CLONING OF THE *SCYTALIDIUM THERMOPHILUM* BIFUNCTIONAL CATALASE / PHENOL OXIDASE GENE AND EXPRESSION IN *ASPERGILLUS SOJAE*

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CLONING OF THE *SCYTALIDIUM THERMOPHILUM*
BIFUNCTIONAL CATALASE / PHENOL OXIDASE GENE AND
EXPRESSION IN *ASPERGILLUS SOJAE*

submitted by **HATİCE ÖZLEM ERÇİN** in partial fulfillment of the requirements for the degree of **Master of Science in Food Engineering Department, Middle East Technical University** by,

Prof. Dr. Canan Özgen _____
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. Zümrüt B. Ögel _____
Head of Department, **Food Engineering**

Prof. Dr. Zümrüt B. Ögel _____
Supervisor, **Food Engineering Dept., METU**

Prof. Dr. Ufuk BAKIR _____
Co-Supervisor, **Chemical Engineering Dept., METU**

Examining Committee Members:

Assoc. Prof. Dr. Candan GÜRAKAN _____
Food Engineering Dept., METU

Prof. Dr. Zümrüt Begüm ÖGEL _____
Food Engineering Dept., METU

Prof. Dr. Ufuk BAKIR _____
Chemical Engineering Dept., METU

Prof. Dr. Mustafa AKÇELİK _____
Biology Dept., Ankara University

Assist. Prof. Dr. Deniz ÇEKMECELİOĞLU _____
Food Engineering Dept., METU

Date: 08.02.2008

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name : Hatice Özlem ERÇİN

Signature :

ABSTRACT

CLONING OF THE *SCYTALIDIUM THERMOPHILUM* BIFUNCTIONAL CATALASE / PHENOL OXIDASE GENE AND EXPRESSION IN *ASPERGILLUS SOJAE*

Erçin, Hatice Özlem

M.Sc., Department of Food Engineering

Supervisor : Prof. Dr. Zümrüt Begüm Ögel

Co-Supervisor: Prof. Dr. Ufuk Bakır

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Scytalidium thermophilum is a thermophilic fungus with an important role in the composting process of mushroom cultivation. An extracellular phenol oxidase of *Scytalidium thermophilum* (STEP) with novel features was previously studied in our laboratory. This enzyme later turned out to be a catalase having phenol oxidase activity.

The aim of this study was to clone *Scytalidium thermophilum* bifunctional catalase/phenol oxidase encoding gene and express the gene in *Aspergillus sojae* for future site directed mutagenesis studies. *Scytalidium thermophilum* catalase gene was first cloned into *E. coli* XL1 Blue MRF' and then heterologously expressed in *Aspergillus sojae* ATCC11906. For that aim, the catalase gene was amplified using specific primers, excluding the signal and pro-peptide coding regions and amplified fragment was then cloned into *E.coli* XL1 Blue MRF' and

sequenced. It was observed that the cloned gene, named as *catpo*, was 10 amino acids different from the amino acid sequence of the *S.thermophilum* catalase gene formerly cloned by Novo Nordisk. The *catpo* gene encoding a mature protein of 681 amino acids was then ligated onto expression vector pAN52-4 and the recombinant plasmid was transformed into *Aspergillus sojae* ATCC11906. Heterologuos expression was observed under the control of the glyceraldehydes 3-phosphate dehydrogenese promoter of *Aspergillus nidulans* and the secretion signal of the glucoamylase gene of *Aspergillus niger*. Catalase activity of the transformants reached at a level of 13206 U/g at the end of the fourth day of cultivation. However, this is still lower than the catalase activity of the gene donor strain of *Scytalidium thermophilum*.

Keywords: catalase, phenol oxidase, gene cloning, *Scytalidium thermophilum*

ÖZ

SCYTALIDIUM THERMOPHILUM ÇİFT AKTİVİTELİ KATALAZ/FENOLOKSİDAZ GENİNİN KLONLANMASI VE *ASPERGILLUS SOJAE*'DE HETEROLOG İFADESİ

Erçin, Hatice Özlem
Yüksek Lisans, Gıda Mühendisliği Bölümü
Tez Danışmanı : Prof. Dr. Zümrüt Begüm Ögel
Ortak Tez Yöneticisi: Prof. Dr. Ufuk Bakır

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Scytalidium thermophilum, termofilik bir küf olup yemeklik mantar olan *Agaricus bisporus*'un üretilmesinde verimi artırıcı önemli bir role sahiptir. Laboratuvarımızda daha önce yapılan çalışmalara göre, *Scytalidium thermophilum* diğer fenol oksidazlarda görülmeyen bazı farklı özelliklere sahip yeni bir hücre dışı fenol oksidaz (STEP) üretmektedir. Daha sonra yapılan çalışmalar sonucunda, *S. thermophilum* hücre dışı fenol oksidaz enziminin aslında bir katalaz olduğu, bunun yanında da fenol oksidaz aktivitesine sahip olduğu belirlenmiştir.

Bu çalışmada, daha sonra yapılacak olan yönlendirilmiş mutasyon çalışmaları için *Scytalidium thermophilum* çift aktiviteli katalaz/fenol oksidaz enzimini kodlayan genin klonlanması ve *Aspergillus sojae*' de heterolog olarak üretimi amaçlanmıştır. *Scytalidium thermophilum* katalaz geni ilk olarak *E.coli* XL1

Blue MRF' e klonlanmış, daha sonra *Aspergillus sojae* ATCC11906' de heterolog olarak ifade edilmiştir. Bu amaçla, sinyal peptit ve pro-peptit bölgesini dışarıda bırakacak şekilde, genin başından ve sonundan tasarlanan spesifik primerler vasıtasıyla katalaz geninin amplifikasyonu gerçekleştirilmiştir. Elde edilen parçalar *E.coli* XL1 Blue MRF'e klonlanmış ve klonlanan gen *catpo* olarak adlandırılmıştır. Dizilim analizi sonucunda, *catpo* geninin kodladığı amino asit dizilimindeki 10 amino asidin, daha önce Novo Nordisk tarafından klonlanmış olan *Scytalidium* katalaz geninin kodladığı amino asit diziliminden farklı olduğu belirlenmiştir. Daha sonra, 681 amino asitten oluşan olgun proteini kodlayan *catpo* geni, pAN52-4 ekspresyon vektörlerine aktarılmış ve elde edilen rekombinant plazmidin *Aspergillus sojae* ATCC11906 suşuna transformasyonu gerçekleştirilmiştir. *Aspergillus niger* glukoamilaz geninin sinyal peptit bölgesi altına takılan *catpo* geninin, *Aspergillus nidulans* gliseraldehit-3-fosfat dehidrogenaz promotörü kontrolünde heterolog ekpresyonu tanımlanmıştır. *Aspergillus sojae* transformantlarında katalaz aktivitesi 13206 U/g değerine ulaşmıştır, fakat bu değer gen donörü olan *Scytalidium thermophilum*'un katalaz aktivitesinden daha düşüktür.

Anahtar Sözcükler: katalaz, fenol oksidaz, gen klonlama, *Scytalidium thermophilum*

To My Parents

Perihan Erçin & Sami Erçin

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CHAPTER 1

INTRODUCTION

1.1 Catalases

Catalases (EC 1.11.1.6), belonging to oxidoreductases, are a group of metalloenzymes with the ability to catalyse the decomposition of hydrogen peroxide into water and molecular oxygen (Goldberg *et al*, 1989) as shown in Reaction 1.

$$2H_2O_2 \longrightarrow 2H_2O + O_2 \qquad \text{(Reaction 1)}$$

This overall reaction appears to be simple; however it consists of two distinct stages. What is involved in each of the stages depends on the type of catalases (Chelikani *et al.,* 2003). The mechanism relevant to a particular catalase will be explained in section 1.1.2.

Catalase is a highly conserved heme-containing enzyme that is found in all aerobic organisms including prokaryotes and eukaryotes (Vetrano *et al*., 2005 and Schrerer *et al*., 2002). It is proposed that catalases play an important role in organisms which have evolved to survive under aerobic conditions because of their wide distribution, evolutionary conservation, and capacity to rapidly degrade hydrogen peroxide (Scandalios, 2005). Catalases have been isolated

from microorganisms, plants and animals (Klotz *et al.,* 1997) and now more than 300 catalase sequences are available in the protein databank. Fungi are reported to be effective producers of catalases and different types of catalases and catalase genes have been isolated from fungi (Isobe *et al*., 2006). Some of the examples of catalases that have been isolated from different fungi: *Alternaria alternata* (Caridis *et al.,* 1991), *Aspergillus fumigatus* (Bemmann *et al*., 1981; Paris *et al*., 2003; Shibuya *et al*., 2006), *Aspergillus nidulans* (Kawasaki *et al*., 1997, 2001, Scherer et al., 2002), *Aspergillus niger* (Kikuchi-Torii *et al*., 1982; Fowler *et al*., 1993; Kulys *et al.,* 2003), *Aspergillus oryzae* (Hisada *et al*., 2005), *Blumeria graminis* (Zhang *et al*., 2004), *Candida albicans* (Wysong *et al*., 1998), *Cladosporium fulvum* (Bussink *et al*., 2001) *Neurospora crassa* (Jacob *et al*., 1979; Pereza *et al*., 2002), *Penicillium marneffei* (Pongpom *et al*., 2005), *Penicillium simplicissimum* (Fraaije *et al*., 1996), *Penicilium vitale* (Vainshtein *et al*., 1986; Kulys *et al*., 2003), *Thermoascus aurantiacus* (Wang *et al*., 1998).

1.1.1 Classification of Catalases

Catalases can be classified into three main groups according to structural (subunit sizes, quarternary structures, heme prosthetic groups, sequences, *etc.*) and functional similarities (Nicholls *et al*., 2001 and Zamocky *et al*., 1999):

1. Monofunctional (typical) catalases
2. Catalase-peroxidases
3. Nonheme catalases

1.1.1.1 Monofunctional (typical) Catalases

Homotetrameric and heme-containing monofunctional catalases comprise the largest and the most extensively studied group of catalases. The predominant activity of this group of enzymes is the decomposition of hydrogen peroxide and any peroxidatic activity is minor and restricted to small substrates. The

monofuntional catalases that have been characterized in greatest detail until now are active as tetramers with a total molecular weight range 200-340 kDa with four prosthetic haem groups (Nicholls *et al*., Hisada *et al*., 2005, Zamocky *et al.,* 1999). Based on the subunit size and heme content, this group can be divided into two subgroups: one containing small subunit enzymes (55 to 69 kDa) with heme *b* associated, one containing large subunit enzymes with heme *d* associated. On the other hand, according to the phylogeny, monofunctional catalases can be divided into three clades, arising from a minimum of two gene duplication events. Clade I catalases generally contain enzymes of plant origin, but also include one branch of bacterial catalases and one algae catalase. Clade II catalases are all large subunit enzymes of bacterial and fungal origin. One archaebacterial Clade II catalase is also present, which may be due to a horizontal transfer event from a *Bacillus* species. Clade III catalases are all small subunit catalases from bacteria, archaebacteria, fungi and animals. In older taxonomic groups; Clade III catalases are not present, therefore it is proposed that they arose much later in evolution as a result of gene duplication in bacteria that then spread by horizontal and lateral transfers among bacteria to archaebacteria and eukaryotes (Klotz *et al*., 1997, Nicholls *et al*., 2001 and Chelikani *et al*., 2003). When combined with the classification according to subunit sizes and heme content; Clade I and Clade III catalases are generally composed of small subunit catalases with heme *b* associated, while Clade II enzymes are all large subunit enzymes with heme *d* associated. Figure 1.1 shows the structural differences in heme *b* and heme *d*.

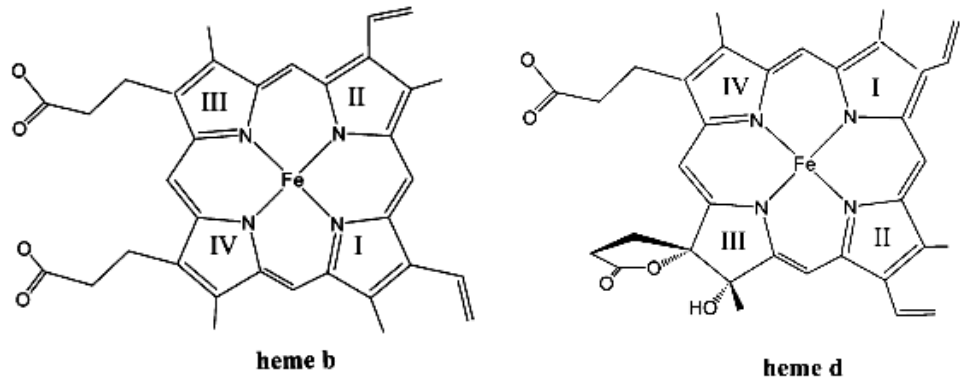


Figure 1.1 Structure of heme *b* and heme *d* (Alfonso-Prieto *et al*., 2007).

1.1.1.2 Catalase-Peroxidases

Catalase-peroxidases, the next largest group of catalases, are also heme containing enzymes and as the name indicated, they exhibit a significant peroxidatic activity in addition to the catalatic acitivity. There is more uniformity in sequence within catalase-peroxidases which contain heme *b* and have subunits larger than 80 kDa with some exceptions. Moreover, catalase-peroxidases show much higher sequence homology with haem peroxidases than with typical catalases. Catalase-peroxidases are active as dimers or tetramers with a total molecular weight range 120-340 kDa. A phylogenetic analysis does not reveal any major subgroupings for them like in the monofunctional catalases (Nicholls *et al,* 2001; Scherer *et al*., 2002; Zamocky *et al*., 1999).

1.1.1.3 Nonheme Catalases

In this group of catalases, the active site contains manganese-rich reaction center instead of a heme group. Because of the lack of heme in the active site, they are also called “pseudo-catalases” (Nicholls *et al*., 2001). Only a few of nonheme

catalases are known and they may have unusual oligomeric structures (homopentamers or homohexamers) with a total molecular weight range of 170-210 kDa (Zamocky *et al*., 1999).

1.1.2 Reaction Mechanism of Catalases

As mentioned in section 1.1, the overall reaction for the degradation of hydrogen peroxide into water and molecular oxygen (Reaction 2) seems simple, but the catalytic process is thought to occur in two stages. For monofunctional, heme containing catalases, the reaction mechanism is as follows:

$H_2O_2 + Enz(Por\text{-}Fe^{III}) \rightarrow H_2O + Compund\ I\ (Por^{+\bullet}\text{-}Fe^{IV}=O)$ (Reaction 2)

$H_2O_2 + Compund\ I\ (Por^{+\bullet}\text{-}Fe^{IV}=O) \rightarrow Enz(Por\text{-}Fe^{III}) + H_2O + O_2$ (Reaction 3)

In the first stage (Reaction 2), heme iron ($Por\text{-}Fe^{III}$) of the resting enzyme is oxidized using hydrogen peroxide as substrate to form an oxyferryl species with a π-cationic porphyrin (Por) radical, which is named as compund I. This stage is followed by the reduction of the compund I coupled to the oxidation of the second hydrogen peroxide molecule by compound I to regenerate the resting state enzyme, water and molecular oxygen (Reaction 3) (Nicholls *et al*., 2001, Kirkman and Gaetani, 2006).

