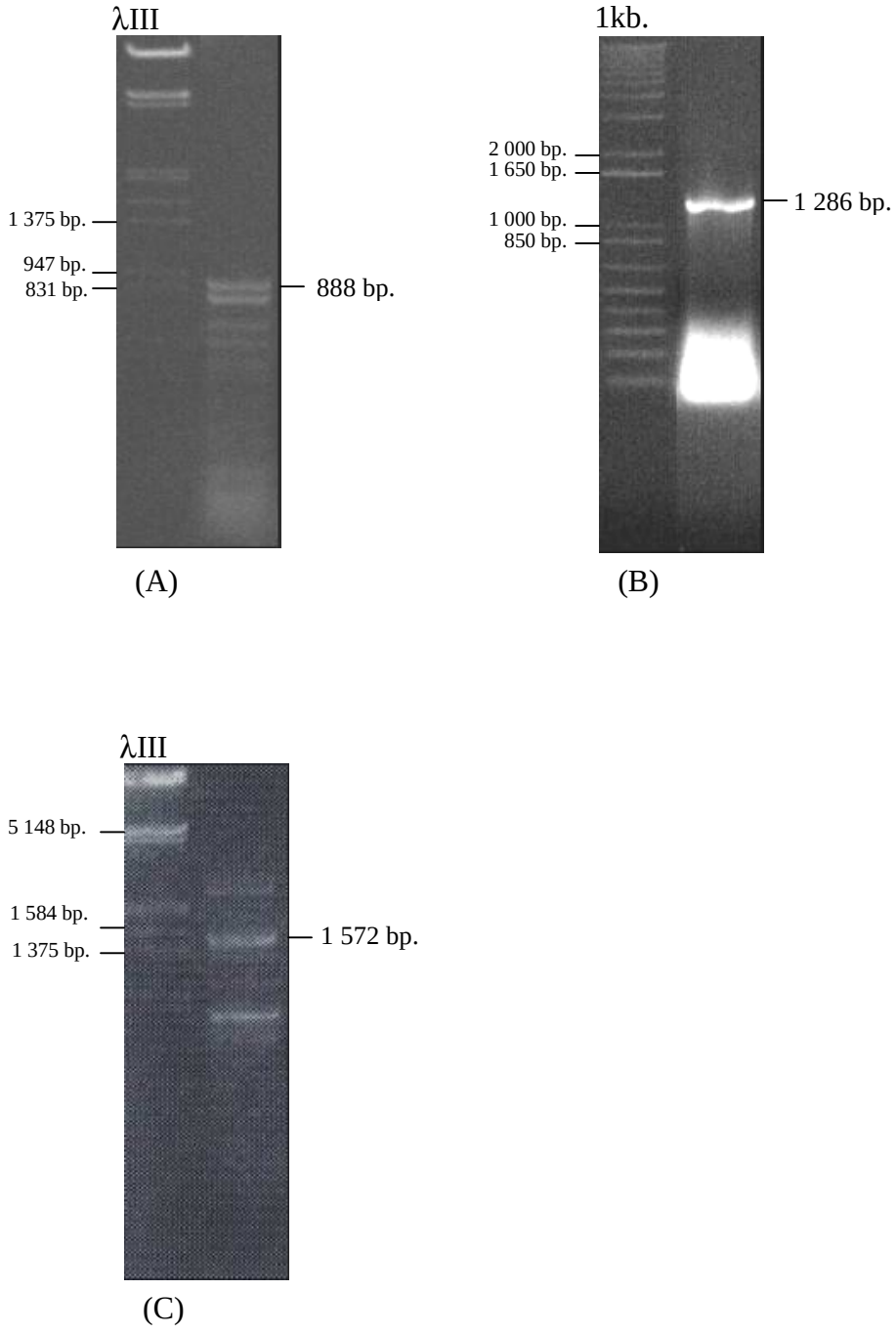


Figure 4.11. Ethidium bromide stained 1% agarose gels showing the PCR products amplified with primer pairs OTP 1 F, TTH 2 R and TAN 2 R.

(A) OTP 1 F and TTH 2 R. The expected product size was 888 bp.

(B) TTH 1 F and TAN 2 R. A PCR product of the expected size of 1 286 bp. was found.

(C) OTP 1 F and TAN 2 R. An expected PCR product with the size of 1 572 bp. was amplified.

All PCR products were cloned into pGEM-T-easy. The plasmids containing the desired PCR products were identified by restriction analysis and sequenced with the primers Sp6 and T7. The fragments were sequenced in duplicate and in both the 5'- 3' direction and the 3'- 5' direction.

Consequently, these fragments contained built in primer sequences corresponding to the sequence of the *A. oryzae* tannase gene. To identify the sequence of the *A. niger* tannase gene matching these regions, four primers were designed, based on the specific gene sequence obtained from the tannase gene from *A. niger* (Table 3.3). These primers were used as sequencing primers (designated FNP 1 and INP 2) on the cloned *A. niger* tannase gene amplified with primer pair OTP 1 F and TAN 2 R. This fragment consisted of the tannase gene start codon and 1 572 bp. of coding sequence. The cloned fragment was sequenced with the designed primers and the corrected sequences were used to replace the *A. oryzae* primer sequences. To amplify and clone the ~195 downstream base pairs of the tannase gene that was unknown as well as the upstream part of the tannase gene, inverse PCR was carried out as described by Ochman *et al.,* (1988). The inverse PCR is a method of *in vitro* amplification of DNA sequences that flank a region of known sequence, in this method it was possible to "walk" outside the region of known sequence without resorting to conventional methods of cloning. Two primers were designed from the *A. niger* tannase gene sequence, these primers were designated as INP 1 and FNP 2 (Table 3.3). Literature suggests that one would digest genomic DNA and allow the DNA to religated upon itself in the hope that monomeric circles would form before PCR amplification would proceed by means of the inverse PCR primers (Figure 4.12), however this method is highly problematic due to large amounts of "junk DNA" reducing the probability of having the fragment containing the gene of interest to ligate upon itself and forming a monomeric circle (Ochman *et al.,* 1988). To minimize the amount of DNA that does not contain the gene of interest the experiment depicted in Figure 4.7 was recreated by digesting the genomic DNA from *A. niger* with *Hind*III, according to the southern blot in Figure 4.7, B lane 10, *Hind*III liberated a single band hybridized fragment. The DNA was isolated from the agarose gel in the region where the single hybridized band was visible, the isolated DNA was ligated and an inverse PCR was performed with the primers INP 1 and FNP 2. A fragment of 4.3 kb correlating to the size found in the southern blot was expected (Figure 4.7, B lane 10), however the inverse primers were

approximately 1.4 kb. apart from each other suggesting that upon amplification of the flanking regions of the tannase gene 1 400 bp. would be excluded in the resultant PCR product. Therefore a positive inverse PCR product would be ~2.9 kb. in size and successful amplification is shown in Figure 4.13.

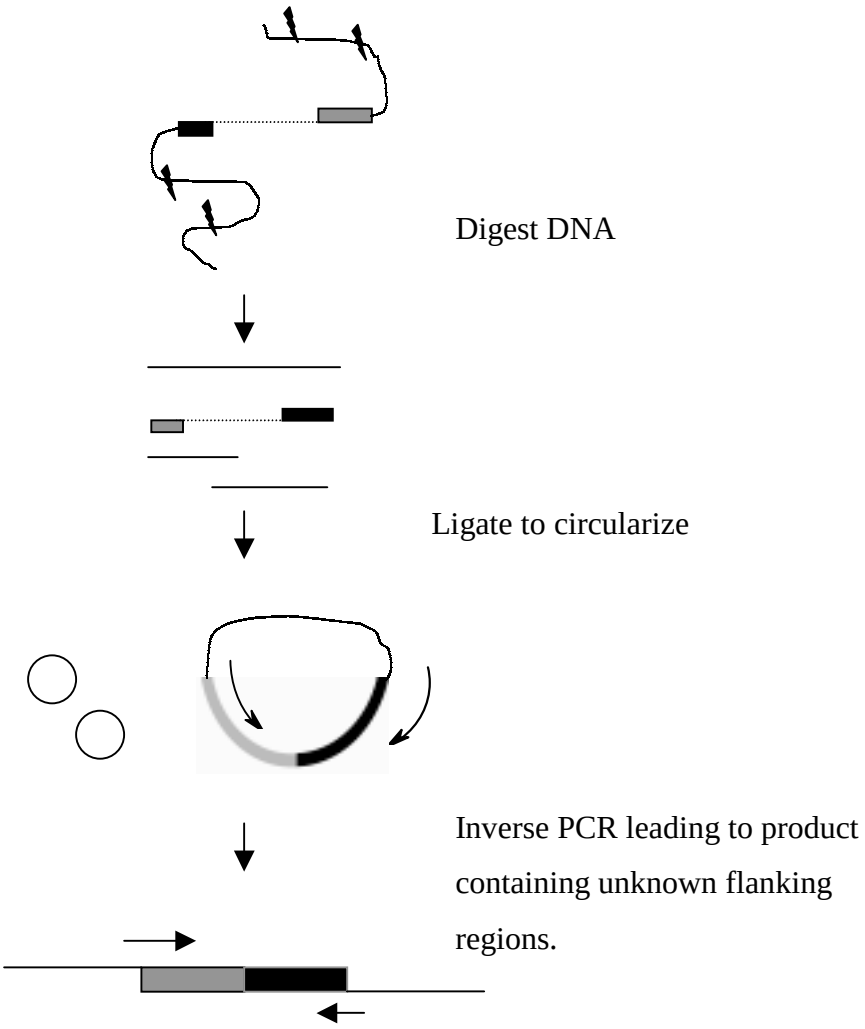


Figure 4.12. A schematic representation of the inverse PCR. The known part of the gene is depicted as a line between two blocks representing the unknown flanking DNA sequence. The DNA is digested with a restriction enzyme, circularised under favourable conditions liberating monomeric circles and subsequently amplified by the inverse PCR primers that anneal to the known core region amplifying the unknown flanking DNA (Adapted from Ochman *et al.*, 1988).

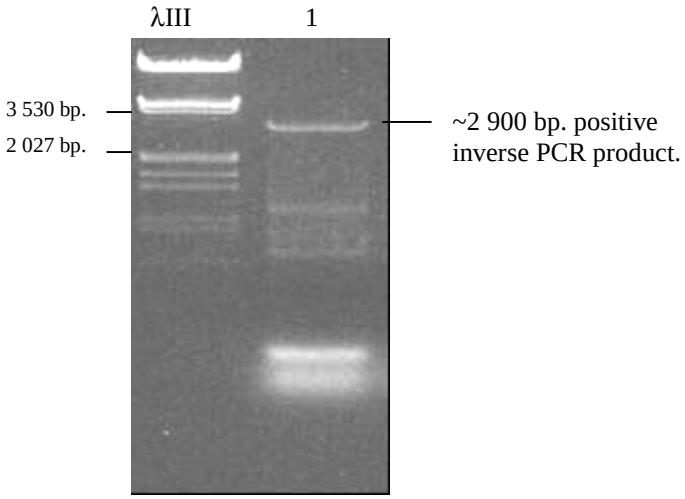


Figure 4.13. An ethidium bromide stained 1 % agarose gel depicting the 2.9 kb. positive inverse PCR product. Lane 1, the 2.9 kb. inverse PCR product corresponds to the unknown flanking regions of the known tannase gene sequence from *A. niger*.