Compund I can also be reduced with a single electron to an inactive compound II (Reaction 4) (Nicholls *et al.,* 2001)*:*

$Compund\ I\ (Por^{+}\text{-}Fe^{IV}=O) + e^{-} \rightarrow Compound\ II\ (Por\text{-}Fe^{IV}=O)$ (Reaction 4)

The overall catalatic reaction pathway of catalase-peroxidases is composed of the same two stages for the monofunctional catalases (Reaction 2 and 3). Although sequence and tertiary/quarternary structure of catalase peroxidases are very

different from monofunctional catalases, similarity in the catalatic reaction pathway may be due to the heme in the structure. In the peroxidatic reaction of catalase peroxidases, compund I is reduced by organic electron donors to the resting state via two electron transfer. The peroxidatic reaction pathway is as follows (Nicholls *et al*., 2001, Kirkman and Gaetani, 2006):

Compund I (Por^{+}-Fe^{IV}=O) + 2AH2 → Enz (Por-Fe^{III}) + 2AH + H_2O (Reaction 5)
Compund I (Por^{+}-Fe^{IV}=O) + AH2 → Compound II (Por-Fe^{IV}-OH) + AH (Reaction 5a)
Compound II (Por-Fe^{IV}-OH) + AH2 → Enz (Por-Fe^{III}) + AH + H_2O (Reaction 5b)

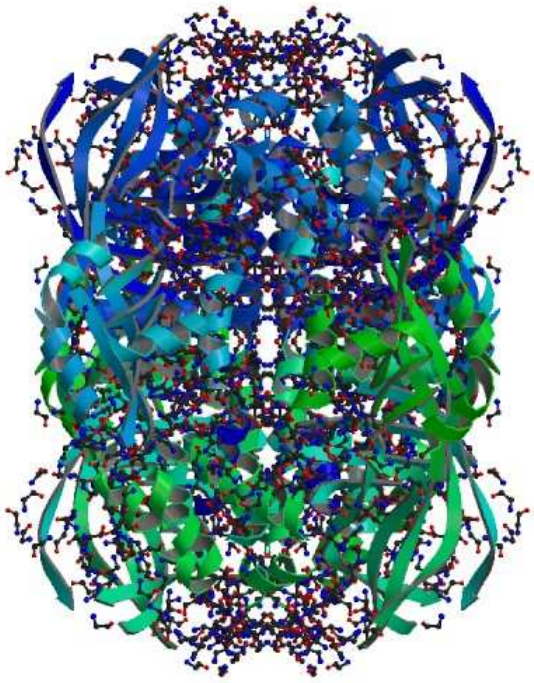
1.1.3 Structure of Monofunctional Catalases

Although there are differences in size of monofunctional catalases, they share a common feature; having a homo tetrameric quarternary structure with the heme group deeply buried in a beta-barrel core structure in each subunit. Monofunctional catalases with large subunit enzymes have extensions at both the amino and carboxyl ends. Extension at carboxyl end has flavodoxinlike structure, a unique His-Tyr bond, a protected cysteine, and a modified heme. NADPH binding and an oxidized methionine are found in small subunit enzymes.

There are four distinct regions in the tertiary structure of small subunit enzymes. Carboxyl terminal domain (flavodoxinlike domain) forms the fifth region in the tertiary structure of large subunit enzymes. The first region is the amino terminal arm, which extends 50 or more residues from the amino terminus almost to the essential histidine residue. In the N-teminal region, there is very little structural similarity. The second region is the antiparallel β-barrel which includes about

250 residues from the essential histidine toward the C-terminus. The third region is the wrapping domain. It includes 110 residues in an extended structure and links the β-barrel and the α-helical region. The fourth region is the α-helical domain and C-terminal of small subunit enzymes is included in this region (Zamocky *et al.,* 1999 and Nicholls *et al.,* 2001). As an example to structure of monofunctional catalases, three-dimensional structures of large subunit catalase from *Penicillium vitale* and large subunit catalase from *Neurospora crassa* are given in Figure 1.2.

(a)



(b)

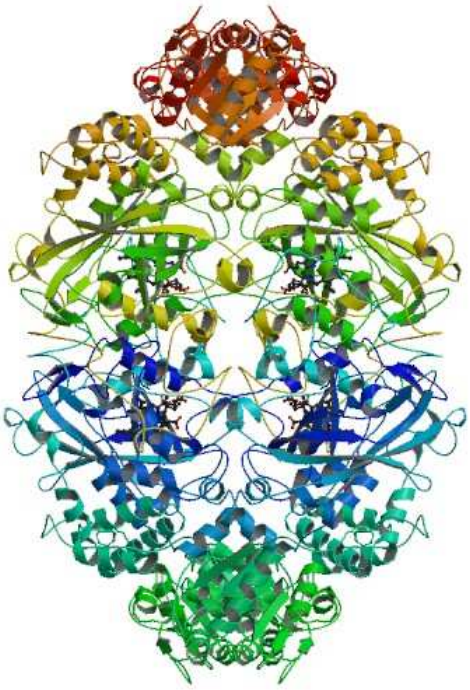


Figure 1.2 (a) Three-dimensional structure of catalase from *Penicillium vitale* (Vainshtein *et al*., 1986). (b) Three-dimensional structure of catalase from *Neurospora crassa* (Cat1) (Diaz *et al.,* 2004).

1.1.4 Industrial Usage of Catalases

Catalases are important industrial enzymes finding a wide range of usage area in industry in which it is required to remove residual hydrogen peroxide from a system to which hydrogen peroxide has been added, such as the use of catalase for the removal of hydrogen peroxide after pasteurization or bleaching. In textile industry, it is used to remove hydrogen peroxide from fabric which is bleached by an alkaline hydrogen peroxide treatment before dyeing. In pulp bleaching, catalase is also used in a similar application. Moreover, catalase is used for the removal of hydrogen peroxide from contact lenses after hydrogen peroxide disinfection (United States Patent, No. 5646025).

1.2 Phenol Oxidases

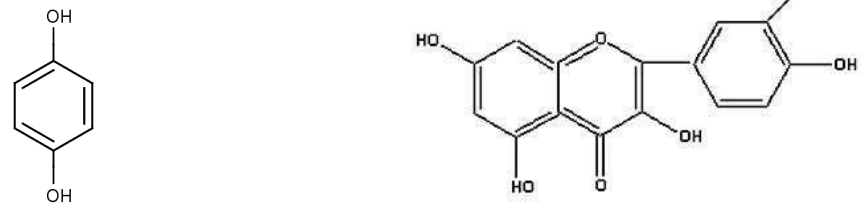
Phenol oxidases comprise a group of copper containing enzymes which catalyze the oxidation of phenolic compounds by molecular oxygen (Ögel *et al*., 2006). A wide range of substrates are used and a considerable overlap exists in the substrate affinities of these enzymes, which makes it difficult to assign precise enzymatic descriptions and categorizations (Burke *et al.,* 2002 and Griffith, 1994).

Phenol oxidase enzymes have a wide range of industrial applications including waste water treatment, production of biomaterials, applications in the alcoholic and non-alcoholic beverage industry and they also have been used for analytical purposes like biosensor preparation (Ögel *et al*., 2006).

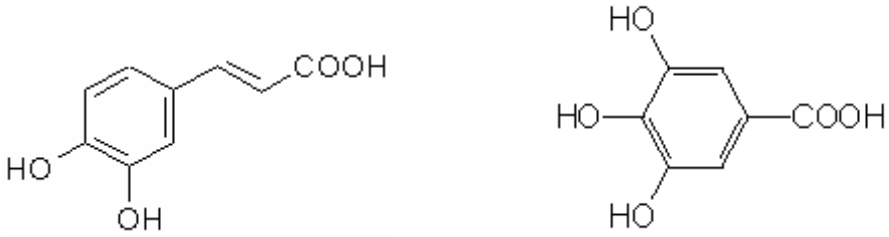
1.2.1 Phenols

Phenols, substrates of phenol oxidases, are chemical compounds consisting of a hydroxyl group attached to the benzene ring. The parent compound of this group is phenol. Phenolics vary in size; such that they include very simple phenolics like hydoxybenzoic acid and large polymers like tannins with high molecular weight (Tomas-Barberan *et al.*, 2001).

Polyphenols are produced as plant secondary metabolites and act as antioxidants due to the ability of readily donating hydrogen to stabilize radical species. Furthermore, they can interact with metal ions to form a chelate through their aromatic hydroxyl groups, which is considered to have a role in metal overload diseases and in all oxidative stress conditions involving a transition metal ion. Because of these properties of polyphenols, they have been proposed to exert effects in a maltitude of disease states, including cancer, cardiovascular disease, and neurodegenerative disorders. However, besides the beneficial effects of polyphenols, it has been reported that some polyphenols promote oxidative damage in the presence of metal ions under certain conditions. The precise mechanisms by which polyphenols exert their beneficial or toxic effects remain unclear. Examples of phenolic compounds are procyanidins (found in apple, grape, peach, etc.), oleuropein (found in olive), capsaicins (pepper), cinnamates, isoflavones, quercetin, caffeic acid, gallic acid, anthocyanins, hydroxybenzoic acid, flavanones (Akagawa et al., 2003, Tomas-Barberan *et al.*, 2001 and Williams *et al.,* 2004).



hydroquinone quercetin



caffeic acid gallic acid

Figure 1.3 Structures of some phenolic compounds (Akagawa *et al*., 2003 and Williams *et al*., 2004).

1.2.2 Classification of Phenol Oxidases

In the literature there is no exact classification for phenol oxidases, however they are generally divided into two classes. First group consists of tyrosinase (monophenol monooxygenase; EC 1.14.18.1) and catechol oxidase (o-diphenol oxidase, EC 1.10.3.1) and the second group consists of laccase (*p*-diphenol: dioxygen oxidoreductase; EC 1.10.3.2) (Ögel *et al*., 2006).

Tyrosinases have a pair of antiferromagnetically coupled copper ions at the active site which is called type 3 (Sanchez-Amat *et al.,* 1997). These enzymes catalyze two types of reactions which are *ortho* hydroxylation of monophenols

(cresolase/monophenolase activity; EC 1.14.18.1) and the subsequent oxidation of *o*-diphenols to *o*-quinones (catechol oxidase/diphenolase activity; EC 1.10.3.1). Catechol oxidase is the name of enzymes that catalyze only the latter reaction such enzymes that have only catecholase activity. Catecholase activity which is the oxidation of *o*-diphenols to *o*-quinones is responsible for the melanin synthesis since the oxidation products, *o*-quinones, undergo self polymerization or react with other substances and form high molecular weight black/brown pigments, called melanins (Marusek *et al*., 2006).

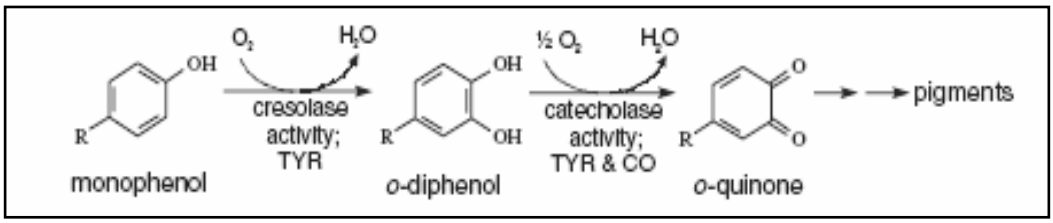


Figure 1.4 Black/brown pigments formation by the oxidation of phenolics via the cresolase activity and the subsequent catecholase activity of the enzymes (Marusek *et al*., 2006).

Laccase is a member of the family of multicopper blue oxidases, since they have three different copper centers type 1 which is the blue center characterized by the absorption around 600 nm, type 2 and type 3 (Sanchez-Amat *et al.,* 1997). Their functions are related to lignin degradation and/or detoxification of lignin degradation products, pigmentation accumulation, sporulation, detoxification of toxic compounds plant pathogenesis and mycelial morphogenesis (Score *et al*., 1996).

As mentioned earlier, it is difficult to assign unique enzymatic descriptions for phenol oxidases due to the overlap in the activities; however laccases and tyrosinases have been differentiated on the basis of substrate specifity and sensitivity to specific inhibitors. In terms of substrate specifity, only laccase is able to oxidize methoxyphenols, such as syringaldazine and only tyrosinase has a capacity to oxidize L-tyrosine. Therefore, incombination with the ability to use syringaldazine as a hydrogen donor and the inability to oxidize L-tyrosine provides an identification of laccase activity (Burke *et al*., 2002 and Sanchez Amat *et al*., 1997).

1.3 Bifunctionality of Catalases and Phenol Oxidases

In the literature, there are some examples of catalases and phenol oxidases with additional enzymatic activities besides their primary functions. Furthermore, these additional activities of both enzymes generally overlap such that; catalases have oxidative activity and phenol oxidases have catalatic activity. This relationship can be explained by the generation of hydrogen peroxide which is the substrate of catalase as a by product of pro-oxidation of polyphenols (Akagava, *et al*., 2003). In contrast to beneficial effects of polyphenols as antioxidants, production of hydrogen peroxide by the oxidation of them promote oxidative damage and an antioxidant enzyme catalase prevents this oxidative damage by modulating the level of hydrogen peroxide (Aoshima *et al*.,2005 and Scandalios, 2005).

Phenol oxidase with an additional catalatic activity was first introduced by Jolley and Russell *et al*. (1973) for mushroom tyrosinase. It was reported that a reaction had been observed between mushroom tyrosinase and hydrogen peroxide yielding a spectroscopically observable product (Russell *et al.*, 1973). Catalatic activity of mushroom tyrosinase was also reported by Yamazaki *et al.* (2004) and Garcia-Molina *et al. (*2005). Furthermore, mushroom tyrosinase has also been

reported to catalyze the oxygenation reaction of phenols with hydrogen peroxide (peroxygenase activity) (Yamazaki *et al.*, 2004). Another example for phenol oxidases with a catalatic activity is one of the isozyme of catechol oxidase from sweet potato (*Ipomoea batatas*). It has been reported that 39 kDa of isozyme exhibit a catalase-like activity when hydrogen peroxide was applied as a substrate (Gerdemann *et al.*, 2001).

Besides the phenol oxidases with an additional catalase activity, only mammalian catalase has been reported to have an oxidase activity (Vetrano *et al*., 2005). As mentioned in section 1.1.1, the primary function of catalase is decomposition of hydrogen peroxide into water and molecular oxygen, which is named as catalatic activity. Second function of catalase is the oxidation of organic electron donors (e.g., ethanol, ascorbic acid) at low concentrations (<1 μM) of hydrogen peroxide (H_2O_2) and is called as peroxidatic activity (Scandalios, 2005). Third and the novel function of catalases is oxidase activity which has been introduced by Vetrano *et al.* (2005) for mammalian catalase. Mammalian catalase which is a homotetrameric enzyme with a 60 kDa subunit size belongs to the group of monofunctional catalases with small subunit size. Besides the catalatic activity of mammalian catalase, it has been reported that mammalian catalase like other catalases exhibit peroxidatic activity at a slow rate in the presence of low concentration of hydrogen peroxide (Kirkman and Gaetani*.,* 2006).

Among microorganisms, the only known bifunctional catalase-phenol oxidase is the enzyme from the thermophilic fungus *Scytalidium thermophilum* (Sutay *et al*., 2008 in press). The enzyme, named as CATPO, is the first bifunctional catalase/phenol oxidase in the literature that is characterized in detail. According to its amino acid sequence data, CATPO is classified as a monofunctional catalase and is a 320 kDa homotetramer of 80 kDa subunits. The mechanism of phenol oxidation remains to be determined.

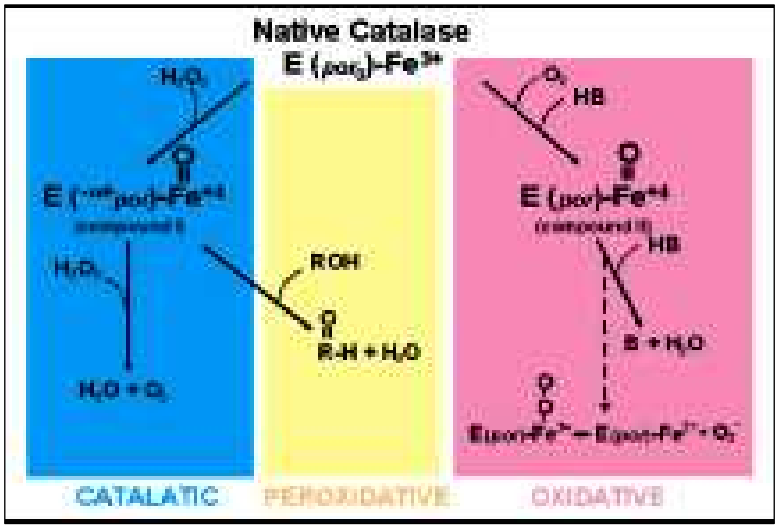


Figure 1.5 Reaction pathways of mammalian catalase; catalatic activity, peroxidatic activity and oxidative activity (Vetrano *et al*., 2005).