The 2.9 kb. inverse PCR product was cloned into the vector pGEM-T Easy and sequenced with the primers Sp6 and T7. From the sequencing results it was possible to distinguish between the overlapping parts of the already sequenced regions of the tannase gene from *A. niger* and the newly sequenced unknown downstream part from these and earlier sequencing results the genomic DNA sequence for the tannase gene from *A. niger* was constructed.

4.6 The tannase gene sequence from *A. niger*

The gene sequence for tannase from *A. niger* was 1 741 bp. in size and compared well with the tannase gene sequence from *A. oryzae* (1 767 bp.) (Hatamoto *et al.*, 1996). The 1 741 bp. coded for a single open reading frame consisting out of 579 amino acid residues. The complete open reading frame for the tannase gene from *A. niger* is shown in Figure 4.14.

```
        10          20          30          40
ATGCGCCAAC  ACTCGCGCAG  TTGTCGCTGC  TCTGGCAGCA
TACGCGGTTG  TGAGCGCGTC  AACAGCGACG  AGACCGTCGT

        50          60          70          80
GCCACTGCCC  AGGCTACTTC  CCTGTCCGAT  CTCTGCACTG
CGGTGACGGG  TCCGATGAAG  GGACAGGCTA  GAGACGTGAC

        90         100         110         120
TTTCCAATGT  CCAGTCCGCC  CTTCCTTCCA  ACGGCACTCT
AAAGGTTACA  GGTCAGGCGG  GAAGGAAGGT  TGCCGTGAGA

       130         140         150         160
CCTGGGCATC  AACTTGATCC  CCTCTGCCGT  CACTGCCAAC
GGACCCGTAG  TTGAACTAGG  GGAGACGGCA  GTGACGGTTG

       170         180         190         200
ACTGTCACGA  TGCCAGtTCT  GGCATGGGCA  GCTCCAGCTC
TGACAGTGCT  ACGGTCaAGA  CCGTACCCGT  CGAGGTCGAG

       210         220         230         240
CTACGACTAC  TGCAACGTCA  CTGTCACCTA  CACCCACACC
GATGCTGATG  ACGTTGCAGT  GACAGTGGAT  GTGGGTGTGG

       250         260         270         280
GGCAAGGGTG  ACAAGGTGGT  CGTAAAGTAC  GCCCTGCCCG
CCGTTCCCAC  TGTTCCACCA  GCATTTCATG  CGGGACGGGC

       290         300         310         320
CTCCTTCTGA  TTTCAAGAAC  CGTTTCTACG  TCGCCGGTGG
GAGGAAGACT  AAAGTTCTTG  GCAAAGATGC  AGCGGCCACC

       330         340         350         360
TGGTGGTTTC  TCTCTCTCCA  GCGATGCTAC  TGGCGGTCTC
ACCACCAAAG  AGAGAGAGGT  CGCTACGATG  ACCGCCAGAG

       370         380         390         400
GAGTACGGTG  CTGCCTCGGG  TGCCACCGAA  GCCGGCTACG
CTCATGCCAC  GACGGAGCCC  ACGGTGGCTT  CGGCCGATGC
```

Figure 4.14. …

```
       410         420         430         440
ACGCCTTCTC  CTACAGCTAC  GACGAAGTCG  TCCTCTATGG
TGCGGAAGAG  GATGTCGATG  CTGCTTCAGC  AGGAGATACC

       450         460         470         480
CaaCggCTCG  ATCAACTGGG  ATGCCACTTA  CATGTTTGGc
GttGccGAGC  TAGTTGACCC  TACGGTGAAT  GTACAAACCg

       490         500         510         520
TACCAGGCTC  TGGGTGAAAT  GACCAAGATC  GCCAAGCCCC
ATGGTCCGAG  ACCCACTTTA  CTGGTTCTAG  CGGTTCGGGG

       530         540         550         560
TGACCCGTGG  CTTTTaCGGT  CTCTCCAGCG  ACAAGAAGAT
ACTGGGCACC  GAAAAtGCCA  GAGAGGTCGC  TGTTCTTCTA

       570         580         590         600
CTATACCTAC  TACGAGGGCT  GTTCCGATGG  TGGTCGTGAG
GATATGGATG  ATGCTCCCGA  CAAGGCTACC  ACCAGCACTC

       610         620         630         640
GGTATGAGTC  AGGTTCAGCG  CTGGGGAGAT  GAATATGACG
CCATACTCAG  TCCAAGTCGC  GACCCCTCTA  CTTATACTGC

       650         660         670         680
GTGTTATCGC  TGGTGCCCCT  GCCTTCCGCT  TTGCTCAGCA
CACAATAGCG  ACCACGGGGA  CGGAAGGCGA  AACGAGTCGT

       690         700         710         720
GCAGGTCCAC  CACGTCTTCC  CTGCCACTAT  CGAACATACC
CGTCCAGGTG  GTGCAGAAGG  GACGGTGATA  GCTTGTATGG

       730         740         750         760
ATGGATTACT  ACCCTCCCCC  TTGCGAGCTT  GACAAGATCG
TACCTAATGA  TGGGAGGGGG  AACGCTCGAA  CTGTTCTAGC

       770         780         790         800
TTAACGCTAC  CATCGAAGCC  TGTGACCCTC  TCGACGCCGT
AATTGCGATG  GTAGCTTCGG  ACACTGGGAG  AGCTGCGGCA

       810         820         830         840
aCCATGGCGT  TGTCTCCCCA  CTGACCTCTG  CATGCTGAAC
tGGTACCGCA  ACAGAGGGGT  GACTGGAGAC  GTACGACTTG

       850         860         870         880
TTCAATCTCA  CCTCCATCAT  CGGCCAGTCC  TACTACTGTG
AAGTTAGAGT  GGAGGTAGTA  GCCGGTCAGG  ATGATGACAC

       890         900         910         920
CTGCAGAGAA  CTACACCTCC  CTGGGCTTCG  GCTTCAGCAA
GACGTCTCTT  GATGTGGAGG  GACCCGAAGC  CGAAGTCGTT

       930         940         950         960
GCGCGCCGAA  GGCAGCACTA  CTAGCTACCA  GCCCGCCCAG
CGCGCGGCTT  CCGTCGTGAT  GATCGATGGT  CGGGCGGGTC


       970         980         990        1000
AATGGCTCCG  TCACCGCCGA  AGGTGTCGCC  CTCGCCCAGG
TTACCGAGGC  AGTGGCGGCT  TCCACAGCGG  GAGCGGGTCC
```

Figure 4.14. …

```
      1010        1020        1030        1040
CCATCTACGA  CGGTCTCCAC  GACTCCAACG  GCAAGCGCGC
GGTAGATGCT  GCCAGAGGTG  CTGAGGTTGC  CGTTCGCGCG

      1050        1060        1070        1080
CTACCTCTCG  TGGCAGATCG  CCGCCGAGCT  GTCTGACGGT
GATGGAGAGC  ACCGTCTAGC  GGCGGCTCGA  CAGACTGCCA

      1090        1100        1110        1120
GACACCGAGT  ATGACTCCAC  CACTGACTCC  TGGACTTTGA
CTGTGGCTCA  TACTGAGGTG  GTGACTGAGG  ACCTGAAACT

      1130        1140        1150        1160
GCATCCCCTC  CACTGGCGGC  GAGTACGTGA  CCAAGTTCGT
CGTAGGGGAG  GTGACCGCCG  CTCATGCACT  GGTTCAAGCA

      1170        1180        1190        1200
GCAGCTTCTT  AACATCGACA  ACCTGGAGAA  CCTCGATAAT
CGTCGAAGAA  TTGTAGCTGT  TGGACCTCTT  GGAGCTATTA

      1210        1220        1230        1240
GTCACCTACG  ACACCCTGGT  CGAGTGGATG  AACATCGGTA
CAGTGGATGC  TGTGGGACCA  GCTCACCTAC  TTGTAGCCAT

      1250        1260        1270        1280
TGATCCGCTA  CATCGACAGT  CTCCAGACCA  CCATTCCCGA
ACTAGGCGAT  GTAGCTGTCA  GAGGTCTGGT  GGTAAGGGCT

      1290        1300        1310        1320
CCTCACCACC  TTCCAGAAGT  CCGGTGGTAA  GATGATCCAC
GGAGTGGTGG  AAGGTCTTCA  GGCCACCATT  CTACTAGGTG

      1330        1340        1350        1360
TACCACGGTG  AATCGGACCC  CAGTATCCCG  ACCGCCTCAT
ATGGTGCCAC  TTAGCCTGGG  GTCATAGGGC  TGGCGGAGTA

      1370        1380        1390        1400
CCGTCCACTA  CTGGCAGGCC  GTCCGTCAGG  CCATGTACCC
GGCAGGTGAT  GACCGTCCGG  CAGGCAGTCC  GGTACATGGG

      1410        1420        1430        1440
CAACACCACC  TACACCCAGT  CGCTTAAGGA  GATGTCCGAC
GTTGTGGTGG  ATGTGGGTCA  GCGAATTCCT  CTACAGGCTG

      1450        1460        1470        1480
TGGTACCAGC  TTTACCTCGT  CCcTGGCGCT  GCCCACTGCG
ACCATGGTCG  AAATGGAGCA  GGgACCGCGA  CGGGTGACGC

      1490        1500        1510        1520
GTACCAACGA  CCTCCAGCCG  GGTCCTTACC  CTGAGGACAA
CATGGTTGCT  GGAGGTCGGC  CCAGGAATGG  GACTCCTGTT


      1530        1540        1550        1560
CATGGAGATT  ATGATCGACT  GGGTTGAGAA  CGGcAACAAG
GTACCTCTAA  TACTAGCTGA  CCCAACTCTT  GCCgTTGTTC
```

Figure 4.14. …

```
      1570        1580        1590        1600
CCTTCCCGTC  TCAACGCCAC  CGTCTCCTCC  GGCTACTATG
GGAAGGGCAG  AGTTGCGGTG  GCAGAGGAGG  CCGATGATAC

      1610        1620        1630        1640
CTGGTGAGAC  CCAGATGCTT  TGCCAGTGGC  CTTCTCGTCC
GACCACTCTG  GGTCTACGAA  ACGGTCACCG  GAAGAGCAGG

      1650        1660        1670        1680
CCTCTGGACC  AGCAACTCCA  GCTTCTCTTG  TGTTCATGAC
GGAGACCTGG  TCGTTGAGGT  CGAAGAGAAC  ACAAGTACTG

      1690        1700        1710        1720
TCCAAGTCCC  TTGCTACTTG  GGACTACACT  TTTGATGCTT
AGGTTCAGGG  AACGATGAAC  CCTGATGTGA  AAACTACGAA

      1730        1740
TCAAGATGCC  CGTTTTCTAA
AGTTCTACGG  GCAAAAGATT
```

Figure 4.14. The sequenced tannase gene (open reading frame) from *A. niger*.

4.7 Homology between the tannase gene sequences from *A. oryzae* and *A. niger*

The tannase gene from *A. niger* was sequenced from the genomic DNA. After analysis of the DNA sequence data it was evident that this organism's tannase gene was similar to the sequence for tannase from *A. oryzae* (Hatamoto *et al.*, 1996). Both the tannase genes did not contain any intron sequences. Alignments of the tannase gene sequences from *A. oryzae* and *A. niger* revealed that there was a 76.76% homology between the nucleotide sequences of the two genes (Figure 4.15). From the amino acid alignment it was calculated that the tannase gene sequence from *A. niger* contained an identity of 71% and a similarity of 10.19% (Figure 4.16).

```
oryzae  1    ATGCGCCAACACTCGCGCATGGCCGTTGCT 30
niger   1    ATGCGCCAACACTCGCGCA--GTTGTCGCT 28

oryzae  31   GCTTTGGCAGCAGGA---GCGAACGCAGCT 57
niger   29   GCTCTGGCAGCAGCCACTGCCCAGGCTACT 58

oryzae  58   TCTTTTACCGATGTGTGCACCGTGTCTAAC 87
niger   59   TCCCTGTCCGATCTCTGCACTGTTTCCAAT 88
```

Figure 4.15. …

```
oryzae  88   GTGAAGGCTGCATTGCCTGCCAACGGAACT 117
niger   89   GTCCAGTCCGCCCTTCCTTCCAACGGCACT 118

oryzae  118  CTGCTCGGAATCAGCATGCTTCCGTCCGCC 147
niger   119  CTCCTGGGCATCAACTTGATCCCCTCTGCC 148

oryzae  148  GTCACGGCCAACCCTCTCTACAA-CCAGTC 176
niger   149  GTCACTGCCAACACTGTCACGATGCCAGTT 178

oryzae  177  GGCTGGCATGGGTAGCACCACTACCTATGA 206
niger   179  --CTGGCATGGGCAGCTCCAGCTCCTACGA 206

oryzae  207  CTACTGCAATGTGACTGTCGCCTACACGCA 236
niger   207  CTACTGCAACGTCACTGTCACCTACACCCA 236

oryzae  237  TACCGGCAAGGGTGATAAAGTGGTCATCAA 266
niger   237  CACCGGCAAGGGTGACAAGGTGGTCGTAAA 266

oryzae  267  GTACGCATTCCCCAAGCCCTCCGACTACGA 296
niger   267  GTACGCCTGCCCGCTCCTTCTGATTTCAA 296

oryzae  297  GAACCGTTTCTACGTTGCTGGTGGTGGTGG 326
niger   297  GAACCGTTTCTACGTCGCCGGTGGTGGTGG 326

oryzae  327  CTTTTCCCTCTCTAGCGATGCTACCGGAGG 356
niger   327  TTTCTCTCTCTCCAGCGATGCTACTGGCGG 356

oryzae  357  TCTCGCCTATGGCGCTGTGGGAGGTGCCAC 386
niger   357  TCTCGAGTACGGTGCTGCCTCGGGTGCCAC 386

oryzae  387  CGATGCTGGATACGACGCATTCGATAACAG 416
niger   387  CGAAGCCGGCTACGACGCCTTCTCCTACAG 416

oryzae  417  CTACGACGAGGTAGTCCTCTACGGAAACGG 446
niger   417  CTACGACGAAGTCGTCCTCTATGGCAACGG 446

oryzae  447  AACCATTAACTGGGACGCCACATACATGTT 476
niger   447  CTCGATCAACTGGGATGCCACTTACATGTT 476

oryzae  477  CGCATACCAGGCACTGGGAGAGATGACCCG 506
niger   477  TGGCTACCAGGCTCTGGGTGAAATGACCAA 506

oryzae  507  GATCGGAAAGTACATCACCAAGGGCTTTTA 536
niger   507  GATCGCCAAGCCCCTGACCCGTGGCTTTTA 536

oryzae  537  TGGCCAGTCCAGCGACAGCAAGGTCTACAC 566
niger   537  CGGTCTCTCCAGCGACAAGAAGATCTATAC 566

oryzae  567  CTACTACGAGGGTTGCTCCGATGGAGGACG 596
niger   567  CTACTACGAGGGCTGTTCCGATGGTGGTCG 596

oryzae  597  TGAGGGTATGAGTCAAGTCCAGCGCTGGGG 626
niger   597  TGAGGGTATGAGTCAGGTTCAGCGCTGGGG 626

oryzae  627  TGAGGAGTATGACGGTGCGATTACTGGTGC 656
niger   627  AGATGAATATGACGGTGTTATCGCTGGTGC 656

oryzae  657  CCCGGCTTTCCGTTTCGCTCAGCAACAGGT 686
niger   657  CCCTGCCTTCCGCTTTGCTCAGCAGCAGGT 686
```

Figure 4.15. …

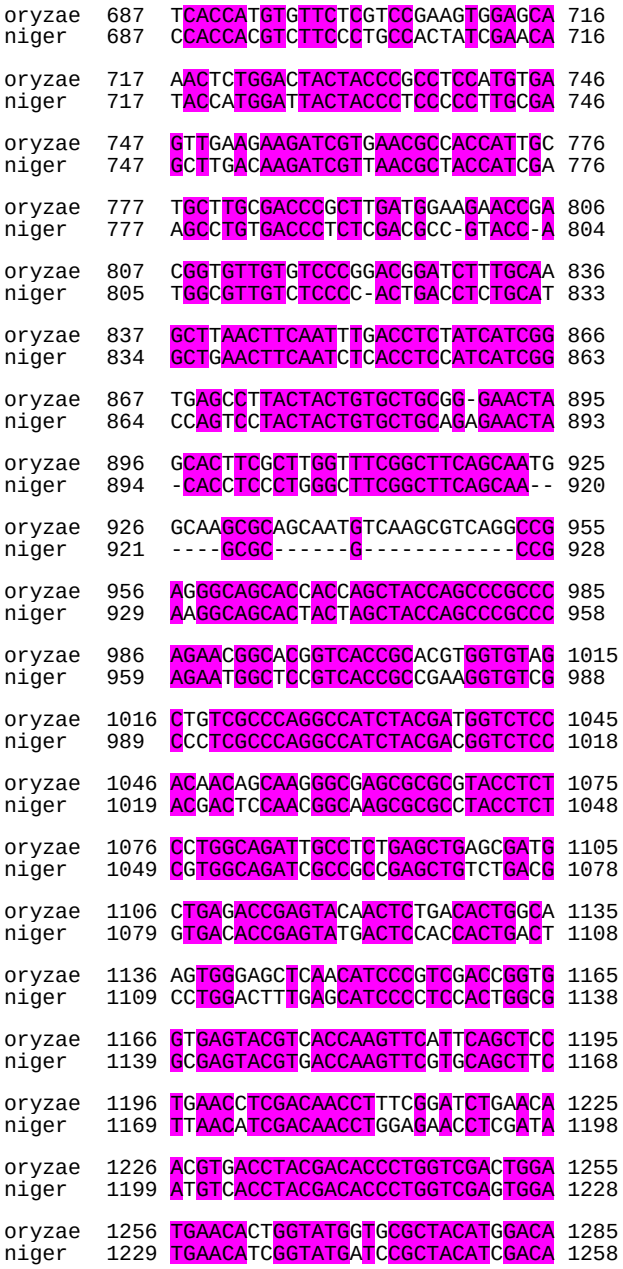

oryzae 687 TCACCATGTGTTCTCGTCCGAAGTGGAGCA 716
niger 687 CCACCACGTCTTCCCTGCCACTATCGAACA 716
oryzae 717 AACTCTGGACTACTACCCGCCTCCATGTGA 746
niger 717 TACCATGGATTACTACCCTCCCCCTTGCGA 746
oryzae 747 GTTGAAGAAGATCGTGAACGCCACCATTGC 776
niger 747 GCTTGACAAGATCGTTAACGCTACCATCGA 776
oryzae 777 TGCTTGCGACCCGCTTGATGGAAGAACCGA 806
niger 777 AGCCTGTGACCCTCTCGACGCC-GTACC-A 804
oryzae 807 CGGTGTTGTGTCCCGGACGGATCTTTGCAA 836
niger 805 TGGCGTTGTCTCCCC-ACTGACCTCTGCAT 833
oryzae 837 GCTTAACTTCAATTTGACCTCTATCATCGG 866
niger 834 GCTGAACTTCAATCTCACCTCCATCATCGG 863
oryzae 867 TGAGCCTTACTACTGTGCTGCGG-GAACTA 895
niger 864 CCAGTCCTACTACTGTGCTGCAGAGAACTA 893
oryzae 896 GCACTTCGCTTGGTTTCGGCTTCAGCAATG 925
niger 894 -CACCTCCCTGGGCTTCGGCTTCAGCAA-- 920
oryzae 926 GCAAGCGCAGCAATGTCAAGCGTCAGGCCG 955
niger 921 ----GCGC------G----------CCG 928
oryzae 956 AGGGCAGCACCACCAGCTACCAGCCCGCCC 985
niger 929 AAGGCAGCACTACTAGCTACCAGCCCGCCC 958
oryzae 986 AGAACGGCACGGTCACCGCACGTGGTGTAG 1015
niger 959 AGAATGGCTCCGTCACCGCCGAAGGTGTCG 988
oryzae 1016 CTGTCGCCCAGGCCATCTACGATGGTCTCC 1045
niger 989 CCCTCGCCCAGGCCATCTACGACGGTCTCC 1018
oryzae 1046 ACAACAGCAAGGGCGAGCGCGCGTACCTCT 1075
niger 1019 ACGACTCCAACGGCAAGCGCGCCTACCTCT 1048
oryzae 1076 CCTGGCAGATTGCCTCTGAGCTGAGCGATG 1105
niger 1049 CGTGGCAGATCGCCGCCGAGCTGTCTGACG 1078
oryzae 1106 CTGAGACCGAGTACAACTCTGACACTGGCA 1135
niger 1079 GTGACACCGAGTATGACTCCACCACTGACT 1108
oryzae 1136 AGTGGGAGCTCAACATCCCGTCGACCGGTG 1165
niger 1109 CCTGGACTTTGAGCATCCCCTCCACTGGCG 1138
oryzae 1166 GTGAGTACGTCACCAAGTTCATTCAGCTCC 1195
niger 1139 GCGAGTACGTGACCAAGTTCGTGCAGCTTC 1168
oryzae 1196 TGAACCTCGACAACCTTTCGGATCTGAACA 1225
niger 1169 TTAACATCGACAACCTGGAGAACCTCGATA 1198
oryzae 1226 ACGTGACCTACGACACCCTGGTCGACTGGA 1255
niger 1199 ATGTCACCTACGACACCCTGGTCGAGTGGA 1228
oryzae 1256 TGAACACTGGTATGGTGCGCTACATGGACA 1285
niger 1229 TGAACATCGGTATGATCCGCTACATCGACA 1258