Vetrano *et al.* summarized three reaction pathways of the mammalian catalase as shown in Figure 1.5. In the oxidation reaction, compound II like intermediate is formed by the interaction of heme iron of resting enzyme with a strong reducing substrate such as benzidine (HB) and molecular oxygen. In subsequent electron transfer the substrate is oxidized to regenerate the resting state of enzyme. In contrast to peroxidatic activity, this oxygen dependent oxidase activity of mammalian catalase did not require hydrogen peroxide or any additional cofactors. According to the spectroscopic analysis of catalase oxidase activity, the reaction involves heme iron and the formation of compound II takes place through the interactions of heme iron and moleculer oxygen. However, the precise mechanicsm of the oxidase reaction is not clear, since the structure of the reaction intermediates and products are unknown. In this study 10-acetyl-3,7-dihydroxyphenoxazine, which is also a peroxidase substrate, was used for the characterization of oxidase activity of mammalian catalase. Furthermore, other

peroxidase substrates, including pyrogallol, catechol and DCFH have been reported to be oxidized by mammalian catalase (Vetrano *et al*., 2005).

Vetrano *et al*. (2005) have reported this oxidase activity in purified catalase obtained from different sources, including mouse and bovine liver, mouse and human keratinocytes, and hamster fibroblasts. Despite the differences in their amino acid sequences, functionally important amino acid sequences were found to be conserved (Kirkman and Gaetani, 2006).

1.4 Oxidative Stress: Oxidants and Antioxidants

Oxidative stress, potentially leading to damage, occurs when there is an imbalance between oxidants and antioxidants in favor of the oxidants. Oxidants are formed during normal metabolic activity, mainly through aerobic respiration; however they can also be produced at elevated rates as a result of various environmental perturbations, such as extreme temperatures, radiation, xenobioitcs, toxins like heavy metals, ionizing and UV radiation, etc. Many inducers of oxidative stress are known carcinogens, mutagens and toxins (Sies, H., 2007, Scandalios, 2005, Aguirre, *et al.,* 2005 and Schrader and Fahimi, 2006).

Several studies indicate that oxidative stress is a common denominator underlying many disease, such as ischemia/reperfusion injury, atherosclerosis, hypertension, cardiovascular disease, rheumatoid arthritis, cystic fibrosis, cancer, type-2 diabetes, or neurodegenerative diseases (Parkinsons's and Alzheimer's disease) (Schrader and Fahimi, 2006 and Valko *et al.,* 2006). Furthermore, oxidative stress has also been linked to aging. Continuous damage of oxidants to cellular macromolecules and incomplete repair of them would lead to its accumulation over time-resulting in age-related deterioration (Bokov *et al*., 2004). Thus, oxygen provides a paradox: Molecular oxygen is the fundamental biological electron acceptor which serves vital roles in the fundamental cellular

functions, however reduced forms of oxygen, namely reactive oxygen species (ROS), has a toxic effect on biomolecules, such as DNA, proteins and lipids. Oxidative damage to DNA can occur in the form of base degradation, single strand breakage, DNA deletions, mutations, translocations or cross-linking of DNA to proteins. Damage to proteins can occur in the form of changed electrical charge, fragmentation of the peptide chain, site-specific amino acid modifications, etc. Oxidative damage to lipids can occur through several mechanisms of ROS reacting with fatty acids in the membrane lipid bilayer, which leads to membrane leakage and cell death. In foods, lipid peroxidation leads to rancidity and development of undesirable odors and flavors (Scandalios, 2005).

1.4.1 Reactive Oxygen Species

Molecular oxygen (O_2), which is essential for aerobic life, can be reduced to water. In the intermediate steps of complete reduction of molecular oxygen, partially reduced species are formed and they are named as reactive oxygen species (ROS). Pathways of molecular oxygen reduction to water are given in Figure 1.6 (Scandalios, 2005).

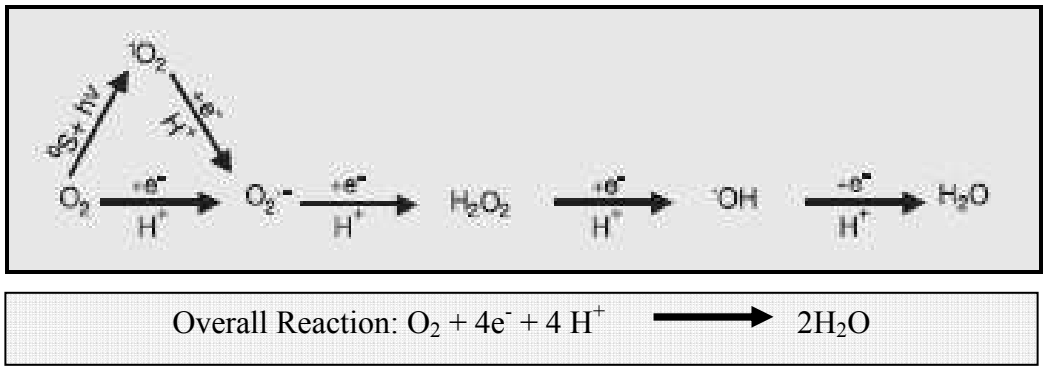


Figure 1.6 Pathways in the reduction of oxygen to water and the generation of various intermediate reactive oxygen species (Scandalios, 2005).

ROS include free radicals, which contain unpaired electrons. Despite the absence of unpaired electrons, thus not being a radical, hydrogen peroxide (H_2O_2) is also described as ROS (Schrader and Fahimi, 2006). As can be seen from the Figure 1.6, in the pathway of molecular oxygen reduction; superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\cdot}$) are formed corresponding to the reduction by one, two and three electrons, respectively and the overall reduction of molecular oxygen requires four electrons. The hydroxyl radical ($OH^{\cdot}$) which is probably the most highly reactive and toxic form of oxygen can also be formed by the interaction of superoxide anion radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) in the presence of metal ion (e.g. iron or copper). Besides the hydroxyl radical ($OH^{\cdot}$), molecular oxygen (O_2) and hydroxyl ion (OH^-) are generated as a result of this metal ion catalyzed reaction (Sies, H., 2007, Scandalios, 2005 and Schrader and Fahimi, 2006).

Oxygen radicals can also occur as alkyl or peroxyl radicals (e.g. RO•, alkoxy and ROO•, peroxy radicals). Furthermore, there are reactive nitrogen species (RNS),

such as nitric oxide ($NO^{\cdot}$), that lead to oxidative damage when excess in amount (Sies, H., 2007and Schrader and Fahimi, 2006).

Recent studies have indicated that besides the harmful effects of ROS on biomolecules, when tightly regulated they play important physiological roles (Scandalios, 2005). In other words, reactive oxygen species (ROS) and reactive nitrogen species (RNS) have a dual role as both deleterious and beneficial species. Some of the useful roles of reactive oxygen species (ROS) are regulation of cell proliferation, cell differentiation, signal transduction, ion transport, apoptosis or necrosis, involvement in the cell wall structure of plants and direct killing of pathogens, etc. (Aguirre, *et al.,* 2005 and Scandalios, 2005). For example, superoxide anion radical ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) acts as intracellular signaling molecules. Particularly, hydrogen peroxide (H_2O_2), which is membrane permeable, diffusible and relatively stable molecule and not harmful until converted to more reactive ROS, is best suited for intra- and even intercellular signaling (Scandalios, 2005 and Schrader and Fahimi, 2006).

1.4.2 Antioxidant Defense Systems

Reactive oxygen species are produced in all aerobic organisms and normally exist in the cell in balance with antioxidant molecules. The reason of oxidative stress is the distruption of this critical balance due to the depletion of antioxidants or excess accumulation of ROS, or both (Scandalios, 2005). Defense mechanisms against free radical-induced oxidative stress involve: preventive mechanisms, repair mechanisms, physical defenses, and antioxidant defenses. Non-enzymatic antioxidants are represented by ascorbic acid (Vitamin C), glutathione (GSH), α-tocopherol (Vitamin E), carotenoids, flavonoids, and other antioxidants. Enzymatic antioxidant defenses include superoxide dismutase (SOD), glutathione peroxidases (GPx) and catalase (CAT) (Valko *et al*., 2007).

Polyphenols are a large group of antioxidants, which are naturally present in fruits and vegetables. They can act as free radical-scavengers, quenching hydroxyl radicals ($OH^{\cdot}$) or superoxide anion radicals ($O_2^{\cdot -}$), etc. However, it has been reported that some polyphenols have detrimental effects on cell death in the presence of metal ions under certain conditions *in vitro*. This effect is suspected to result from the pro-oxidant action, after which hydrogen peroxide (H_2O_2) is generated. This property of polyphenols is related to their structures; only *o*- and *p*-phenolic compounds undergo auto-oxidation under certain conditions (Akagawa *et al.*, 2003 and Aoshima *et al.,* 2005).

Enzymatic defenses convert reactive oxygen species to less reactive species, thus protect the cell by directly scavenging ROS. Superoxide anion radical ($O_2^{\cdot -}$) is dismutated by superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2), which is then, destroyed by catalase (CAT) and/or peroxidases, such as glutathione peroxidases (GPx) (Aguirre *at al*., 2005 and Scandalios, 2005).

1.5 Thermophilic Fungi

Thermophilic and thermotolerant fungi, the most heat stable eukaryotic organisms, have the ability to thrive at temperatures between 45°C and 55°C. They can be distinguished on the basis of their growth temperature minimum and growth temperature maximum, which are at or above 20°C and at or above 50°C, respectively (Maheshwari *et al.,* 2000). The enzymes of thermophilic fungi are thermostable, which is an important feature for industrial utilization, and generally active at neutral to alkaline pH (Ögel *et al*., 2001).

1.5.1 *Scytalidium thermophilum*

Scytalidium thermophilum (Figure 1.7) is a member of *Deuteromycetes* which have no known sexual state in their life cycle. *Scytalidium thermophilum*, also known as *Humicola insolens*, *Humicola grisea* var. *thermoidea* and *Torula thermophila,* is commonly found in mushroom composts and in soil. Like some other thermophilic fungi, it grows massively, from the spores survived after the pasteurization, during the last phase of the composting process. *Scytalidium thermophilum* contribute significantly to the quality of the compost, because it is beneficial for the selective growth of *Agaricus bisporus*. The effects on the growth of the mushroom mycelium have been described at three distinct levels. First, this fungus decreases the concentration of ammonia in the compost, which otherwise would counteract the growth of the mushroom mycelium, and second it immobilizes nutrients in a form in order to become available to the mushroom mycelium. Third, it has a growth-promoting effect on the mushroom mycelium, which has also been demonstrated for several other thermophilic fungi. This growth-promoting effect has been observed both with live and dead cells (Wiegant *et al.,* 1992 and Maheshwari, *et al*., 2000).

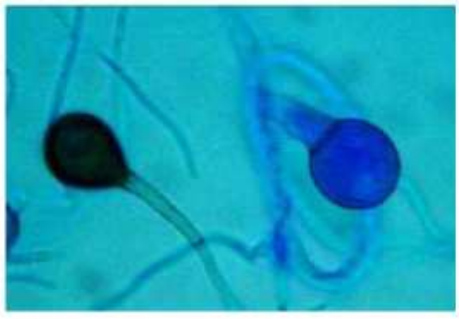


Figure 1.7 *Humicola insolens* spores (type 1) under light microscope (Hamilton and Gomez, 2002).

1.6 Catalase of *Scytalidium thermophilum*

Scytalidium catalase gene was first cloned by Novo Nordisk Bio Tech, Inc in 1997. In this study, partial peptide sequences obtained from an isolated *S.thermophilum* catalase protein were used to design two degenerate primers which were then used in PCR reactions against genomic DNA of *S.thermophilum.* PCR product obtained from this reaction was cloned into a plasmid. After isolation of fragment from the plasmid, it was labelled and used to screen genomic libraries. One of the hybridizing fragments was shown to contain the entire coding region of the catalase gene and screening of cDNA library using PCR with exact match primers yielded cDNA subclones. As a result, it was found that the catalase gene of *S .thermophilum* is encoded by 2791 base pairs, with 7 introns and 717 amino acids encodes a prepro-catalase with a 19 amino acid signal sequence, and a 17 amino acid proregion. The sequence of the *S.thermophilum* catalase gene and the predicted amino acid sequence are shown in Appendix D.

S.thermophilum catalase was determined as a tetrameric protein with total molecular weight of 320 kDa and subunit molecular weight of 80 kDa. Isoelectric point (pI) of the protein was verified as 5.0 in our laboratory. Kulys *et al.* (2003) has reported that according to the estimation from amino acid composition subunit size of homotetrameric *S.thermophilum* catalase was 74.9 kDa and according to the SDS-PAGE experiment it was determined as 86 kDa. It has also been reported that calculated isoelectric point of the protein was found as 5.1, while it was determined as 4.0 using isolelectric focusing (Kulys *et al*., 2003). The catalase has activity pH optimum at about 7 and exhibits stability in a broad range pH from 4 to 9 (United States Patent, No. 5646025).

1.7 Heterologous Protein Production

Filamentous fungi can secrete a broad range of different enzymes and this provides them to be able to use many organic compounds as the source for their nutrients (Gouka *et al*., 1997). A number of filamentous fungal strains and their products have been used in the food industry and have resulted in a so-called GRAS (generally regarded as safe) for some of their products except the related species which produce toxins. The development of molecular biological techniques has provided a recent application of filamentous fungi as cell factories for heterologous and homologous protein production (Archer, 2000 and Gouka *et al*., 1997). Heterologous protein production in *Aspergilli* was started with *Aspergillus nidulans*, however; other species, *A.niger, A.awamori, A.oryzae, A.sojae*, have been used more frequently because of the higher levels of recombinant protein production (Gouka *et al*., 1997).

1.7.1 *Aspergillus sojae*

Aspergillus sojae, which has never been isolated from the field, is generally agreed to be a domesticated variant of *A. parasiticus. Aspergillus sojae* contains homologues of several aflatoxin biosynthetic genes, however; it is unable to produce aflatoxins due to a probable defect in the aflatoxin pathway regulatory gene homologue, *aflR.* Therefore, *Aspergillus sojae* is generally reconized as safe (Takahashi *et al.,* 2002).

A new expression host from *A. sojae* ATCC strain was developed by Margreet Heerikhuisen, Cees van den Hondel and Peter Punt, TNO Nutrition and Food Research, Dept. of Microbiology (Zeist, The Netherlands). An auxotrophic marker system, which involves the genes complementary to the pre-existing mutations and lead to prototrophic growth, was developed for *A. sojae* involving

the usage of *pyrG* as a selection marker. Orotidine-5'-monophosphate decarboxylase (=pyrG-) mutants of *A. sojae*, which lacks the ability to synthesize uridine was used as host organism. By co-transformation of pAMDSPYRG, with *amd*S and *pyrG* genes encoded by the expression vector, the transformants could grow in the minimal medium while untransformed *pyr*G- mutants could not grow in the absence of uracil or uridine from the selective medium as they were blocked in pyrimidine biosynthesis due to the lack of orotidine-5'-phosphate-decarboxylase (Punt *et al.,* 1992 and Timberlake*,* 1991).

1.8 Scope of the Study

In this study, it was aimed to clone and express the *Scytalidium thermophilum* bifunctional catalase/phenol oxidase gene in *Aspergillus sojae*. For this purpose *Scytalidium* catalase gene was first subcloned into *E.coli* XL1 Blue MRF' cells on vector pCR2.1® and sequenced. Then, sub-cloned catalase gene was ligated onto expression vector pAN52-4, in which the catalase gene was placed under the control of the glyceraldehyde-3-phosphate dehydrogenase promoter as a strong and constitutive promoter and glucoamylase preprosequence of *Aspergillus niger* was used to target the secretion of the catalase. Recombinant plasmids carrying the catalase gene in correct orientation were transformed into *Aspergillus sojae* ATCC11906 (pyrG-), which is an auxotrophic mutant lacking the ability to synthesize uridine, by protoplast transformation method. The marker plasmid pAMDSPYRG containing *pyr*G gene from *Aspergillus niger* was co-transformed for the selection of the transformants by uridine prototrophy.