Figure 4.15. …

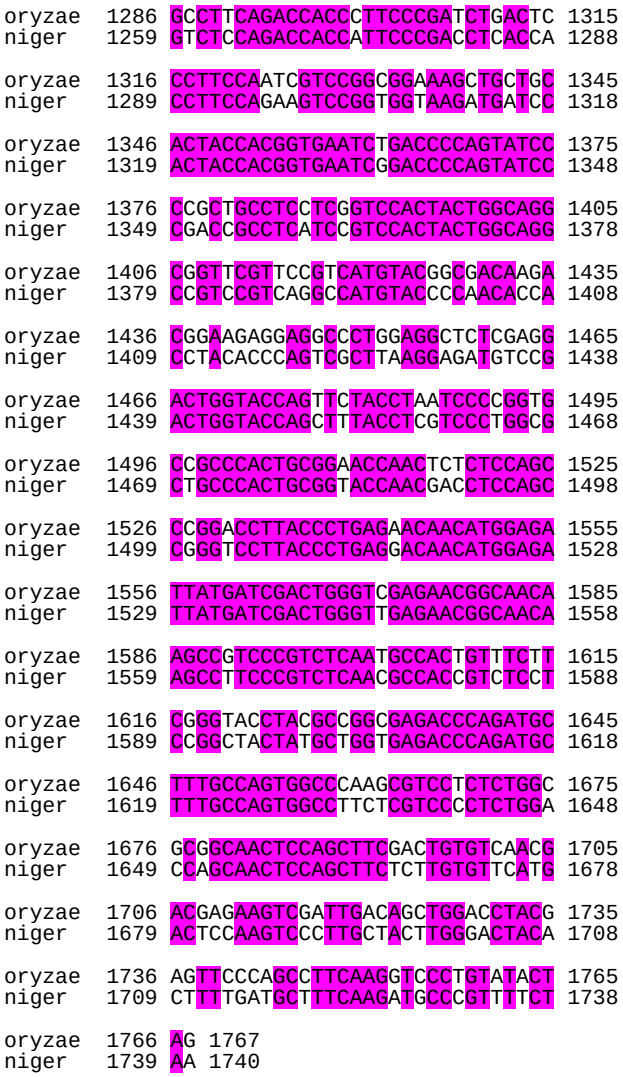


Figure 4.15. A DNA sequence alignment between the tannase genes from *A. oryzae* and *A. niger*.

The amino acid sequence for tannase from *A. niger* suggested the occurrence of a signal peptide sequence between amino acids 1 to 16 (Figure 4.16. B) in contrast to the known leader peptide sequence for tannase from *A. oryzae* found between amino acids 1 to 18 (Figure 4.16. A).
According to the SignalP signal peptidase cleavage site prediction program (http://www.cbs.dtu.dk/services/SignalP.html) the probability of finding a peptidase cleavage site at position 16 for *A. niger's* tannase was only 15.4%, which is very low. This could mean that the protein is expressed with its leader peptide sequence still linked to the protein and not removed by a signal peptidase. However to answer this question the protein would have to be isolated and an N-terminal sequence determined.

The amino acid alignment between the translated tannase genes revealed a 6 + 2 amino acid deletion at positions 307-312 and from 315-316 in tannase from *A. niger*. According to Hatamoto *et al.,* (1996) the tannase gene from *A. oryzae* is translated as a single polypeptide sequence of 588 a.a from which an 18 a.a signal peptide is cleaved. Subsequently a KEX-II like protease cleaves the tannase peptide into two subunits at position 315 to 316 between two dibasic residues with the sequence Lys(K)-Arg(R) (Carrez *et al.,* 1990). The dibasic residues Lys(K)-Arg(R) are found at position 307 and 308 (Figure 4.16. C) in the tannase amino acid sequence for *A. niger*. Therefore it is also hypothesized that the tannase gene from *A. niger* is transcribed as a single polypeptide chain of 579 a.a. The peptide sequence is cleaved into two chains at the KEX-II like protease cleavage site (Figure 4.16. C) (Lys-Arg) to liberate two subunits that will be linked by disulfide bonds to liberate a catalytically active enzyme (Figure1.5.).

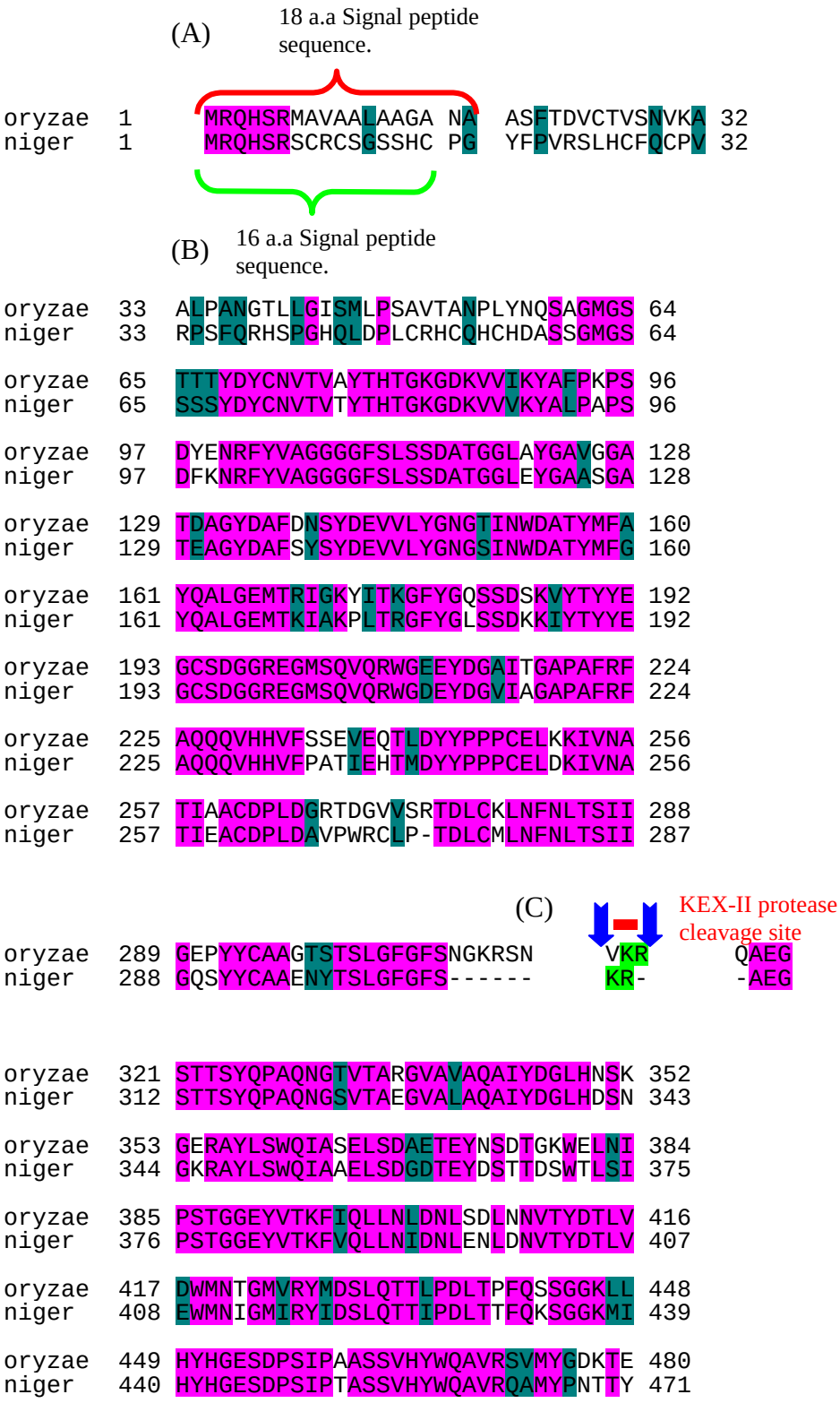

(A)
18 a.a Signal peptide sequence.
oryzae 1 MRQHSRMAVAALAAGA NA ASFTDVCTVSNVKA 32
niger 1 MRQHSRSCRCSGSSHC PG YFPVRSLHCFQCPV 32
(B)
16 a.a Signal peptide sequence.
oryzae 33 ALPANGTLLGISMLPSAVTANPLYNQSAGMGS 64
niger 33 RPSFQRHSPGHQLDPLCRHCQHCHDASSGMGS 64
oryzae 65 TTTYDYCNVTVAYTHTGKGDKVVIKYAFPKPS 96
niger 65 SSSYDYCNVTVTYTHTGKGDKVVVKYALPAPS 96
oryzae 97 DYENRFYVAGGGGFSLSSDATGGLAYGAVGGA 128
niger 97 DFKNRFYVAGGGGFSLSSDATGGLEYGAASGA 128
oryzae 129 TDAGYDAFDNSYDEVVLYGNGTINWDATYMFA 160
niger 129 TEAGYDAFSYSYDEVVLYGNGSINWDATYMFG 160
oryzae 161 YQALGEMTRIGKYITKGFYGQSSDSKVYTYYE 192
niger 161 YQALGEMTKIAKPLTRGFYGLSSDKKIYTYYE 192
oryzae 193 GCSDGGREGMSQVQRWGEEYDGAITGAPAFRF 224
niger 193 GCSDGGREGMSQVQRWGDEYDGVIAGAPAFRF 224
oryzae 225 AQQQVHHVFSSEVEQTLDYYPPPCELKKIVNA 256
niger 225 AQQQVHHVFPATIEHTMDYYPPPCELDKIVNA 256
oryzae 257 TIAACDPLDGRTDGVVSRTDLCKLNFNLTSII 288
niger 257 TIEACDPLDAVPWRCLP-TDLCMLNFNLTSII 287
(C)
KEX-II protease cleavage site
oryzae 289 GEPYYCAAGTSTSLGFGFSNGKRSN VKR QAEG
niger 288 GQSYYCAAENYTSLGFGFS------ KR- -AEG
oryzae 321 STTSYQPAQNGTVTARGVAVAQAIYDGLHNSK 352
niger 312 STTSYQPAQNGSVTAEGVALAQAIYDGLHDSN 343
oryzae 353 GERAYLSWQIASELSDAETEYNSDTGKWELNI 384
niger 344 GKRAYLSWQIAAELSDGDTEYDSTTDSWTLSI 375
oryzae 385 PSTGGEYVTKFIQLLNLDNLSDLNNVTYDTLV 416
niger 376 PSTGGEYVTKFVQLLNIDNLENLDNVTYDTLV 407
oryzae 417 DWMNTGMVRYMDSLQTTLPDLTPFQSSGGKLL 448
niger 408 EWMNIGMIRYIDSLQTTIPDLTTFQKSGGKMI 439
oryzae 449 HYHGESDPSIPAASSVHYWQAVRSVMYGDKTE 480
niger 440 HYHGESDPSIPTASSVHYWQAVRQAMYPNTTY 471

Figure 4.16. …

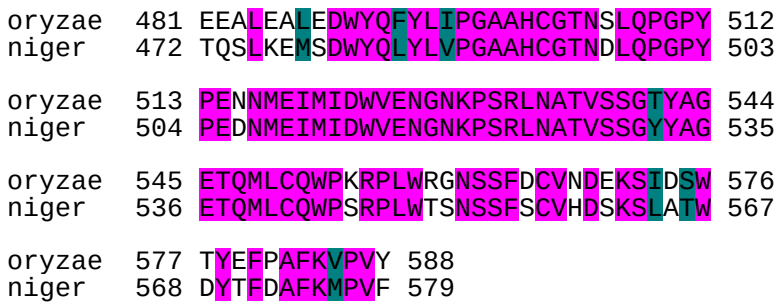


Figure 4.16. Amino acid alignment between the translated tannase genes from *A. oryzae* and *A. niger*, showing amino acid identity (purple) and similarity (green). (A) The proposed signal peptide sequence for *A. oryzae's* tannase (Hatamoto *et al.*, 1996). (B) The proposed signal peptide sequence for *A. niger's* tannase. (C) Proposed KEX-II like protease cleavage site in the peptide sequence for tannase.

The molecular weight and pI for tannase from *A. oryzae* and *A. niger* were calculated with the "Calculate pI\MW tool" on the ExPasy server at http://www.expasy.ch/tools/pi_tool.html (Table 4.2).

Table 4.2. Calculated pI/MW for tannase from *A. niger* and *A. oryzae.*

Organism	**Before KEXII protease and peptidase cleavage.**		**After KEXII protease and peptidase cleavage into 2 subunits.**	
	pI	**MW (Da)**	**MW (Da) Subunit 1**	**MW (Da) Subunit 2**
A. oryzae (Hatamoto *et al.*, (1996).	4.76	64 000	33 000	30 000
A. niger	5.07	64 179	33 700	30 400

The calculated molecular weights for the tannase subunits as well as the proposed pI values for the genes correlated well with each other as well as with the pI values of *Chryphonectria parasitica's* tannase being between 4.6 – 5.1 and for *A. oryzae* tannase being near to pH 4.0 (Aoki *et al.*, 1976, Iibuchi *et al.*, 1968). To compare the calculated molecular weights to other organism's tannase was problematic due to the fact that the reported molecular weights were determined by SDS-PAGE, therefore

including the molecular sizes of the glycosylated proteins, making it impossible to compare the data.

4.7.1 The postulated active site for tannase

Adachi *et al.,* (1971) proposed that the active site for tannase from *A. flavus* contained an active serine residue. They further postulated that the active site for tannase were very similar to that of subtilisin. Therefore suggesting that the sequence for the active site for tannase was threonine-serine-methionine. However, later Parthasarathy and Bose (1976) hypothesised that the active site for tannase contained two amino acids that are o-glycosidically linked and therefore should be threonine and serine.

4.7.2 Proposed N- and O- linked glycosylation sites in tannase from A. niger

From literature it is known that tannase is a glycoprotein (Hatamoto *et al.,* 1996, Parthasarathy & Bose, 1976, Aoki *et al.,* 1976, Barthomeuf *et al.,* 1994, Niehaus *et al.,* 1997). Figure 4.17 represents the proposed N- and O- linked glycosylation sites for tannase from *A. niger*.

MRQHSRSCRCSGSSHCPGYFPVRSLHCFQCPVRPSFQRHSPGHQLDPLCRHCQHCHDASSGMG
SSSSYDYCNVTVTYTHTGKGDKVVVKYALPAPSDFKNRFYVAGGGGFSLSSDATGGLEYGAA
SGATEAGYDAFSYSYDEVVLYGNGSINWDATYMFGYQALGEMTKIAKPLTRGFYGLSSDKKI
YTYYEGCSDGGREGMSQVQRWGDEYDGVIAGAPAFRFAQQQVHHVFPATIEHTMDYYPPPC
ELDKIVNATIEACDPLDAVPWRCLPTDLCMLNFNLTSIIGQSYYCAAENYTSLGFGFSKRAEGS
TTSYQPAQNGSVTAEGVALAQAIYDGLHDSNGKRAYLSWQIAAELSDGDTEYDSTTDSWTLS
IPSTGGEYVTKFVQLLNIDNLENLDNVTYDTLVEWMNIGMIRYIDSLQTTIPDLTTFQKSGGKM
IHYHGESDPSIPTASSVHYWQAVRQAMYPNTTYTQSLKEMSDWYQLYLVPGAAHCGTNDLQ
PGPYPEDNMEIMIDWVENGNKPSRLNATVSSGYYAGETQMLCQWPSRPLWTSNSSFSCVHDS
KSLATWDYTFDAFKMPVF

Figure 4.17. Amino acid sequence for tannase from *A. niger*, showing predicted glycosylation sites [N-linked glycosylation sites , O-linked glycosylation sites] (Balloou, 1982).

Piater, (1999) indicated that *A. niger* tannase is a glycoprotein with its polypeptide moiety being relatively small. Further investigation is however needed to fully elucidate the carbohydrate composition of tannase from *A. niger*.

4.8 Cloning of the tannase gene from *A. oryzae* for expression studies

Although the tannase gene from *A. oryzae* has been cloned no expression studies have been performed on this cloned gene in other microorganisms or even in a deletion mutant of the same species (Hatamoto *et al.,* 1996). This led to the investigation of the expression of the tannase gene, cloned from *A. oryzae* in the yeast *Saccharomyces cerevisiae*. Due to time constraints and the time taken to sequence the tannase gene from *A. niger*, it was decided not to express the tannase cloned from *A. niger*.

It is not yet known if the tannase enzyme is transported to the extracellular space, although the 18 amino acid signal peptide sequence is hypothesized to be involved in directing the protein extracellularly (Hatamoto *et al.,* 1996). Primers were therefore designed to amplify the coding region of the *A. oryzae* tannase gene including the signal peptide sequence in order to express the recombinant enzyme in *S. cerevisiae*. The already cloned tannase gene from section 4.3 in the pGEM-T Easy plasmid was used for subcloning and expression purposes. The cloned tannase gene consisted of a 1 767 bp. region starting at the ATG start codon and ending at the TAG stop codon, including an 18 amino acid signal sequence from nucleotide 1-54 and the protein coding sequence from nucleotide 55-1 767 (*oryTAH*).

4.9 Expression of the recombinant tannase in *S. cerevisiae*

4.9.1 PCR amplification of the PDC1 promoter

To express the *oryTAH* gene in *S. cerevisiae* it was necessary to include a promoter sequence upstream of the tannase gene start codon. To facilitate high-level expression a *PDC1* promoter region was used. The *PDC1* promoter was PCR amplified from the

genome of *S. cerevisiae* W303-A1 (*MAT α leu2-3/112 ura3-1 trp1-1 his3-11/15)*. The PCR was performed on isolated genomic DNA from *S. cerevisiae* W301-A1 with the primers PDC1-F and PDC1-R (Section 3.8.1) (Kellermann *et al.*, 1986). This led to the amplification of a 1 193 bp. fragment consisting of the promoter region of *PDC1*, starting 7 bp. upstream from the *PDC1* initiation codon (Figure 4.18). The primers introduced the restriction sites for *Eco*RI and *Bam*HI to the amplified PCR product to assist in cloning the *PDC1* promoter into the expression vector, and for easy ligation and introduction of the tannase gene downstream from the promoter sequence. The *PDC1* promoter has been intensively studied. From literature it was evident that for the expression of the recombinant tannase the glucose induced *PDC1* promoter would be suitable. Due to availability of the primers and the yeast strain used to amplify the promoter the *PDC1* promoter was used in the pRS426 expression shuttle vector Hohmann, (1991).

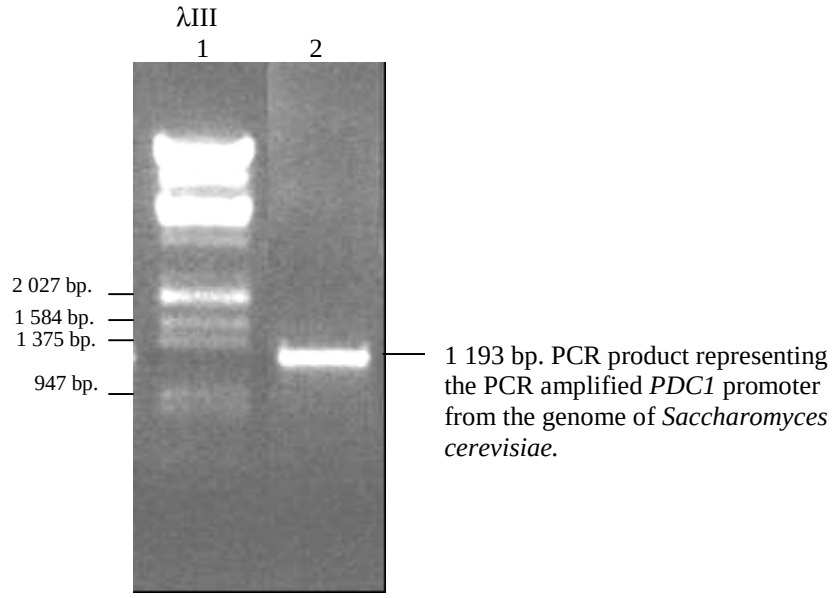


Figure 4.18. An ethidium bromide stained 1% agarose gel showing the PCR amplified *PDC1* promoter. Lane 1, λIII DNA marker. Lane 2 the 1 193 bp. PCR amplified *PDC1* promoter with the introduced *Eco*RI and *Bam*HI restriction sites.

4.9.2 Cloning of the PDC1 promoter into the expression vector pRS426

To express the *oryTAH* gene in *S. cerevisiae* the plasmid pRS426 (ATCC Number: 77107) was chosen as shuttle vector. pRS426 is a multicopy YE-type (episomal) shuttle vector, containing Ampicillin (Amp^R) and uracil (*URA*3) markers, the plasmid also contained an f1 replicon and *Lac* Z start codon, operon sequence and operator for cultivating the plasmid selectively in bacteria.