Scytalidium thermophilum catalase was studied as a novel phenol oxidase at the initial stages of the research in our laboratory. After the purification and amino acid sequencing studies, the enzyme was determined as a catalase with an additional phenol oxidase acitivity (Sutay *et al*., 2008 in press). Thus, *Scytalidium* catalase is a novel bifunctional enzyme; having the phenol oxidase

activity besides the catalatic acitivity. The aim of cloning and heterologous production in *Aspergillus sojae* under the control of the constitutive *gpdA* promoter was to overproduce the *Scytalidium thermophilum* bifunctional catalase for further site-directed mutagenesis studies and for the characterization of the oxidase activity of this enzyme at the molecular level.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Fungal Strains

Scytalidium thermophilum (type culture Humicola insolens) was kindly provided by Dr. Mehmet BATUM from ORBA Inc.

Aspergillus sojae ATCC11906 (pyrG-) strain was kindly supplied by TNO Nutrition and Food Research, Department of Microbiology Holland. This strain has a low proteolytic activity and is a uridine auxotrophic mutant. This means that it is unable to produce the enzyme orotidine-5'-monophosphate decarboxylase which is involved in the biosynthesis of uridine.

2.1.2 Bacterial Strains

Eschericia coli XL1 Blue MRF' was provided in the ZAP Express® cDNA Gigapack® III Gold Cloning Kit (Stratagene).

2.1.3 Chemicals, Enzymes and Kits

The list of chemicals, enzymes and kits used and their suppliers are given in Appendix A.

2.1.4 Growth Media, Buffers and Solutions

The preparation of the growth media, buffers and solutions used are given in Appendix B.

2.1.5 Plasmids, Molecular Size Markers

Plasmids and molecular size markers used are given in Appendix C.

2.2 Methods

2.2.1 Maintenance and Cultivation of the Strains

Scytalidium thermophilum was inoculated onto YpSs agar plates (Appendix B) and incubated at 45 °C for 4-5 days until sporulation, followed by storage at 20ºC for maximum 2 months. Spores from these stock cultures were inoculated into preculture media, which consisted of YpSs broth (Appendix B) including 1% (w/v) glucose as a carbon source instead of starch. After incubating at 45 °C for 24 hours, preculture was transferred into main culture medium. Preculture volume was 2 % of the main culture volume. For DNA isolation, the main culture medium was YpSs broth. However, if enzyme production was required, main culture medium was modified YpSs broth (Appendix B) which included

4% glucose (w/v) and copper sulphate. All cultures were incubated in a shaker incubator at 45 °C and 155 rpm.

Stock cultures of *Aspergillus sojae* ATCC11906 strain were grown on complete medium agar plates (Appendix B) which was supplemented with uridine and uracil at 30°C for 3-7 days until sporulation, followed by storage at 4 °C. For protoplast preparation, spores from stock cultures were inoculated into 250 ml complete medium broth (Appendix B) with uridine and uracil in 500 ml erlenmeyer flasks and incubated at 30 °C and 150 rpm for 16-18 hours.

Aspergillus sojae transformants were grown on minimal medium agar plates (Appendix B) at 30°C for 3-7 days until sporulation and maintained at 4 °C. For the DNA isolation of transformants, spores of *A.sojae* transformants were inoculated into 200 ml YpSs broth (Appendix B) in 500 ml erlenmeyer flasks at 30 °C and 155 rpm for 24 hours. For the determination of catalase activitiy, transformants were grown in complete medium without uridine and uracil (Appendix B) as described above, first at 30 °C and 155 rpm for 24 hours to prepare the preculture and next at 30 °C and 155 rpm for the main culture.

Stock cultures of *Eschericia coli* XL1 Blue MRF' were grown on LB Tetracycline agar plates (Appendix B) at 37°C and stored at 4°C up to 3 weeks. For the preparation of competent cells, *E. coli* XL1 Blue MRF' was inoculated in 5 ml LB medium (Appendix B) and incubated at 37°C and 155 rpm overnight. Then, 100 ml of LB medium was inoculated with 1 ml of overnight culture of *E. coli* XL1 Blue MRF' and incubated at 37°C until the absorbance at 550 nm reached 0.4-0.5.

2.2.2 Nucleic Acid Isolation

2.2.2.1 Genomic DNA Isolation

Fungal strains were cultivated as explained in section 2.2.1. Mycelia were filtered through Whatman 3MM filter paper and dried at 45°C for 30 minutes. Dried mycelia were frozen in liquid nitrogen and ground into powder with a mortar and pestle. Powdered mycelia were transferred to a 50 ml falcon tube. 25 ml TTE buffer (Appendix B) was added to powdered mycelia and centrifuged at 3000 rpm for 10 minutes. The supernatant was discarded. 10 ml of lysis buffer (Appendix B) was added to the pellet and mixed by inversion. The mixture was incubated at 37°C for 15 minutes. Then, an equal volume (10 ml) of phenol:chloroform:isoamylalcohol (25:24:1) was added to the mixture and centrifuged at 5500 rpm for 8 minutes. After centrifugation, upper phase was transferred to a new falcon tube. Phenol:chloroform:isoamylalcohol extraction was repeated until the interphase became clear. The upper phase from the last extraction was transferred into a Sorvall tube. 0.1 volume sodium acetate (3 M, pH 5.2) (Appendix B), and 2.5 volume cold absolute ethanol was added to the collected upper phase in the Sorvall tube. The mixture was left overnight at -20°C. Then, the tubes were centrifuged at 12 000 rpm for 10 minutes at 4°C and the supernatant was discarded. The pellet was then washed with 10 ml 70% ethanol (Appendix B) and the tubes were centrifuged at 6000 rpm for 3 minutes. The pellet was air-dried for 20 minutes or dried at 45 °C for 10 minutes in order to evaporate all of the residual ethanol. After drying, the pellet was dissolved in 100 µl sterile double distilled water at 4°C overnight. Dissolved DNA solution was transferred into sterile 1.5 ml polypropylene tubes. For 100 µl dissolved DNA solution, 10 µl, 10 mg/ml DNase free RNase (Appendix B) was added and incubated at 37°C for 2 hours. DNA was stored at -20 °C.

The concentration of DNA was determined by running on an agarose gel (Appendix B) with a marker of known concentration (λDNA/*Eco*RI *Hin*d III or λDNA/*Hin*d III) and comparing the intensity of the DNA band with corresponding marker band.

The concentration of DNA was calculated in µg/µl according to the following formula:

$$\text{DNA concentration } (\mu g/\mu l) = \frac{L_{\text{FRAGMENT}}}{TL_{\text{MARKER}}} \times C_{\text{MARKER}} \times V_{\text{MARKER}} \times \frac{1}{V_{\text{DNA}}} \times I_{\text{BAND}}$$

(Equation 1)

$L_{FRAGMENT}$: length of the fragment
TL_{MARKER}: total length of the marker
C_{MARKER}: concentration of marker
V_{MARKER}: volume of marker
I_{BAND}: intensity of the band
V_{DNA}: volume of loaded DNA

2.2.2.2 Plasmid Isolation

5 ml LB broth (Appendix B) with ampicillin at a concentration of 100µg/ml was prepared in 50 ml falcon tubes and inoculated with one colony of *E. coli* XL1 Blue MRF' cells which were expected to contain the vector with insert. After incubation at 37 °C overnight in a shaker incubator, cells were centrifuged at 6000 rpm for 8 minutes and the supernatant was discarded. The pellet was resuspended in 200 µl of solution 1 (Appendix B) and was transferred into sterile 1.5 ml polypropylene tube. The cells in solution 1 were incubated at room temperature for 15 minutes. Then, 200 µl of solution 2 (Appendix B) was added and gently mixed by inverting the tubes for 7- 8 times, followed by an incubation for exactly 5 minutes on ice. After that, solution 3 (Appendix B) was added,

gently mixed by inverting the tubes for 7-8 times and incubated on ice for 15 minutes. After centrifugation at 13000 rpm for 10 minutes at 4 °C, the supernatant was transferred into sterile 1.5 ml polypropylene tube and 2 volume of cold absolute ethanol was added and incubated at –20 °C for 1 hour. The tubes were centrifuged at 13000 rpm for 10 minutes at 4 °C and the supernatant was discarded. The pellet was resuspended in 200 µl NE buffer (Appendix B) and incubated on ice for 1 hour. After incubation, the tubes were centrifuged at 13000 rpm and 4 °C for 15 minutes and the supernatant was transferred into another sterile 1.5 ml polypropylene tube. Then, 400 µl cold ethanol (70%) was added and incubated at –20 °C for 1 hour. After centrifugation at 13000 rpm for 10 minutes, the supernatant was discarded. The pellet was air dried at room temperature or in a vacuum concentrator until the evaporation of all ethanol. Finally, plasmid was dissolved in 15 µl sterile double distilled water.

For sequence analysis, plasmid isolation was carried out by using Plasmid Miniprep Purification Kit (GeneMark) to guarantee the purity of the plasmid. According to the instructions given in the kit; *E. coli* XL1 Blue MRF' cells containing the plasmid were cultivated in 3 ml LB broth with ampicilin at a concentration of 100µg/ml at 37 °C overnight. Pellet was obtained from 1-3 ml of cells by centrifugation at top speed (14000 rpm) for 1 minute in a microcentrifuge. Supernatant was poured off and the excess media was removed. Cells were suspended in 200 µl of Solution I. Then, 200 µl of Solution II was added and mixed by inverting the tube 5 times. The cell suspension became clear immediately. After that, 200 µl of Solution III was added and mixed by inverting the tube 5 times. The lysate was centrifuged at top speed (14000 rpm) in a microcentrifuge for 5 min and a compact white pellet was formed along the side or at the bottom of the tube. All the cleared lysate was transferred into a spin column mounted on a collection tube and was spun down at top speed (14000 rpm) for 1 minute in a microcentrifuge. The filtrate in the collection tube was discarded and 700 µl of Wash solution (include 80 % ethanol) was added and

centrifuged spin for 1 minute at top speed (14000 rpm) and the filtrate was discarded. Washing step was carried out for one more time. Residual ethanol was removed by centrifugation for 3 minutes at top speed (14000 rpm). Finally, 50-100µl of H_2O (pH7.0-8.5) was added into the column and centrifuged at top speed for 1 minute to elute the plasmid.

2.2.2.3 Recovering DNA from Agarose Gels

After the analysis of PCR products by agarose gel electrophoresis (Appendix B), desired DNA band was cut out and recovered from the gel according to the instructions given in the Gel Elution Kit (GeneMark). Gel slice was transferred into a 1.5 ml sterile microcentrifuge tube and an equal volume of Binding Solution was added to the gel slice, and incubated at 60 °C for 5-15 minutes until all the agarose was dissolved. After the gel slice was completely melted, the DNA/agarose solution was transferred into spin column mounted on a collection tube and centrifuged for 1 minute at top speed (14000 rpm). The filtrate in the collection tube was discarded and 700µl of Wash Solution was added and centrifuged for 1 minute at top speed. Washing step was repeated for one more time and the filtrate was discarded again. In order to remove the residual trace of ethanol centrifugation was done for 2 minutes at top speed. Then, spin column was transferred into a new microcentrifuge tube and incubated at 45°C oven for 5 minutes to evaporate all of the ethanol. Finally, 30-50 µl of double distilled H_2O preheated to 65°C was added and centrifuged for 1 minute to elute the DNA and eluted DNA was stored at -20 °C until usage.

2.2.3 Primer Design

For the amplification of catalase gene, two different specific primers (catS1, catS2) were designed using the sequence of *Scytalidium* catalase gene (United States Patent, No. 5646025). *Hind*III restriction enzyme sites were added to the

5’ ends of both primers, which were required to ligate onto vector pAN52-4. catS1 corresponds to the downstream of the start codon and cleavage by *Hind*III restriction enzyme resulted in the exclusion of signal peptide and propeptide regions, because the expression vector pAN52-4 includes glucoamylase gene of *Aspergillus niger* signal peptide and propeptide regions. catS2 corresponds to the 3’ region stop codon.

Two universal specific primers, called 315 and 316, and two designed specific primers, called right315 and left316, were used for the sequencing process of the cloned gene. 315 corresponded to the upstream and 316 corresponded to the downstream of the insert in the recombinant pCR2.1CatS plasmid. Right315 and Left316 were designed in between the catalase gene in order to complete the sequencing process of the whole target gene.

In addition, another pair of specific primers, namely Acat1 and Acat2, were designed from *Scytalidium* catalase gene and used in PCR amplifications from genomic DNA of *Aspergillus sojae* transformants in order to confirm the presence of *catpo* gene in the transformants. Sequences of all primers are given in Table 2.1.

Table 2.1 Sequence of primers designed based on nucleotide sequence of the catalase gene

Name of the Primer	Sense or Antisense	Number of Bases	Sequence of Primer (5'to 3')*
catS1	sense	24	**A : A G C T T C A G G A C A G T C G C C A C T T G** *Hind*III cut site
catS2	antisense	27	**A : A G C T T C T A G G A G T C G A G A G C A A A C C G** *Hind*III cut site
Right315	antisense	22	**A G C C G T C T T G T T G T T G T G G T A G**
Left316	sense	21	**A T G A G C T A A C G T G T G T G T G T G**
315	sense	20	**A G G G T T T T C C C A G T C A C G A C**
316	antisense	19	**G A T A A C A A T T T C A C A C A G G**
Acat1	sense	21	**G T G T C G C C A A C A T T C A A C G A C**
Acat2	antisense	21	**C G T C C A C A A A G A T C T G C A A C G**

* Restriction enzyme cut sites are shown in bold

2.2.4 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was carried out using Taq DNA Polymerase (Fermentas) when plasmid DNA was used as a template and 50 µl reaction mixture contained:

- sterile double distilled water to give a final volume of 50 µl
- 1X PCR Buffer minus Mg from 10X PCR Buffer stock (Fermentas)
- 1-3 mM $MgCl_2$ from 25mM or 50mM stock solution
- 0.2 mM dNTP mixture from 10mM or 25mM stock solution
- 25-50 pmoles from each specific primers
- 2 units Taq DNA polymerase (Fermentas)
- 10-20 ng plasmid DNA

The components of the PCR reaction were mixed in a 0.2 ml thin-walled PCR tube and amplifications were performed according to the following cycle:

95 °C 2 min
95 °C 1 min
Ta °C 1 min
72 °C 1 min/kb
72 °C 10 min

where Ta is the annealing temperature at which the primers anneal to the complementary regions in the template strands and ranges from 45°C to 60°C according to the melting temperatures of primers.

PCR for the purpose of gene cloning was carried out with Platinum *Pfx* DNA Polymerase (Invitrogen). A 50 µl reaction mixture contained:

- sterile double distilled water to give a final volume of 50 µl
- 1X *Pfx* Amplification Buffer from 10X *Pfx* Amplification Buffer stock (Invitrogen)
- 1X PCRx Enhancer Solution from 10X PCRx Enhancer stock solution (Invitrogen)
- 1.5 mM Magnesium Sulfate from 50 mM $MgSO_4$ stock solution
- 0.3 mM dNTP mixture from 10mM or 25mM stock solution
- 50 pmoles from each specific primers
- 1.25 unit *Pfx* DNA Polymerase (Invitrogen)
- 0.25 µg genomic DNA

The components of the PCR reaction were mixed in a 0.2 ml thin-walled PCR tube and amplifications were performed according to the following cycle:

94 °C 2 min
94 °C 15 sec
Ta °C 30 sec
68 °C 1 min/kb

2.2.5 Visualization and Photography of Nucleic Acids

Visualization and analysis of the genomic DNA, plasmids and PCR products was carried out by agarose gel electrophoresis using 0.8% (w/v) agarose gel in 1X TAE Buffer (Appendix B).

Samples were mixed with a loading dye (Fermentas) at a ratio of 6:1 and loaded into the wells. Electrophoresis was carried out at 5-7 V/cm for 45-60 minutes.

Then, the gel was visualized under UV at 312 nm and photographed by a Gel Documentation System (BioRad).

2.2.6 Transformation of *E. coli* XL1 Blue MRF'

2.2.6.1 Preparation of Competent Cells

E. coli XL1 Blue MRF' cells were cultivated as explained in section 2.2.1. After the optical density at 550 nm reached 0.4-0.5, cells were dispensed into two falcon tubes. The tubes were chilled on ice for 10 minutes and centrifuged at 6000 rpm for 5 min at 4°C. The supernatant formed after centrifugation was discarded. Then, the cells were resuspended in a total volume of 50 ml (25 ml for each tube) ice-cold solution A (Appendix B) and kept on ice for 15 minutes. Centrifugation at 6000 rpm for 5 min at 4°C was carried out and the supernatatant was discarded again. The cells were resuspended in a total volume of 7 ml (3.5 ml for each tube) ice-cold Solution A and 100% sterile glycerol was added to a final concentration of 20% (700 µl for each tube). Finally, 300 µl aliquots were dispensed into sterile 1.5 ml polypropylene tubes and stored at -80°C.

2.2.6.2 Transformation of Competent Cells

0.5-2 µl plasmid was mixed with TE buffer (Appendix B) to give a final volume of 50 µl. This mixture was added to one batch of competent cells (300 µl) and kept on ice for 30 minutes. The competent cells and plasmid mixture were transferred into a 42°C water bath and incubated there for precisely 2 minutes, followed by incubation on ice for 2 minutes. Then, 1 ml LB medium (Appendix B) was added and the mixture was incubated at 37°C in a shaker incubator. 50-200 µl aliquots were spread on LB ampicilin agar plates (Appendix B), of which ampicilin concentration was 100 µg/ml, and incubated overnight at 37°C.

2.2.7 Restriction Enzyme Digestion

For the best activity of the enzyme, the restriction enzyme buffer was chosen according to the suggestions of the supplier (Fermentas). A 20 µl reaction mixture contained:

- sterile double distilled water to give a final volume of 20 µl
- 1X Restriction Enzyme Buffer from 10X Restriction Enzyme Buffer stock (Fermentas)
- 0.1 to 5 µg plasmid DNA obtained from plasmid isolation
- 10 to 15 units appropriate restriction enzyme (Fermentas)

The components of the reaction were mixed in a 0.2-0.5 ml thin-walled PCR tube and the reaction mixture was incubated at 37ºC for minimum 2 hours to overnight.

2.2.8 Ligation

2.2.8.1 Ligation onto Vector pCR2.1®

The amplifications from genomic DNA were carried out by using Platinum *Pfx* DNA Polymerase (Invitrogen) as described in section 2.2.3. Direct cloning of DNA amplified by Platinum *Pfx* DNA Polymerase (Invitrogen) into pCR®2.1 is difficult because of the 3´ to 5´ exonuclease proofreading activity of the enzyme. This activity removes the 3´ A-overhangs which is necessary for TA Cloning®. Therefore, 3´ A-overhangs were added to the PCR products before ligation onto vector pCR2.1®. The PCR products were cleaned by using Gel Elution Kit (GeneMark) as described in section 2.2.2.3, prior to the addition of 3´ A-overhangs addition.

For the addition of 3´ A-overhangs, a 10 µl reaction contained:

- 1X PCR Buffer minus Mg from 10X PCR Buffer stock (Fermentas)
- 2 mM $MgCl_2$ from 25mM stock solution
- 0.2 mM dNTP mixture from 10mM or 25mM stock solution
- 6 units Taq DNA polymerase (Fermentas)
- amplified PCR product to give a final volume of 10 µl

The components of the reaction were mixed in a 0.2 ml thin-walled PCR tube and incubated at 72 ºC for 30 minutes.

Before ligation, 3´ A-overhangs added products were cleaned again by using Gel Elution Kit (GeneMark). The volume of the insert needed to ligate with 50 ng of pCR2.1® vector was determined according to the following equation:

$$\text{X ng PCR product} = \frac{(\text{Y bp PCR product}) \times (50\,\text{ng pCR®2.1 vector})}{(\text{size in bp of the pCR®2.1 vector:} \sim 3900)} \qquad \text{(Equation 2)}$$

where X ng is the amount of 3´ A-overhangs added PCR product of Y base pairs to be ligated for a 1:1 (vector:insert) molar ratio as described in TA Cloning® Kit (Invitrogen) protocol.

According to TA Cloning® Kit (Invitrogen) protocol, 10 µl ligation reaction contained:

- sterile double distilled water to give a final volume of 10 µl
- X ng fresh 3´ A-overhangs added insert
- 10X Ligation Buffer to give a final concentarion of 1X (Invitrogen)
- 50 ng pCR®2.1 vector (25 ng/µl)
- 4.0 Weiss units T4 DNA Ligase (Invitrogen)

The ligation reaction was mixed in a 0.2 ml thin-walled PCR tube and incubated at 14°C overnight.

2.2.8.2 Ligation onto Vector pAN 52-4

Plasmid DNA and the insert to be ligated were digested using appropriate restriction enzyme(s) as described in section 2.2.6. The insert to be cloned onto vector pAN52-4 was obtained after subcloning onto vector pCR®2.1. Therefore, restriction enzyme digestion not only provided that the ends of the vector pAN52-4 and the insert were complementary but also provided the separation of the insert and the vector pCR®2.1. After restriction digestion, the insert was recovered from agarose gels by using Gel Elution Kit (GeneMark) with the method described in section 2.2.2.3.

Before ligation, digested plasmid DNA was dephosphorylated with alkaline phosphatase. The alkaline phosphatase treatment was perfomed in the reaction mixture in which cleavage by restriction endonuclease was carried out. The reaction mixture for the dephosphorylation of plasmid DNA contained:

- 20 µl reaction mixture containing the digested plasmid (1-20 pmol termini)
- sterile double distilled water to give a final volume of 50 µl
- 1X reaction buffer for calf intestine alkaline phosphatase from 10X buffer
- 1 to 2 units calf intestine alkaline phosphatase (Fermentas)

The reaction mixture was incubated at 37°C for a minimum of four hours up to overnight. After the dephosphorylation step, the plasmid was cleaned by using Gel Elution Kit (GeneMark) by the method described in section 2.2.2.3.

Ligation was carried out in a 0.2 ml thin-walled PCR tube and a 10 µl reaction mixture contained:

- sterile double distilled water to give a final volume of 10 µl
- 1X ligation buffer for T4 DNA ligase from 10X ligation buffer stock (Roche)
- 0.5 mM ATP
- 1 unit T4 DNA ligase (Roche)
- amount of insert (I) to be ligated for 1:1 or 1:3 molar ratio was calculated according to the following equation:

$$\text{I}\ (\mu\text{g}) = \frac{fragmentsize(bp)}{vectorsize(bp)} \times \frac{3}{1} \times 0.1\mu g plasmid \qquad \text{(Equation 3)}$$

The reaction mixture was incubated at 16°C overnight.

2.2.9 Identification of Recombinant Clones

2 µl ligation mixture was transformed into *E. coli XL1 Blue MRF'* competent cells by the method described in section 2.2.5.2 and 50-200 µl of transformed cultures were spread on LB ampicilin agar plates (Appendix B) as described. However, for blue-white colony selection, agar plates also contained 40 µl 100 mM IPTG and 40 µl 2% X-gal (Appendix B) besides 100 µg/ml ampicillin. IPTG and X-gal solutions were spread on LB ampicilin plates and incubated at 37°C for 30 minutes before plating the transformed cultures. After incubation at 37°C for the growth of transformants, white colonies were picked and the presence of insert was confirmed by plasmid isolation (Section 2.2.2.2), followed by restriction enzyme digestion (Section 2.2.6) and polymerase chain reaction (Section 2.2.3).

2.2.10 Transformation of *Aspergillus sojae*

2.2.10.1 Strains

Aspergillus sojae ATCC11906 (pyrG-) strain was used for the heterologous expression. As mentioned in section 2.1.1, *Aspergillus sojae* ATCC 11906 (pyrG-) strain is unable to produce the enzyme orotidine-5'-monophosphate decarboxylase which is involved in the biosynthesis of uridine. This system provides a higher transformation frequency and is a commonly used auxotrophic selection method for fungal transformantions.

2.2.10.2 Selective Plasmids

As a selective marker, pAMDSPYRG plasmid was used in the co-transformation studies. This plasmid includes *amd*S gene of *Aspergillus nidulans* and *pyr*G gene of *Aspregillus niger*. The *amdS* gene encodes an enzyme that confers the ability to use acetamide and acrylamide as nitrogen and carbon sources and the *pyr*G gene encodes orotidine-5'-monophosphate decarboxylase which is involved in the biosynthesis of uridine. Therefore, it is possible to select the transformants for uridine prototrophy after the co-transformation using pAMDSPYRG plasmid as a selective marker.

2.2.10.3 Expression Vectors

Fungal vector pAN52-4 was used as an expression system. This vector comprises the promoter region of glyceraldehydes-3-phoshate dehydrogenase (*gpd*A) as a strong and constitutive promoter and *trp*C terminator from *Aspergillus nidulans* (Punt *et al*., 1992).

Table 2.2 Expression and selection vectors used in the *Aspergillus sojae* co-transformation experiments.

Expression vector	Expression cassette	Reference
pAN52-4	*gpd A* promoter and *trpC* terminator of *A.nidulans*	Punt *et al*; 1992

Selection vector	Selection Marker	Reference
pAMDSPYRG	amdS of *A.nidulans* pyrG of *A.niger*	Verdoes *et al.,* 1993

2.2.10.4 Transformation Method

Fungal co-transformations were carried out using pAN52-4 fungal expression vector carrying the insert to be cloned and pAMDSPYRG selective plasmid containing the *pyr*G selection marker by the method described by Punt PJ and van den Hondel CA (1992).

First of all, protoplasts were prepared from the strain *Aspergillus sojae* ATCC11906 (pyrG-). For protoplast formation, 250 ml complete medium broth (Appendix B) supplemented with uridine and uracil in 500 ml erlenmeyer flasks was inoculated with 10^6 – $5x10^8$ spores per flask from the stock cultures of *A.sojae* ATCC11906 and incubated at 30°C and 150 rpm for 16-18 hours. Mycelium was separated using a sterile nylon cloth and washed with 20 ml lytic solution (Appendix B) per 1g mycelium. 0.8 g lysing enzyme from *Trichoderma*

harzianum (Sigma) was dissolved in 20 ml lytic solution with a final enzyme concentration of 40 mg/ml and filter sterilized. Then, mycelium was added to this lytic solution containing the lysing enzyme and mixed gently. Mycelium in the lytic solution was incubated at 30°C with slow agitation for 3-5 hours. After 3 hours incubation, protoplast formation was followed by using an optical microscope for every half an hour. When many free protoplasts were observed (more then $1x10^8$) the mycelial debris were removed by filtration through a sterile glass wool located in a funnel and the protoplasts were transferred on ice. Protoplast suspension was centrifuged at 3000 rpm for 5 minutes at 4°C. The supernatant was discarded carefully and the pellets were resuspened gently in 5 ml STC (Appendix B). Then, protoplast suspension was centrifuged again at 3000 rpm for 5 minutes at 4°C. The washing step in STC was repeated and followed by centrifugation at 3000 rpm for 5 minutes at 4°C to form into pellets. After washing, protoplasts were resuspended in STC such that the final concentration was $1x10^8$ protoplasts per ml. Then, 200µl protoplast suspension was mixed with 2µg pAMDSPYRG selective plasmid and 20µg recombinant plasmid pAN52-4CatS (carrying the catalase/phenoloxidase gene) in a 15 ml falcon tube, so the ratio was 1 to 10 for the selective plasmid and the vector. 50 µl of PEG buffer (Appendix B) was added to the transformation mixture and mixed gently, followed by incubation at room temperature (RT) for 20 minutes. Then, 2 ml PEG buffer was added, mixed gently and incubated at RT for another 5 minutes. 4 ml STC was added and mixed gently again. Then, transformation mixture was added into stabilized minimal medium top agar (Appendix B) which was melted and stored at 45°C water bath until use. The tube was mixed by inverting several times and the mixture was poured onto 15 cm MMS-plates (Appendix B). The plates were incubated at 30°C for 3 days.

2.2.11 Enzyme Assay

Catalase activity was assayed by the continuous spectrophotometric rate determination method. In this method, catalase-catalyzed decomposition of H_2O_2 was monitored by the decrease of absorbance at 240 nm. Decrease in absorbance was followed continuously in a spectrophotometer and absorbance data was taken at 30 seconds intervals for 5 to 10 minutes.

In enzyme assay, substrate solution included 50 mM potasssium phosphate buffer (pH 7.0) and 0.036% (w/w) hydrogen peroxide solution (H_2O_2). Absorbance of the substrate solution at 240nm should be between 0.550 and 0.520 absorbance units. If the value was out of this range, hydrogen peroxide from 30% stock solution was added to increase the absorbance or 50 mM potasssium phosphate buffer (pH 7.0) was added to decrease the absorbance. Catalase activity was measured at 25 °C by mixing 1.45 ml substrate solution with 0.5 ml suitably diluted enzyme to give a final volume of 1.5 ml and 50 mM potasssium phosphate buffer (pH 7.0) was used as a blank. The decrease in absorbance at 240 nm was monitored for 5 or 10 minutes. Enzyme activity was determined using the initial rate of the reaction and the extinction coefficient for H_2O_2 was taken as 39.4 M^{-1} cm^{-1} (Merle *et al*., 2007). One enzyme unit was defined as the amount of enzyme that catalyzes the decomposition of 1 µmol H_2O_2 per minute and was calculated by the following formula:

$$U/ml = (\Delta OD/\Delta t)*(1/\varepsilon)*(1000)*(V_K/V_S)*(\text{Dilution Factor}) \qquad \text{(Equation 4)}$$

ΔOD : change in optical density

Δt : change in time (minute)

ε : extinction coefficient of substrate (39.4 $M^{-1}cm^{-1}$)

V_K : total volume of solution in cuvette

V_S : total volume of supernatant in cuvette

The coefficient 1000 in the formula is used to adjust the extinction coefficient from 1/ (mole/L) to 1/ (μmole/ml).

2.2.12 Biomass Measurement

Amount of dry biomass was determined by filtering the cultures through Whatman no.1 filter paper and drying the mycelium at 60 °C for minimum of 16 hours until constant dry weight was reached.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Experimental Strategy for *Scytalidium thermophilum* Catalase/ Phenol Oxidase Gene Cloning

In earlier studies, Ögel *et al.* (2006) have analyzed the production and properties of an extracellular phenol oxidase of *Scytalidium thermophilum* (STEP). The enzyme was able to oxidize both *ortho*- and *para*-diphenols. *Ortho*-diphenol (catechol) is a substrate of catechol oxidases, while *para*-diphenol (hydroquionone) is a substrate of laccases. It was therefore difficult to classify STEP into one of known classes of phenol oxidases; however, the enzyme most notably resembled catechol oxidases. Later, Sutay *et al.* (2008 in press) characterized STEP as a bifunctional catalase/phenol oxidase and named the enzyme as CATPO. It appears that the bifunctional nature of catalases is not limited to *S. thermophilum* but others, including mammalian catalases, also possess an oxidase activity, independent of H_2O_2 (Sutay *et al.,* 2008 in press and Vetrano *et al.,* 1997). In order to study the oxidase mechanism of catalase, this thesis was aimed to clone the bifunctional catalase/phenol oxidase encoding gene of the thermophilic fungus *Scytalidium thermophilum*. The overall experimental strategy is shown in Figure 3.1.