The PCR amplified *PDC1* promoter containing the *Eco*RI and *Bam*HI flanking restriction sites were ligated into the *Eco*RI/*Bam*HI digested pRS426 plasmid. To verify that the promoter was inserted into the multiple cloning site of the plasmid, the plasmid was digested with the restriction enzymes *Eco*RI and *Bam*HI (Figure 4.19). A band corresponding to the vector being 5.725 kb. in size and the cloned *PDC1*

promoter being 1.193 kb. in size, verified that the *PDC1* promoter was cloned into the pRS426 plasmid, therefore the shuttle vector was designated as pRS426-*PDC1*.

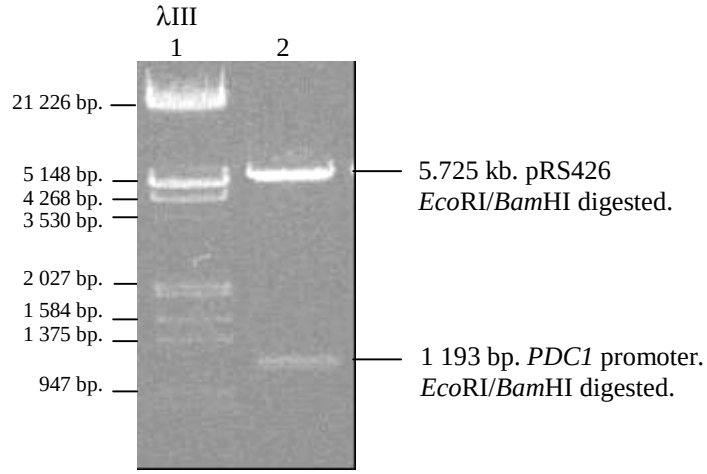


Figure 4.19. An ethidium bromide stained 1% agarose gel showing the digested *PDC1* promoter from the multiple cloning site of the vector pRS426 with *Eco*RI and *Bam*HI. Lane 1, λIII DNA marker. Lane 2, the digested pRS426 vector 5.725 kb. in size, and the 1 193 bp. *PDC1* promoter.

4.9.3 Cloning of the oryTAH gene into the expression vector pRS426-PDC1

To clone the *oryTAH* gene into the expression vector, the tannase gene had to be ligated directly downstream of the promoter sequence. The plasmid pRS426-*PDC1* was digested with the restriction enzyme *Eco*RI and to avoid self-ligation of the plasmid the restriction sites were dephosphorylated. The *oryTAH* fragment was excised from the pGEM-T Easy plasmid with the enzyme *Eco*RI, in order to allow the *Eco*RI restriction compatible sites to be ligated into each other, thus cloning the *oryTAH* gene fragment downstream to the *PDC1* promoter (Figure 4.20).

Furthermore for expression the tannase gene had to be inserted downstream to the *PDC1* promoter in the correct orientation, with the start codon of the gene sequence directly downstream to the promoter sequence in the plasmid. To determine the orientation of the inserted tannase gene in respect to the *PDC1* promoter a restriction map was compiled to establish the size of fragments that would have been liberated if the gene was in the correct orientation. The restriction enzymes that were chosen for the restriction analysis were *Xho*I and *Kpn*I (Figure 4.21). A positive clone was selected by subjecting various clones to restriction analysis with these restriction enzymes, a clone with the expected fragment sizes were selected (6 bands – 6 032 bp., 235 bp., 202 bp., 149 bp., 15 bp. and 10 bp.) (Figure 4.22). Figure 4.22 shows that only four bands were visible, this was due to the fact that the smallest two fragments were 10 and 15 base pairs in size, and thus too small to visualize on the gel. After identification of a clone containing the transcriptionally viable tannase gene, the plasmid was transformed into *S. cerevisiae* (strain L5529, *MAT*α, *ura*3-52, *his3::hisG)* for expression and characterization of the recombinant tannase.

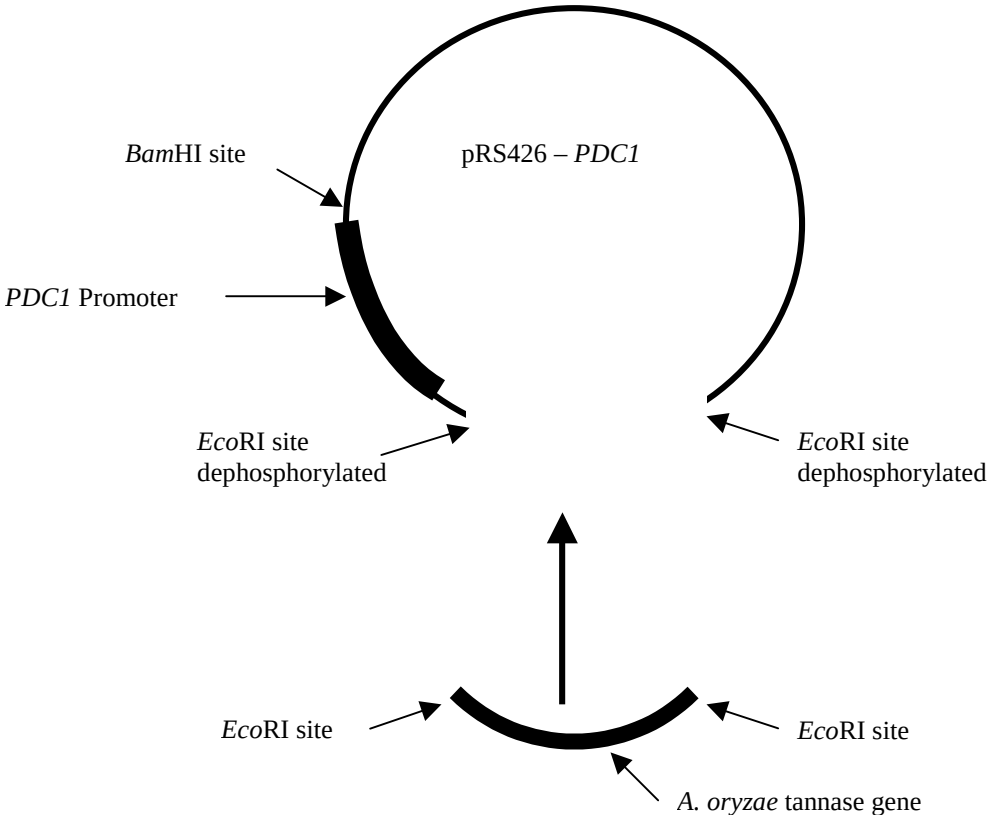


Figure 4.20. A schematic representation of the construction of the pRS426-*PDC1-oryTAH* expression shuttle vector, showing the ligated *PDC1* promoter and the dephosphorylated *Eco*RI sites for the ligation of the tannase gene from *A. oryzae.* The *PDC1* introduced *Bam*HI and *Eco*RI restriction sites are also shown.

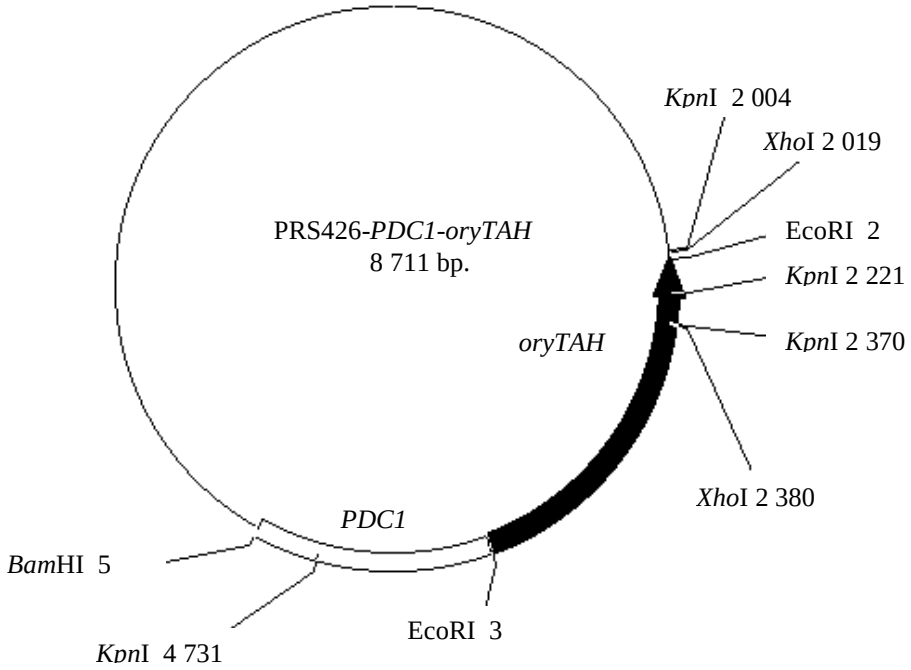


Figure 4.21. A schematic representation of the expression vector pRS426 containing the ligated *PDC1* promoter and tannase gene in the right transcriptional orientation to the promoter sequence. The relevant restriction sites are also shown.

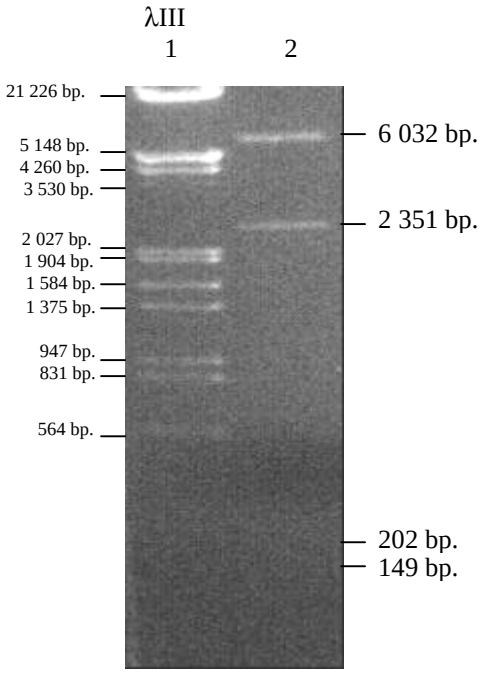


Figure 4.22. An ethidium bromide stained 1% agarose gel depicting the restriction profile of the plasmid pRS426-*PDC1-oryTAH* with the restriction enzymes *Kpn*I and *Xho*I. Lane 1, λIII DNA marker. Lane 2, the 4 expected restriction fragments liberated upon restriction of the pRS426-*PDC1-oryTAH* plasmid, with the tannase gene ligated in the correct transcriptional orientation to the *PDC1* promoter.

4.9.4 Transformation of the constructed pRS426-PDC1-oryTAH plasmid into S. cerevisiae

S. cerevisiae transformed with the pRS426-*PDC1-oryTAH* were selectively grown on uracil deficient media. The incorporated *PDC1* promoter is active in the presence of glucose and because the selective growth media contained 2% glucose, the recombinant tannase was expressed constitutively.

To visualize the expression of the recombinant tannase gene a tannase plate assay was performed (Bradoo *et al.*, 1998). The plate assay was based on the complex formation

and precipitation of 0.5% quinine hydrochloride in the presence of tannic acid (Aoki *et al.*, 1978). The quinine and tannic acid was added to YNB media used to cultivate the transformed yeasts. Upon expression of the recombinant tannase gene the tannic acid is hydrolysed into gallic acid and glucose, which leads to a drop in pH and the formation of water-soluble complexes that leads to the solubilisation of the quinine and subsequently the formation of a clear zone around the tannase expressing yeasts (Figure 4.23).

An appropriate control was an untransformed yeast strain, also grown on the plate assay media, to check that the untransformed yeasts did not express the protein in question. From the formation of a clear zone around the colony, it was concluded that the recombinant protein was excreted, most probably due to the leader peptide sequence, which was included in the sequence of the tannase gene.

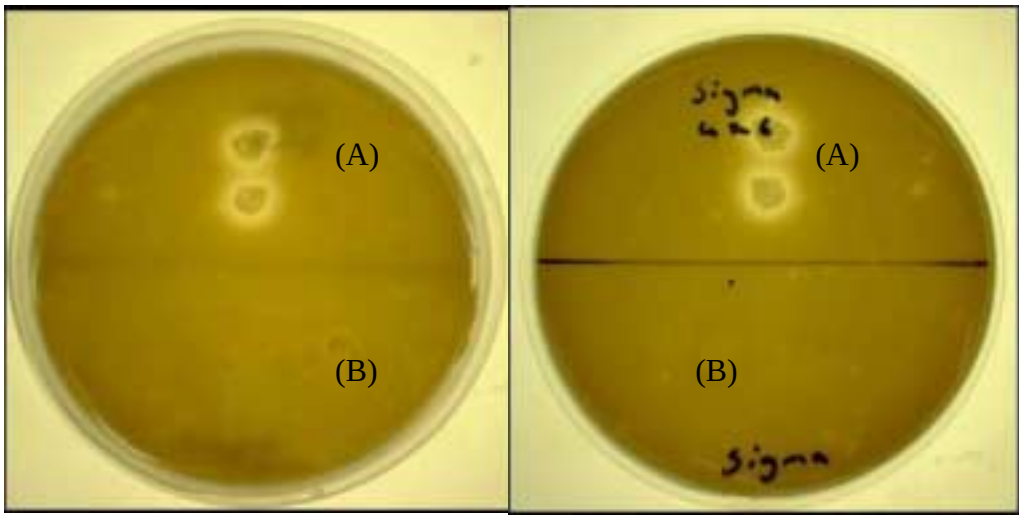


Figure 4.23. A photograph of the pRS426-*PDC1-oryTAH* transformed yeasts, labeled A in the photograph. Around the transformed yeast strains clear zones were visible, indicating expression of the recombinant tannase. The untransformed control cells (B) served as negative control.

4.9.5 Characterization of the recombinant tannase enzyme as expressed by S. cerevisiae

The recombinant tannase was expressed in very low amounts. The low activity was not anticipated due to the inclusion of the *PDC1* promoter directly upstream to the tannase gene in the pRS426 expression vector. The *PDC1* promoter is inducible by the presence of glucose. According to literature the *PDC1* promoter drives transcription when cells are actively growing (Kellerman, 1986). Therefore cells growing exponentially have a higher respiration rate, which meant that there is a constant flow of glucose into the cell for glycolysis to take place. The presence of glucose influx induces the *PDC1* promoter that actively drives transcription of the recombinant tannase (Hohmann, 1991). With this in mind high levels of tannase expression were anticipated however the opposite was found.

To try to isolate a larger amount of tannase, a one litre culture was grown and the growth rate of the yeast cells were carefully monitored (Figure 4.24). The cells were harvested in late exponential growth phase (21 hours), and the extracellular fraction was concentrated to a final volume of 2 ml by ultra-filtration through an Amicon ultra-filtration membrane. The sample of total extracellular proteins were tested for tannase activity, using the standard tannase enzyme assay as described in Section 3.7.2.

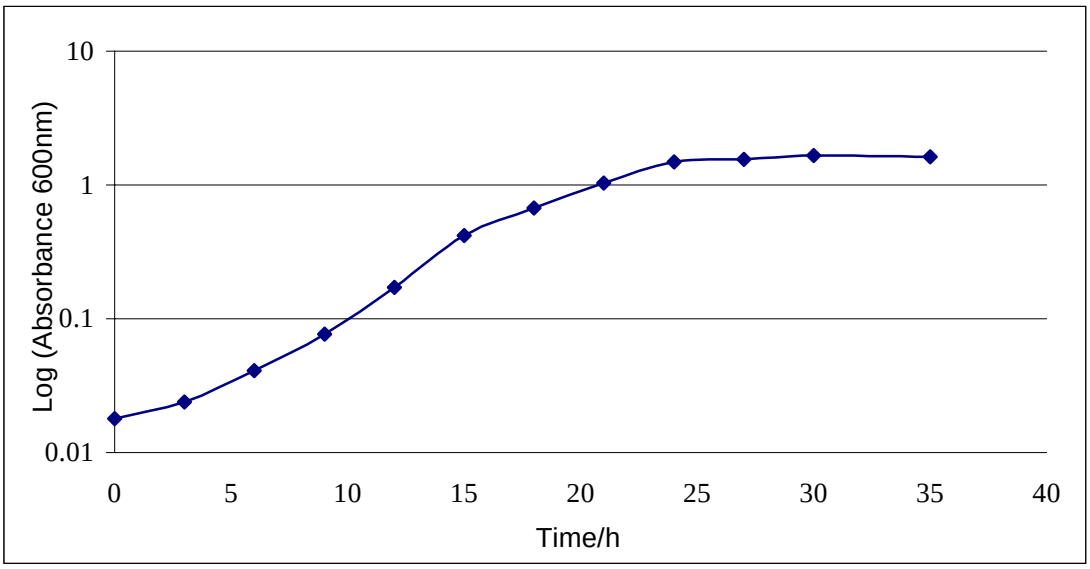


Figure 4.24. A graph representing the growth curve of the transformed yeast *S. cerevisiae* with the expression vector pRS426-*PDC1-oryTAH.* Late exponential growth phase was reached at approximately 21 hours.

To explain the low level of tannase expression the following possibilities need to be investigated. Does *S. cerevisiae* cleave the leader peptide sequence from the protein during posttranslational modification and is the protein channelled extracellularly. Furthermore is the recombinant tannase cleaved by a KEX-II like protease upon expression and is the tannase subunits correctly linked by a disulfide bond formation (Section 4.7). To verify this hypothesis the recombinant protein would have to be isolated and the chains N-terminally sequenced to determine whether the recombinant protein was cleaved by the signal peptidase and the KEX-II protease. Another factor that could influence the level of enzyme expression was the (nuclear) codon usage of *S. cerevisiae* to translate mRNA to amino acids, however according to Ohama *et al.*, (1993) yeasts including *Saccharomyces cerevisiae, Candida azyma, Candida diversa, Candida magnoliae, Candida rugopelliculosa, Yarrowia lipolytica,* and *Zygoascus hellenicus,* use the standard (nuclear) code. Therefore the hypothesis of having low expression due to differences in nuclear codon usage is highly unlikely.

Yeasts however are known to hyper-glycosylate glycoproteins. According to literature the expression of recombinant proteins containing aspargine-linked or O-linked carbohydrates can be problematic. The subsequent modification of side chains are quite different than the modifications found in other eukaryotic cells. Instead of the "extensive cleavage" of the oligosaccharide core and subsequent addition of sialic acid, galactose and *N*-acetylglucosamine, the secreted yeast glycoproteins undergo less extensive modification and cleavage of the core, and even further elongation of the outer chains of mannose residues. As a result secreted recombinant proteins from yeasts may be inactive or different from the natural proteins (Balloou, 1982). To confirm this, the glycosylation patterns in the secreted recombinant proteins will have to be determined.

4.9.6 Optimum pH of the expressed recombinant tannase enzyme

The optimum pH for the expressed tannase enzyme from *A. oryzae* as expressed by *S. cerevisiae* is shown in Figure 4.25. The expressed tannase has an optimum pH value of pH 5.75 which is identical to that reported for the native enzyme.

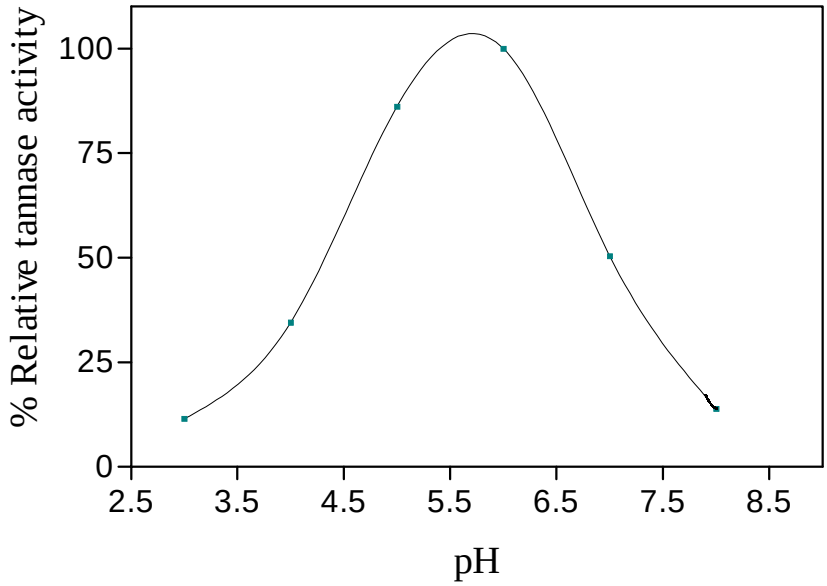


Figure 4.25. Optimum pH of *A. oryzae* tannase as expressed by *S. cerevisiae*, with (-)-epigallocatechin-3-ol-gallate as substrate.

The pH optimum for tannase isolated from *A. niger* was shown to be between 5.0 and 6.0 (Iibuchi *et al.,* 1968). Barthomeuf *et al.,* (1994) showed that the tannase from *A. niger* contained both esterase and depsidase activity with the esterase activity peaking at a pH of 5.0 with the tannase activity peaking at a pH of 5.0. Iibuchi *et al.,* (1968) showed that the tannase isolated from *Chryphonectria parasitica* had an optimum pH of 5.5. Piater, (1999) showed that tannase isolated from *A. alliaceus* had an optimum pH of 5.67, correlating well with the optimum pH of 5.75 found in the recombinant expressed tannase. She also reported that 2 different optimum pH values were found when (-)-eigallocatechin-3-ol-gallate and propyl gallate was used as substrate, suggesting the presence of tannase activity for (-)-eigallocatechin-3-ol-gallate as substrate and esterase activity when propyl gallate was used as substrate (Haslam & Tanner, 1970).

4.9.7 Kinetic parameters of the expressed recombinant tannase enzyme

The kinetic data for the expressed recombinant tannase is shown in Figure 4.26. The substrate relationship is represented in the form of a Michaelis-Menten plot. The Km and Vm values were calculated with (-)-epigallocatechin-3-ol-gallate as substrate. Regression analysis was performed using Graph Pad Prism® computer program version 3.0 Graph Pad software incorporated Copyright 1994 – 1999.