Scytalidium thermophilum catalase gene (*catpo*) was cloned first by Novo Nordisk (United States Patent, No. 5646025) into *Aspergillus oryzae,* therefore,

the gene sequence was available. Thus, specific primers were designed based on the nucleic acid sequence of the *Scytalidium* catalase gene excluding the signal peptide and pro-peptide region. The gene of interest was amplified using Platinum *Pfx* polymerase and A-overhangs were added to the fragment for TA cloning. Then, the amplified gene fragment with A-overhangs was ligated onto vector pCR®2.1 and cloned into *E. coli* XL1 Blue MRF' cells. Following plasmid isolation, sequencing was carried out in order to control the specifity of the amplified fragment. Alignment between the sequencing results and the target gene sequence assigned that the sequence of the amplified fragment corresponds to sequence of the target gene except difference in 10 amino acids. After subcloning into pCR®2.1, recombinant plasmids, pCR2.1CatS, and vector pAN52-4 were cut with *Hind*III restriction enzyme and the catalase gene was ligated, followed by transformation into *E. coli* XL1 Blue MRF' cells. Recombinant plasmids, pAN52-4CatS, were again determined by PCR using gene-specific primers. The orientation of the ligation was confirmed by *Nco*I restriction enzyme digestion. Finally, the recombinant plasmid, pAN52-4CatS, was transformed into *Aspergillus sojae.* A total of 12 transformants showed catalase activity. Presence of the catalase gene was confirmed by PCR using the genomic DNA of the transformants and enzyme activity analysis.

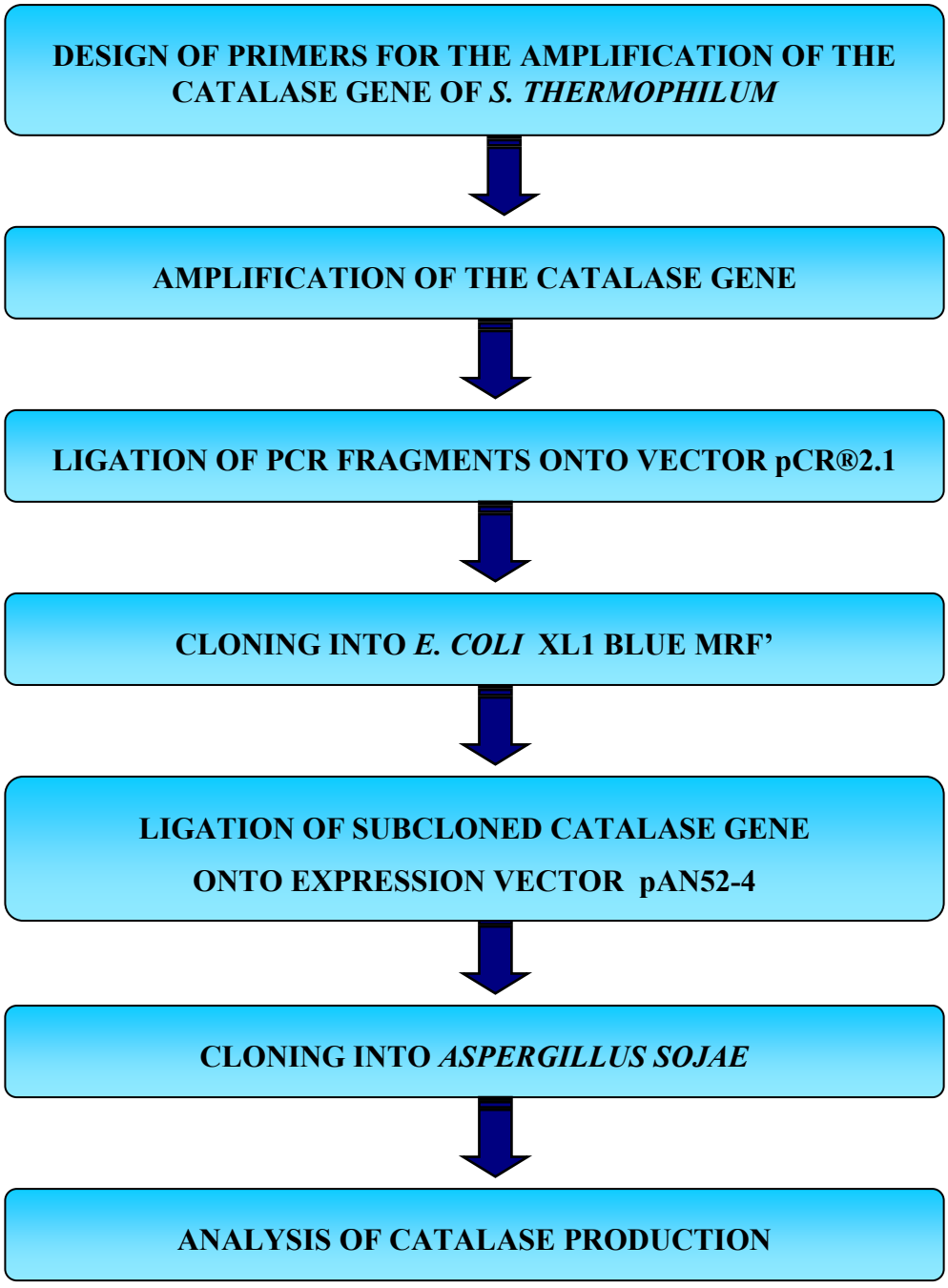


Figure 3.1 Flowchart of the experimental strategy

3.2 PCR Cloning of the *Scytalidium thermophilum* Catalase / Phenol Oxidase Gene

3.2.1 Isolation and Purification of *Scytalidium thermophilum* Genomic DNA

Scytalidium thermophilum total genomic DNA was isolated according to the procedure given in section 2.2.2.1. The resulting DNA samples were visualized by agarose gel electrophoresis (Figure 3.2). Concentration of DNA was calculated according to the formula given in the section 2.2.2.1.

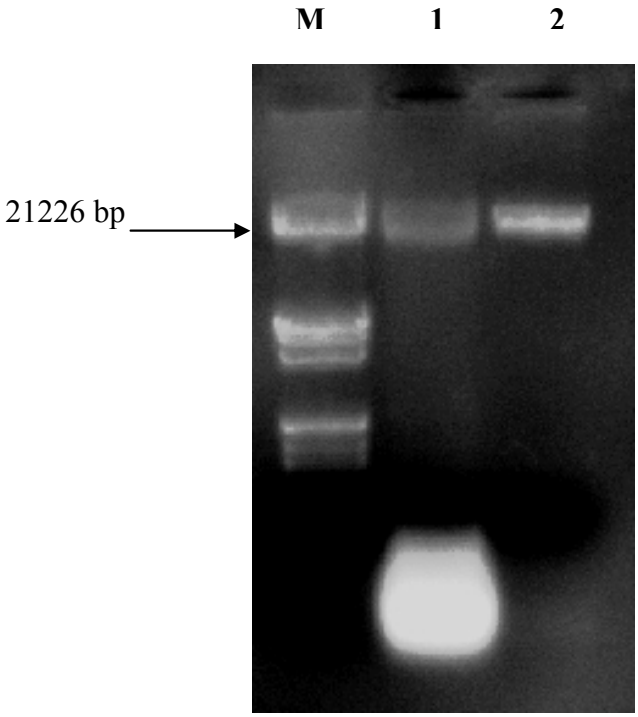


Figure 3.2 Genomic DNA of *Scytalidium thermophilum.* M, Molecular size marker: λDNA/*Eco*RI+*Hind*III (0.5µg/µl); lane 1, 0.1µg/µl DNA (before RNAse treatment); lane 2, 0.1µg/µl DNA (after RNAse treatment).

3.2.2 Amplification of *Scytalidium thermophilum* Catalase / Phenol Oxidase Gene

S. thermpohilum catalase gene was amplified from the genomic DNA using two specific primers, namely catS1 and catS2 (Figure 3.3). Amplification with catS1 and catS2 primers yielded a PCR product of an expected size of about 2697bp (Figure 3.4). Although the amplification primers, catS1 and catS2, were designed according to the expression vector pAN52-4, the amplified gene fragments, obtained by using those primers, were first used in the ligation process onto vector pCR®2.1 for sub-cloning.

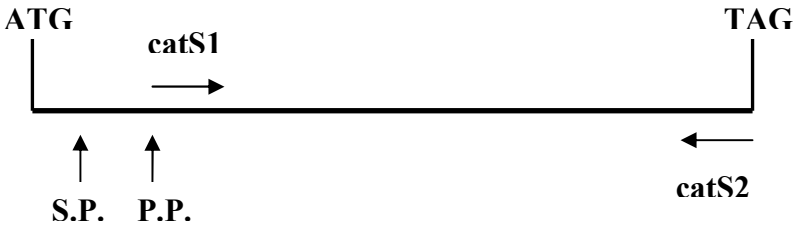


Figure 3.3 The schematic illustration of the location of primers catS1 and catS2, signal peptide cleavage site (S.P) and propeptide cleavage site (P.P) on the catalase gene.

For the optimization of PCR reactions, different annealing temperatures and different concentrations of $MgCl_2$ or $MgSO_4$, which ranges from 1.5 mM to 3.0 mM, were tested. In the beginning, *Taq* DNA polymerase did not yield a PCR product, since the target gene was too large. Therefore, amplification was carried out with *Pfx* DNA polymerase (Invitrogen) and PCRx Enhancer Solution of the

kit was also used for higher primer specificity. Best amplification was at 55°C with 1.5 mM $MgSO_4$ (Figure 3.4).

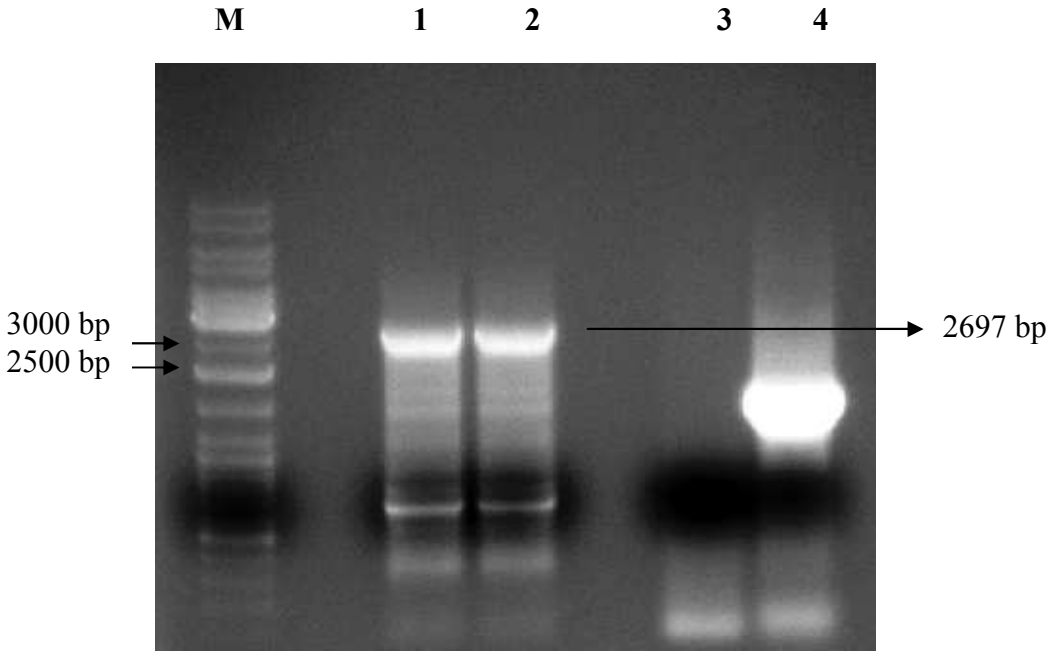


Figure 3.4 Amplification of catalase gene from *S.thermophilum* using primers cats1 and catS2. PCR amplifications yielded a major band of approximately 2697 bp size. M, GeneRuler™ DNA Ladder Mix (100-10000 bp, Fermentas), lane 1&2; amplified gene fragments; lane 3, negative control; lane 4, positive control.

3.2.3 Ligation of the Putative Catalase / Phenol oxidase Gene onto Vector pCR®2.1

Nontemplate-dependant activity of *Taq* polymerase, which adds single deoxyadenosine (A) to the 3' ends of PCR products, allows PCR inserts to ligate efficiently onto vector pCR®2.1 which is a TA Cloning® vector, having a single 3'deoxythymidine (T) residues at each end. In this study; however, PCR amplifications of the catalase gene were carried out with the enzyme *Pfx* DNA polymerase (Invitrogen) which has a proofreading activity, so it does not add 3' A-overhangs required for the ligation. Therefore, 3´ A-overhangs were added to the PCR products by the method described in section 2.2.8.1. The resulting insert used in the ligation is shown in Figure 3.5.

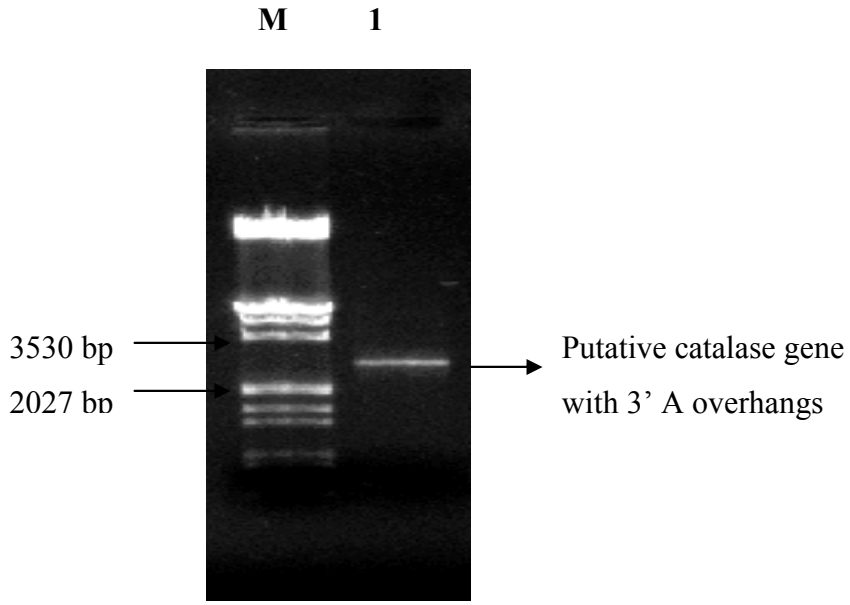


Figure 3.5 The PCR fragment carrying the putative catalase gene of *S.thermophilum* following addition of 3' A-overhangs and purification. M, Molecular size marker: λDNA/*Eco*RI+*Hind*III; lane 1, putative catalase gene fragment.

3.2.4 Isolation of Recombinant Plasmids Containing the Catalase / Phenol Oxidase Gene

Following ligation and selection of *E.coli* transformants, the isolated recombinant plasmid was named as pCR2.1CatS (approximate size 6626 bp) (Figure 3.6). Plasmid isolates (Figure 3.7) were first analyzed for the presence of insert by PCR reactions and restriction enzyme digestion. Out of 22 plasmids (15 of them are shown in Figure 3.7), only pCR2.1CatS5 and pCR2.1CatS8 were likely to carry the catalase gene. PCR amplification with primers catS1 and catS2 from the plasmid pCR2.1CatS5 yielded the expected 2697 bp fragment. Also, *Hind*III restriction enzyme digestion of pCR2.1CatS5, yielded two fragments, one corresponding to pCR®2.1 vector (3929bp) and the other corresponding to the insert (2697 bp) as expected. It was concluded that recombinant plasmid pCR2.1CatS5 carried the putative catalase/phenol oxidase gene (Figure 3.8).

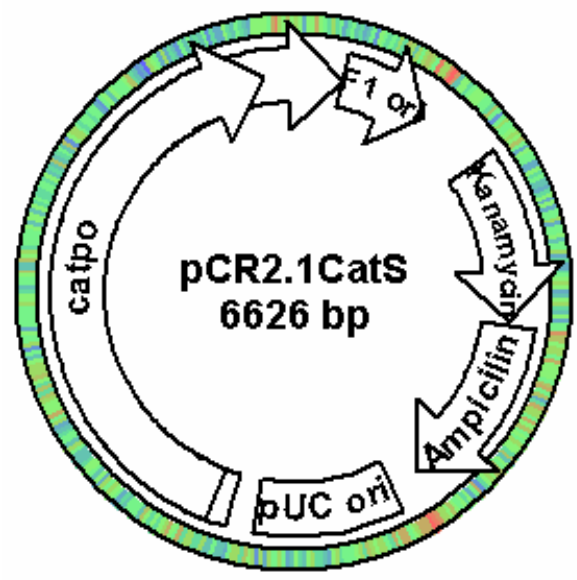


Figure 3.6 Recombinant pCR2.1CatS plasmid.

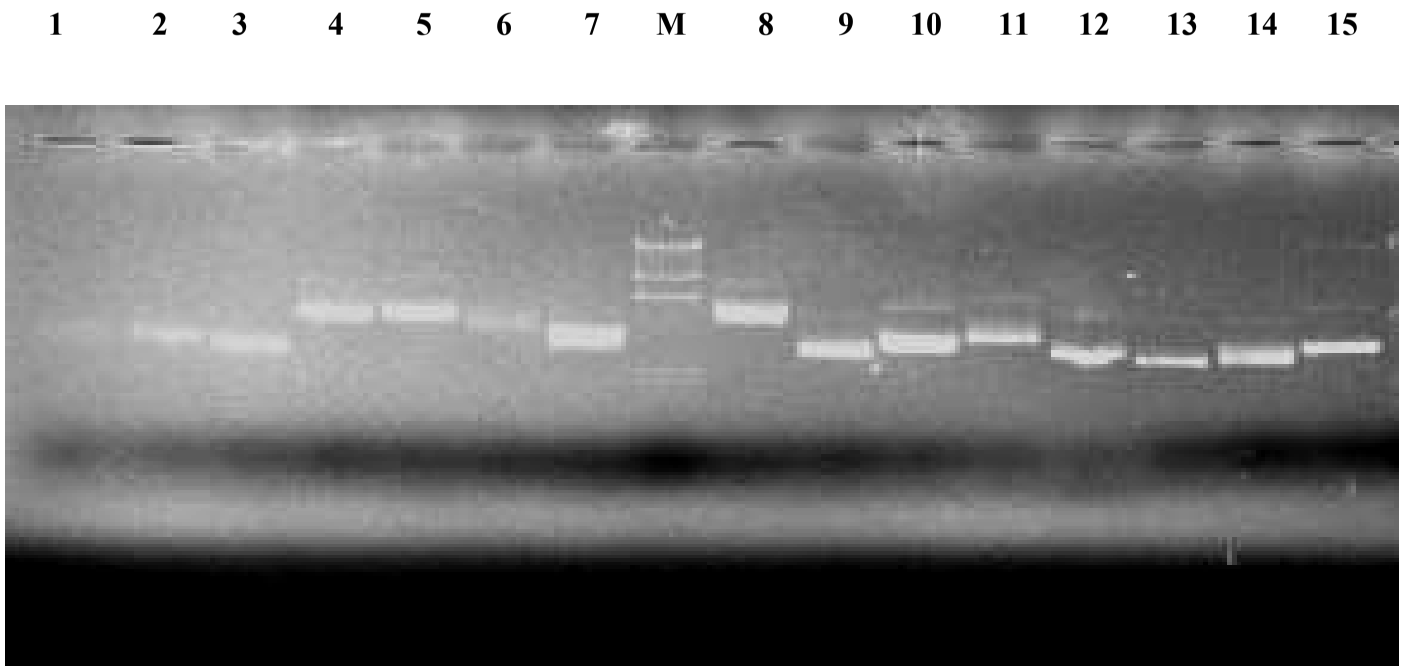


Figure 3.7 Isolated plasmids from *E.coli* pCR2.1CatS transformants. M, Molecular size marker: λDNA/*Hind*III (0.5µg/µl); lane 1-15 analysis of the isolated plasmid DNAs pCR2.1CatS1 to pCR2.1CatS15, respectively.

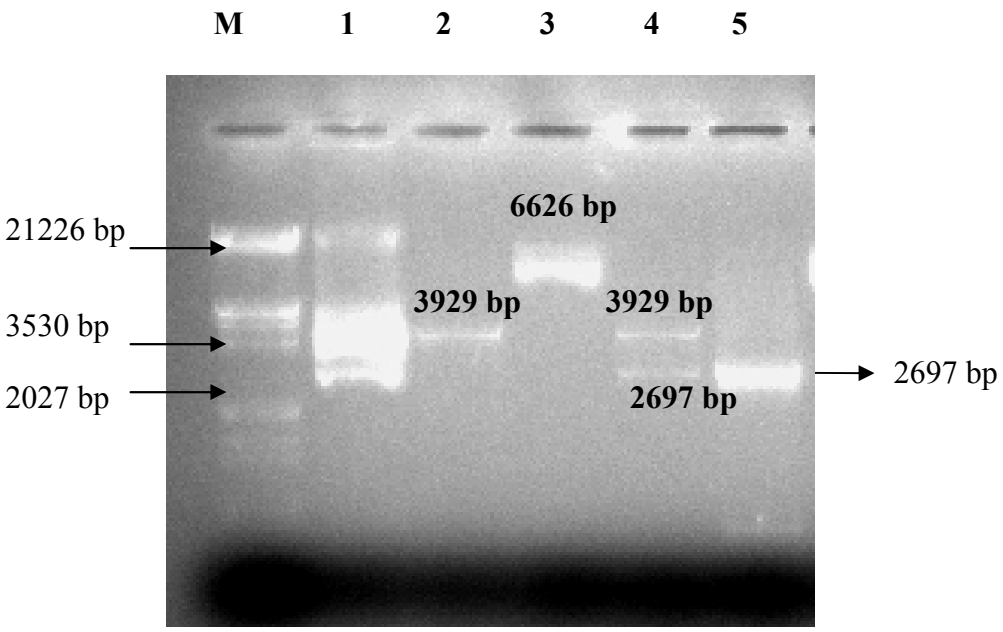


Figure 3.8 M, Molecular size marker: λ DNA/*Eco*RI+ *Hind*III; lane 1, vector pCR®2.1; lane 2, linearized vector pCR®2.1 after restriction digestion; lane 3, recombinant plasmid pCR2.1CatS5; lane 4, *Hind*III restriction enzyme digestion of the recombinant plasmid pCR2.1CatS5; lane5, PCR amplification from pCR2.1CatS5. The expected size of fragments are indicated on top of the bands.

The insert on purified recombinant plasmid, pCR2.1CatS5, was sequenced in order to confirm that the insert is indeed the catalase/phenol oxidase gene and to control the presence of any unwanted mutations. The gene cloned in this study was indeed the catalase gene of *S. thermophilum* and was named as *catpo*.

3.2.5 Characterization of the *catpo* Gene

As indicated before, CATPO encoding gene was amplified such that the native signal and propeptide regions were excluded. According to sequence data, the *catpo* gene encoding the mature CATPO region has 681 amino acids with 7

introns. Amino acids for heme binding and active site amino acids were predicted according to the amino acid sequence of *Neurospora crassa* Cat3, whose active site amino acids and amino acids for heme binding has been reported by Michan *et al*. (2002) (Figure 3.9). Theorotical pI value and molecular weight (MW) of each subunit of the protein was calculated based on the amino acid composition of the c*atpo* gene and found as 5.37 and approximately 75 kDa, respectively. Sutay *et al.* (2008 in press) has determined the pI value of the protein as 5.0 on Isoelectric Focusing Gel and has determined the MW of each subunit of the protein as approximately 80 kDa by SDS-PAGE and MW of the protein as approximately 320 kDa by native-PAGE.

As compared to the amino acid sequence of *S. thermophilum* catalase, determined by Novo Nordisk in 1997 (United States Patent, No.5646025), *catpo* gene also encodes a mature protein of 681 amino acids, although 10 amino acids of the *catpo* gene were different (Table 3.1). Amino acid sequence alignment of the *catpo* gene with the amino acid sequence of the catalase gene, revealed by Novo Nordisk, is given in Figure 3.10.

Differences in amino acid sequences may come from the probable unwanted mutations during PCR. In this study, PCR for the purpose of gene cloning was carried out using the enzyme Platinum® *Pfx* DNA Polymerase (Invitrogen). On the other hand, in subcloning studies carried out by Novo Nordisk, *Taq* DNA Polymerase obtained from Boehringer Mannheim was used in PCR and these fragments were sequenced using M13 and catalase specific primers for the determination of predicted amino acid sequence od *Scytalidium* catalase. *Taq* DNA polymerase is the predominant polymerase used in PCR; however, its lack of proofreading activity results in inaccurate nucleotide incorporation and generation of mutant molecules. Platinum® *Pfx* DNA Polymerase has proofreading 3′ to 5′ exonuclease activity that edits mismatched nucleotides and it has been shown to provide higher fidelity than any thermostable DNA

polymerase tested (Takagi *et al*., 1997 and Lackovich *et al*., 2001). In conclusion, 10 amino acid differences between the amino acid sequences of catpo gene and *Scytalidium* catalase gene, revealed by Novo Nordisk, may be the result of mismatch mutations during PCR. Because of the existence of proofreading activity of Platinum® *Pfx* DNA Polymerase used in this study, it is expected that the amino acid sequnce of *catpo* gene is more likely to be the correct amino acid sequence of *Scytalidium* catalase.

Table 3.1 Amino acid differences between the amino acid sequence of *catpo* gene and the amino acid sequence of *Scytalidium* catalase gene which was determined by Novo Nordisk.

Amino acid in the *catpo* gene	**Amino acid in the *Scytalidium* catalase gene, determined by Novo Nordisk**
A_{148}	T_{184}
R_{161}	S_{197}
T_{186}	A_{222}
I_{301}	V_{337}
A_{426}	V_{462}
K_{477}	R_{513}
G_{502}	A_{538}
V_{522}	L_{558}
D_{647}	N_{683}
M_{659}	K_{695}

Figure 3.9 Nucleotide sequence of the *catpo* gene and the encoded amino sequence. Intron positions are also indicated in the figure. Active site amino acids are highlighted by green and amino acids for heme binding are highlighted by blue. (each amino acid is encoded by the triplet under the position of it)

```
a.a.    233 RQGKASLVWEEAQVLSGKNADFHRQDLWDAIESGNGPEWDVCVQIVDES
nuc.   1191 ccgagacgtgggcgctgaaggtcccgctggagtgagcgtggtgcagggt
            gagacgttgaacattcgaacatagaatgactacgagcagatgtattaac
            cgaggtacgagggtttcgtcccctgccgtttgcgcaaagtcccgtctgc

a.a.    282 QAQAFGFDLLDPTKIIPEEYAPLTKLGLLKLDRNPTNYFAETEQVMFQP
nuc.   1338 cgcgtgtgtcgcaaaacggtgctaacgctacgcacaattggagcgatcc
            acactgtattaccattcaaacctcatNttatagaccaatcacaatttac
            ggactcccggcgagcccggcccggaggcgggtctgccccc ggggcgcac

a.a.    331 GHIVRGVDFTEDPLLQGRLFSYLDTQLNRNGGPNFEQLPINMPRVPIHN
nuc.   1487 gcagcgggtaggccccgcctttcgaccacaggcatgcccaaaccgcaca
            gattggtatcaacttaggttcatacatagaggcataatctatcgtctaa
            ttaccccccggtcgagatccgctcgggcgtcgcctgggcccgggggtcc

a.a.    380 NNRDGAGQMFIHRNKYP          Y:Y[tac]        TPNTLN
nuc.   1634 aacggggcatacaaatcTGTAAGTA  Intron 6   CAGACacaaca
            aagagcgatttagaaac <1-----[1686 : 1739]-1>  ccacta
            ttcccccggcccgcgtt                         tcccgc

a.a.    404 SGYPRQANQNAGRGFFTAPGRTVSGALVREVSPTFNDHWSQPRLFFNSL
nuc.   1760 agtcccgacaggcgttagcgcagaggcgcggtcatagcttcccc ttatc
            ggacgacaaacgggttcccggctggcttgatccctaaagcacgtttact
            tttggaccatcacaccagtctccctccctgggaaccccgggctcccccc

a.a.    453 TPVEQQFLVNAMRFEISLVKSEEVRKNVLTQLNRVSHDVAVRVAAAIGL
nuc.   1907 acggcctcgagactgaacgatgggaaagcaccacgacggggcggggagc
            cctaaatttactgtatgttacaatgaattcatagtgaatctgtccctgt
            tccaggcccccgccacctgggaacggcgccgcccccttgcgcgcctccc

a.a.    502 AAPDADDTYYHNNKTAGVSILGSGPLPTIKTLRVGILATTSESSALDQA
nuc.   2054 ggcggggattcaaaagggtacgagctcaaaaccggacgaaagtagcgcg
            cccacaacaaaaaaccgtcttgggctcctactgtgttcccgacgctaac
            cgccgccacccccggtccgctacgcgtccgtcccccgtcgcggcggtga

a.a.    551 AQLRTRLEKDGLVVTVVAETLREGVDQTYSTADATGFDGVVVVDGAAAL
nuc.   2201 gcccaccgaggcggaggggaccggggcattagggagtgggggggggggc
            catgcgtaaagtttcttcactgagtaacacccaccgtagttttagccct
            cgccctgagcgtgcgtgtaggcggacgacgggtcgtccctttgcggggg
```

Figure 3.9 (continued).

```
a.a.     600 FASTASSPLFPTGRPLQIFVDAYRWGKPVGVCGGKSSEVLDAADVPENG
nuc.    2348 tgaagttcttcagactcatgggtctgacgggtggataggtgggggcgag
             tcgccccctccggctatttacagggactgtgggacgattaccatcaag
             tcccgggggcggcggggctgcgtggaggctgttgggcgggtggttgatg


a.a.     649 DGVYSEESVDKFVEEFEKGLATFR                        FT
nuc.    2495 gggttggtggatgggtgagtgataGTGAGTC  Intron 7   TAGta
             agtacaactaattaataagtcctg<0-----[2567 : 2660]-0>tc
             cggtgggggcgtgggtggggttcg                        tc


a.a.     675 DRFALDS
nuc.    2667 gctgcgt
             agtctac
             tgtgccc
```

Figure 3.9 (continued).