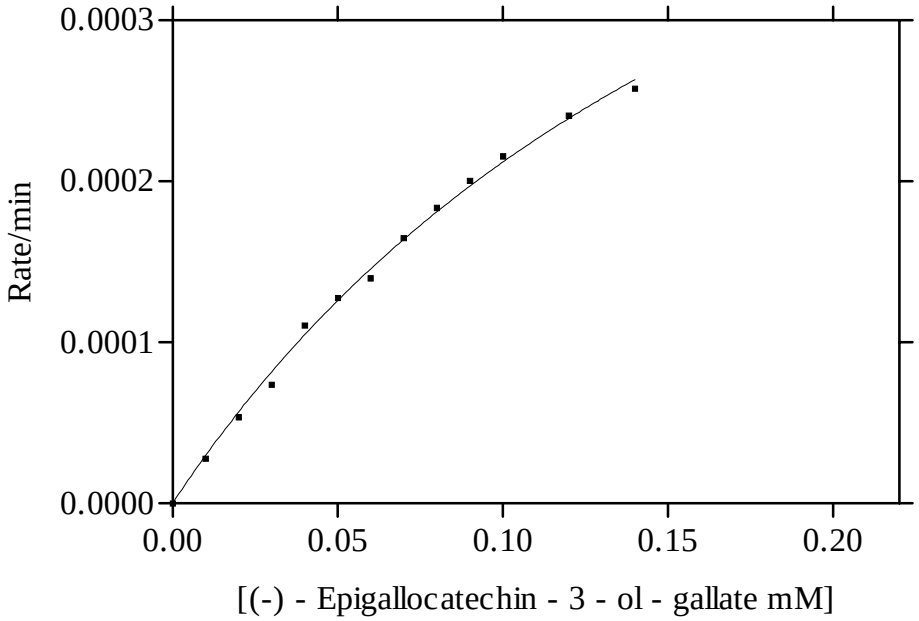


Figure 4.26. Michaelis-Menten kinetics of the recombinant tannase from *A. oryzae* with (-)-epigallocatechin-3-ol-gallate as substrate.

The K_m and V_m values for the substrate was calculated to be V_m=2,35X10^{-4} (units) and K_m: 0.02354 mM (-)-epigallocatechin-3-ol-gallate. Unfortunately, we consistently observed what resembled substrate inhibition at higher concentrations. The estimate of K_m is therefore not very reliable but it does correspond very well with the value of 0.019 obtained for the same substrate by Piater, (1999). This phenomenon could also be a result of incorrect glycosylation by the yeast. Only two K_m values were found in literature from *A. flavus* and *Penicillium chrysogenum.* The values available for the two species are for different substrates, and not for the natural substrate on which the

organism grows, which made it very difficult to compare the calculated K_m and V_m values of the recombinant expressed tannase in comparison to those calculated from literature. Tannase from *P. chrysogenum* had a calculated K_m value of 0.48 X 10^{-4} mM for tannic acid as substrate (Rajakumar & Nandy, 1983) and in the case of *A. flavus* the K_m values were 0.5 X 10^{-4} M for tannic acid as substrate, and 1.4 X 10^{-4} M for glucose – 1 – gallate (Yamada *et al.*, 1968). Therefore the calculated K_m and V_m values for the recombinant tannase could not be compared to any other published values, thus further investigation needed to be conducted with various other substrates.

4.10 General conclusions

A comparison between the cDNA sequence for tannase from *A.* oryzae with the genomic DNA sequence for tannase from *A. niger* revealed that no introns were present in the genes. The tannase genes from *A. oryzae* and *A. niger* proved to be very similar. The tannase gene from *A. niger* was 1 740 bp. in size contrast to the tannase gene from *A. oryzae* being1 767 bp. in size. DNA sequence alignment studies between the tannase gene sequences revealed that there was a 76.76% homology between the two genes on nucleotide level. Amino acid alignments revealed that the ORF for tannase from *A. niger* showed a 71.5% identity and a similarity of 10.19% to the amino acid sequence for tannase from *A. oryzae*. During posttranslational modification the peptide sequence for tannase from *A. oryzae* is cleaved into two subunits by a KEX-II like protease at position 315 and 316, liberating to peptide subunits of 30 000 Da and 33 000 Da in size. The KEX-II like protease site is identifiable in the protein sequence of *A. niger* at positions 307 and 308 where the KEX-II protease cleaves the carboxyl side of dibasic residues (Lys-Arg). Therefore it was hypothesized that the tannase from *A. niger* also undergoes KEX-II like protease cleavage liberating two subunits of 33 700 Da and 30 400 Da.

The tannase gene from *A. oryzae* was PCR amplified and cloned into an expression vector, pRS426 containing a PCR amplified *PDC1* glucose induced promoter (Hohmann, 1986). Low amounts of catalytically active tannase was expressed by *S. cerevisiae* and it was hypothesized that the recombinant tannase was hyper-

glycosylated by the expression host, resulting in secreted recombinant proteins that may be inactive or different from the natural proteins (Balloou, 1982). To confirm this hypothesis further investigation is however needed. To characterise the expressed recombinant tannase, further investigation is needed, with possibly using an *Aspergillus* tannase mutant strain as expression host.

Chapter 5

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Chapter 6

Summary

The aim of the study was to investigate *Aspergillus* spp. for tannase activity, to sequence the genes of positive isolates and to use the knowledge available to express a tannase gene from one of the *Aspergillus* isolates in *Saccharomyces cerevisiae,* together with the characterization and comparison of the recombinant tannase with published properties of other tannases.

Tannin acyl hydrolase (E.C. 3.1.1.20) is commonly referred to as tannase. Tannase is responsible for the hydrolysis of ester and depside linkages in hydrolysable tannins to liberate gallic acid and glucose. Tannase have been isolated from a wide range of organisms including bacteria, fungi and yeasts. *A. alliaceus, A. fumigatus, A. niger* and *A. oryzae* were positively identified as tannase producers with tannic acid as sole carbon source.

The tannase gene from *A. niger* was cloned and sequenced. The ORF for tannase from *A. niger* consisted of 1 740 bp. Tannase genes from *A. niger* and *A. oryzae* revealed that the genes were 76.76% identical on nucleotide level. The 1 740 bp. tannase gene from *A. niger* coded for a 579 amino acid sequence containing a possible 16 amino acid leader peptide sequence. On amino acid level the tannase gene from *A. niger* showed a 71% identity and a 10.19% similarity with the known amino acid sequence for tannase from *A. oryzae.* Analysis of the amino acids sequence for tannase from *A. niger* revealed the occurrence of a KEX-II like protease cleavage site. It was hypothesized that the tannase gene from *A. niger* was translated as a single polypeptide chain of 579 a.a long. Upon expression the signal peptide was cleaved followed by disulfide bond formation and subsequent cleavage of the single polypeptide chain into two subunits by the KEX-II protease. The protein sequence for tannase from *A. niger* showed high probabilities for N- and O- linked glycosylation sites, revealing the possible glycoprotein nature of the tannase from *A. niger.* The tannase gene from *A. oryzae* was cloned and expressed in *S. cerevisiae.* The recombinant tannase had an optimum pH of 5.75 with (-)-

epigallocatechin-3-ol-gallate as substrate. The K_m and V_m values for the substrate was calculated to be V_m=2,35X10^{-4} (units) and K_m: 0.02354 mM (-)-epigallocatechin-3-ol-gallate. The recombinant tannase was expressed in very low amounts probably due to the phenomenon exhibited in yeasts to hyper glycosylate glycoproteins, therefore expressing proteins that are often catalytically inactive or different than the native enzymes.

To fully elucidate the kinetics of the recombinant expressed tannase future expression will be required in an established *Aspergillus* expression host.

Chapter 7
Opsomming

Die doel van die studie was om *Aspergillus* spp. te ondersoek vir tannase aktiwiteit, die geen volgorde van positiewe isolate te bepaal en om van bestaande kennis gebruik te maak om ‘n rekombinante tannase van een van die *Aspergillus* isolate uit te druk in *Saccharomyces cerevisiae*. Asook om die rekombinante ensiem te karaktiriseer en om die data te vergelyk met reeds gepubliseerde data van tannase.

Tannien asiel hidrolase (E.C. 3.1.1.20) is algemeen bekend as tannase. Tannase hidroliseer ester en depsied bindings in hidroliseerbare tanniene om vrystelling van galliensuur en glukose te bewerkstellig. Tannase is reeds geïsoleer van ‘n wye reeks organismes, insluitend bakterieë, fungi en giste. *A. alliaceus, A fumigatus, A niger* and *A. oryzae* is geïdentifiseer as tannase produseerders op tanniensuur as enigste koolstofbron.

Die tannase geen van *A. niger* is gekloneer en die DNS volgorde is bepaal. wat uit ‘n oop leesraam van 1740 bp. bestaaan. Nukleotied vergelykings tussen die tannase geen van *A. niger* en *A. oryzae* het 76.76% identiteit op nukleotied vlak getoon. Die 1 740 bp. tannase geen van *A. niger* kodeer vir ‘n 579 aminosuur volgorde met ‘n moontlike 16 aminosuur leier peptied volgorde. ‘n Identiteit van 71% en ‘n ooreenkoms van 10.19% is waargeneeem tussen *A. niger* en *A. oryzae* tannase.’n Analise van die aminosuur volgorde dui op die teenwoordigheid van ‘n KEX-II protease snydingssetel. Daar word dus voorgestel dat die tannase geen van *A. niger* getransleer word as ‘n enkel polipeptied ketting van 579 aminosure. Na verwydering van die seinpeptied en vormingvan disulfied bindings, is gespekuleer dat die enkel polipeptied ketting gesny word in twee subeenhede deur ‘n KEX-II protease. Die tannase van *A. niger* is waarskynlik ‘n glikoproteïen as gevolg van die hoë waarskynlikheid van N- en O- gebonde glikosilerende setels op die proteïen volgorde van tannase.

Die tannase geen van *A. oryzae* is gekloneer en uitgedruk in *S. cerevisiae*. Die rekombinante tannase het ‘n optimum pH van 5.75 getoon met (-)-Epigallocatechin-3-ol-

gallate as substraat. Die K_m en V_m waardes vir die betrokke substraat, was V_m=2,35X10^{-4} (eenhede) and K_m: 0.02354 mM (-)-epigallocatechin-3-ol-gallate.. Lae vlakke van tannase uitdrukking is waargeneem in *S. cerevisiae*, weens die geneigdheid van giste om glikoproteïne te hiperglikosileer. Dit kan lei tot die uitdrukking van rekombinante proteïne wat katalities onaktief is. Ten slotte, om die rekombinante tannase volledig te karakteriseer, sal gebruik gemaak moet word van ‘n *Aspergillus* uitdrukkings gasheer.