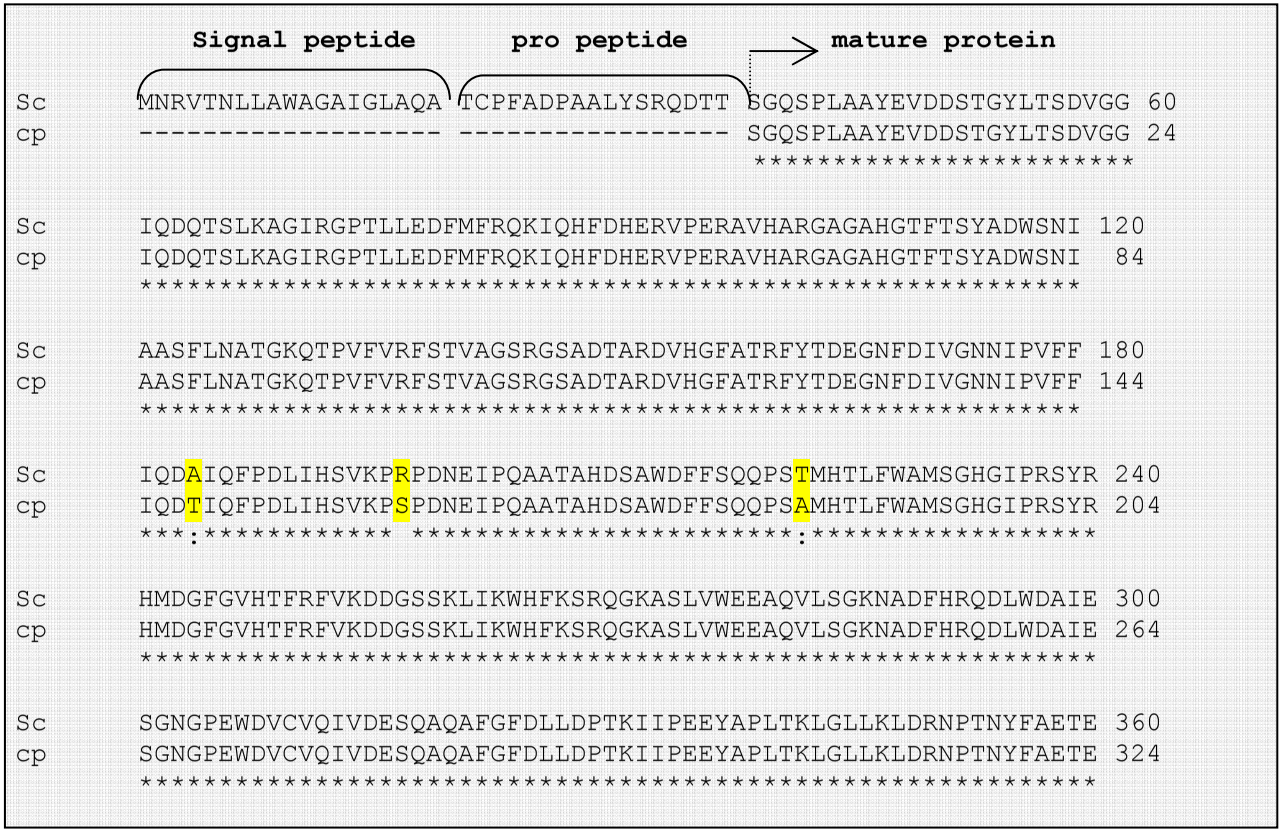


Figure 3.10 Alignment of the amino acid sequence revealed from the *catpo* gene sequence and the catalase gene sequence determined by Novo Nordisk. Sc indicates the amino acid sequence of *S. thermophilum,* determined by Novo Nordisk and cp indicates the amino acid sequence of *catpo* gene. (amino acid differences are highlighted in yellow)

Figure 3.10 (continued).

3.3 Cloning of the *catpo* Gene onto the *Aspergillu sojae* Expression Vector pAN52-4

3.3.1 Primer Design

Primers (catS1 and catS2) used for the amplification of the *catpo* gene from *Scytalidium* genomic DNA were actually designed to allow cloning into the *Aspergillus sojae* expression vector pAN52-4. As mentioned in section 2.2.3, *Hind*III restriction enzyme sites were added to the 5' ends of both primers and amplified fragments lacked the native signal peptide and pro-peptide regions of the catalase gene. The expression vector pAN52-4 possesses the signal and pro-peptide sequence of the glucoamylase gene (*gla*A) of *Aspergillus niger*. It was decided to fuse the catalase gene to the signal and pro-peptide region of *gla*A with the possibility of enhancing the secretion process (Punt *et al.,* 1992). The catS1 primer was designed such that two additional nucleotides were added to the 5' end of the primer downstream of the *Hind*III restriction enzyme site in order to conserve the actual reading frame (Figure 3.10). Signal peptide sequence (dark blue), propeptide sequence (blue), *Bam*HI restriction enzyme site (orange), *Hind*III restriction enzyme site (pink) and the additional nucleotides (green) are indicated in the figure. The underlined nucleotide in the figure was changed to prevent hairpin–loop formation; however, change in this nucleotide was chosen such that it did not cause any change in the amino acid sequence of the catalase gene.

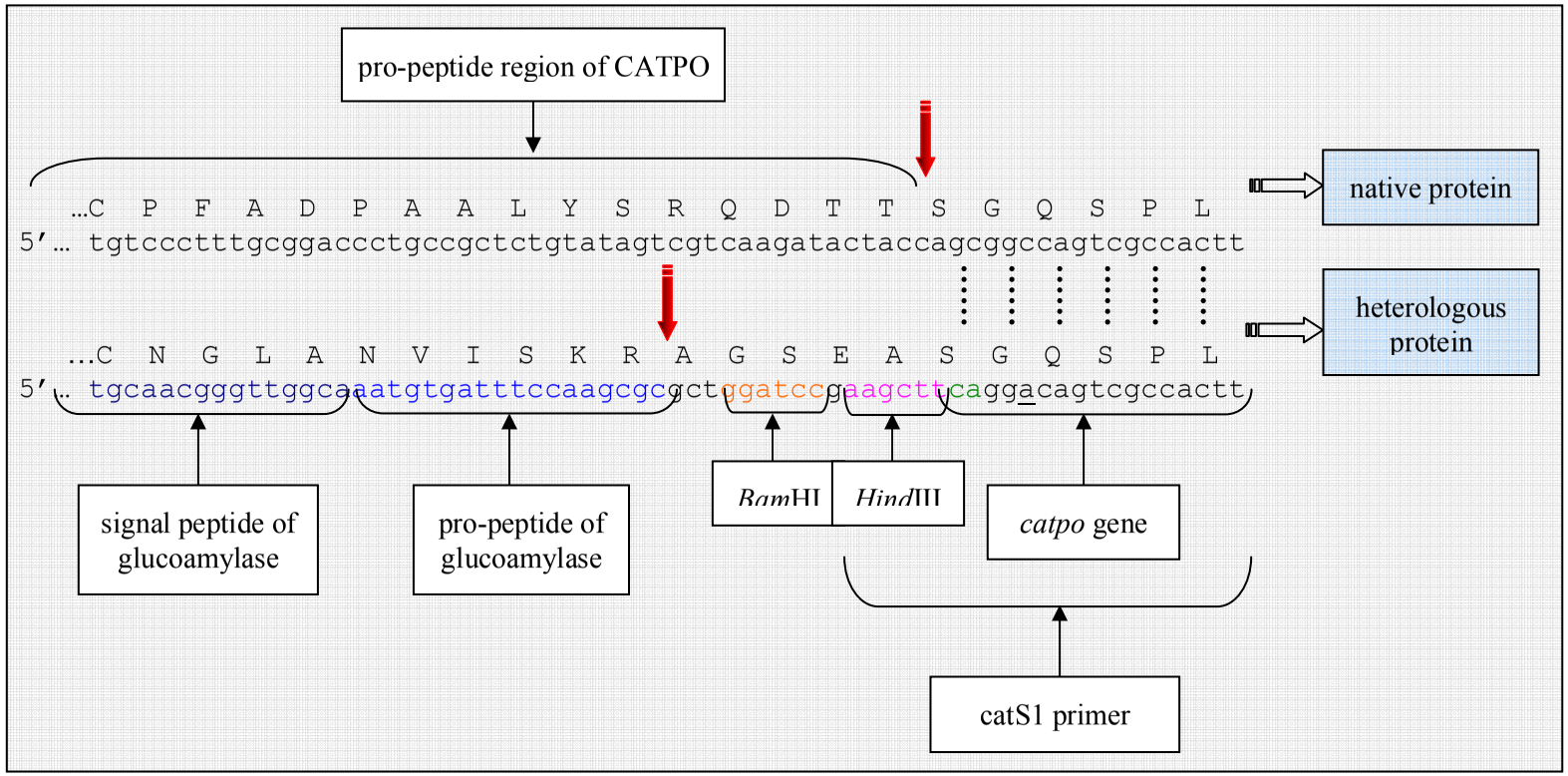


Figure 3.11 The vector-insert junction region in the recombinant plasmid pAN52-4CatS containing the *catpo* gene of *S.thermophilum* on vector pAN52-4 (heterologous protein) and pro-peptide-mature protein junction region of *S.thermophilum* catalase (native protein). Red arrows indicate the mature protein start sites in both the heterologous and native protein. Signal peptide sequence (dark blue), propeptide sequence (blue), *Bam*HI restriction enzyme site (orange), *Hind*III restriction enzyme site (pink) and the additional nucleotides (green) are indicated in the figure.

3.3.2 Restriction Digestion of the Recombinant Plasmid pCR2.1CatS5

After sub-cloning onto vector pCR®2.1 and sequence analysis, the *catpo* gene was cloned onto the expression vector pAN52-4 by using the unique *Hind*III restriction enzyme cut site. The pCR2.1CatS5 recombinant plasmid was therefore cut with *Hind*III restriction enzyme and the 2697 bp gene fragment was recovered from the agarose gel by the method described in section 2.2.2.3. Expression vector pAN52-4 was also cut with the *Hind*III restriction enzyme and dephosphorylated to prevent religation by the method explained in section 2.2.8.2.

3.3.3 Ligation of the *catpo* Gene onto Vector pAN52-4 and Transformation into *E. coli* Blue MRF'

The recombinant plasmid, containing the *catpo* gene on vector pAN52-4, was named as pAN52-4CatS. (approximate size 8457 bp) (Figure 3.11).

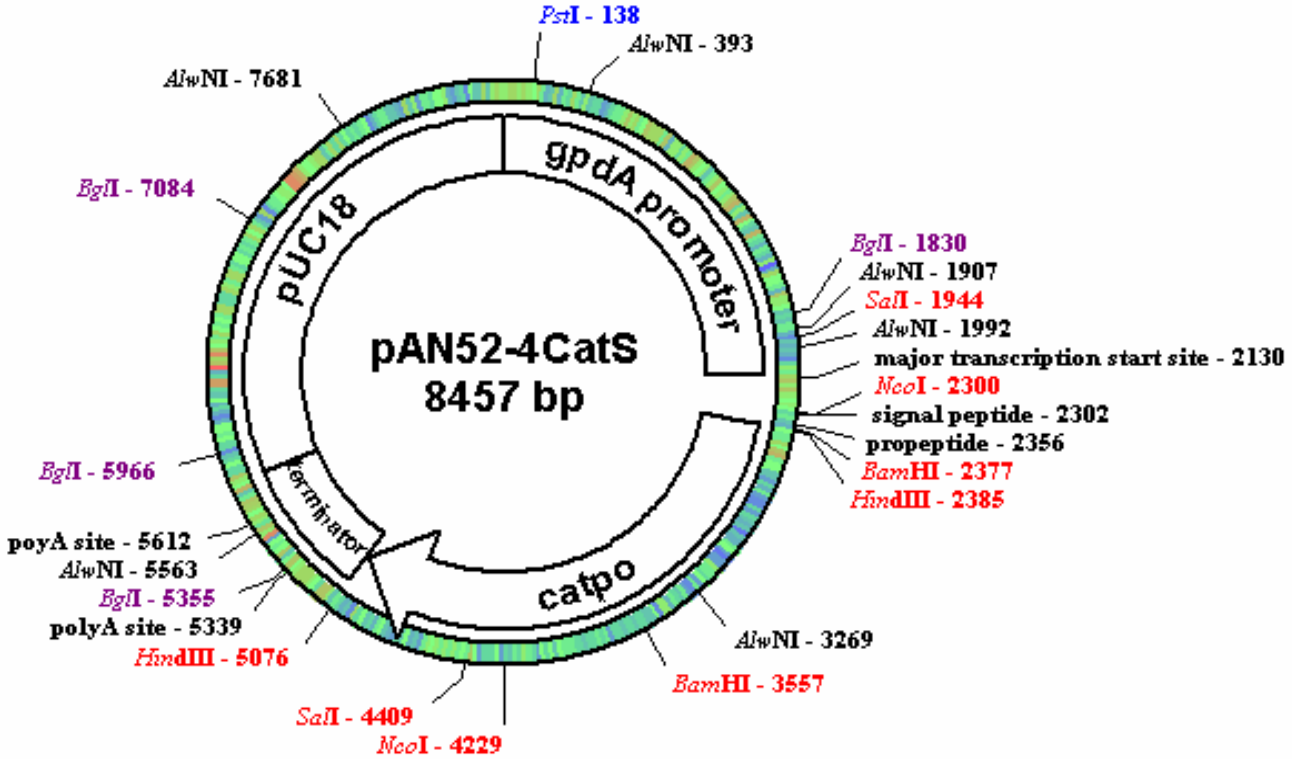


Figure 3.12 Recombinant pAN52-4CatS plasmid.

3.3.4 Isolation of the Recombinant Plasmids Containing the *catpo* Gene

After the transformation into *E.coli* XL1 Blue MRF' cells, only white colonies were formed due to the lack of a screening marker, therefore the recombinant clones were selected randomly. 45 white colonies were isolated and analyzed by agarose gel electrophoresis (20 of them are shown in the Figure 3.12). Since the orientation of the insert in the recombinant plasmid was important for expression, *Nco*I restriction enzyme digestion was carried out on 19 plasmid isolates, which had the expected size of the recombinant plasmid. If the insert was in the correct orientation, *Nco*I restriction enzyme digestion would yield two fragments with the size of 7524 bp and 933 bp (Figure 3.13). Only 3 recombinant plasmids, pAN52-4CatS28, pAN52-4CatS32 and pAN52-4CatS40, had the *catpo* gene in the correct orientation. The presence of the *catpo* gene was also confirmed by gene-specific PCR amplifications, using primers catS1 and catS2 (Figure 3.14). Recombinant plasmid pAN52-4CatS28 was used for further transformation into *Aspergillus sojae* ATCC 11906.

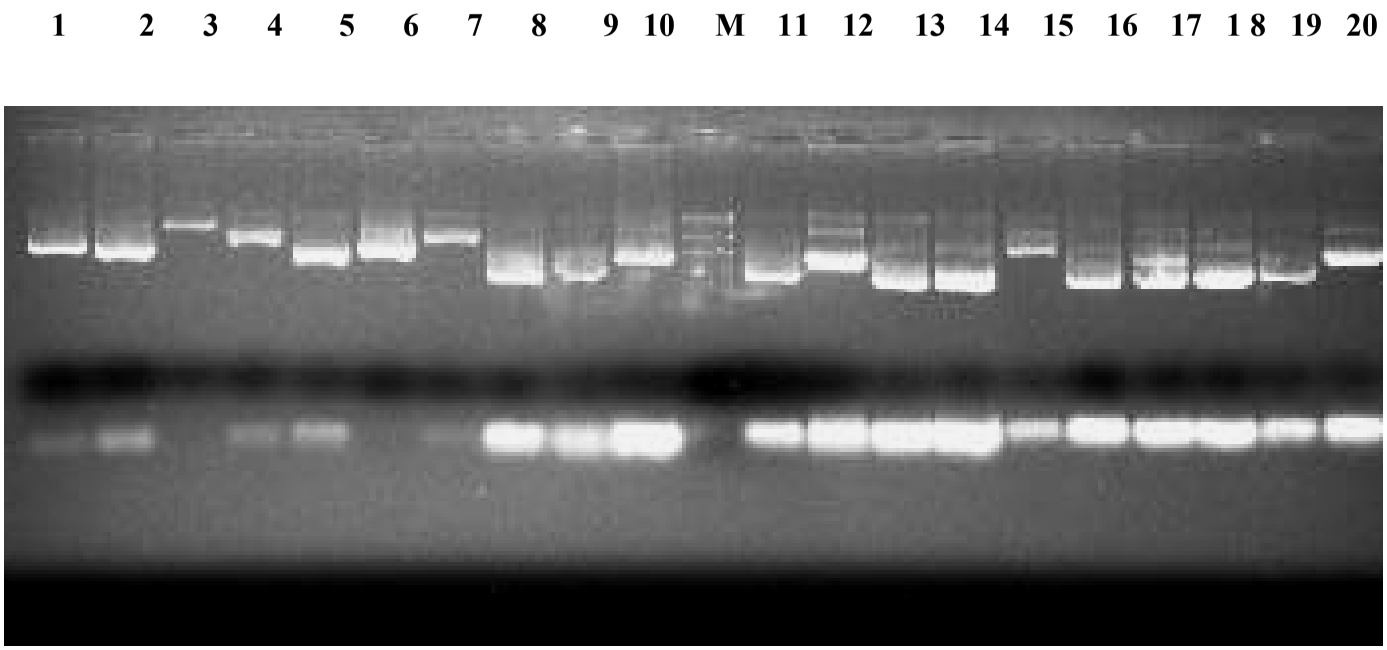


Figure 3.13 Isolated plasmids after ligation of *catpo* gene on vector pAN52-4.
lane 1 – lane 10, plasmids 26-35; lane 11-lane 20, 36-45; M, Molecular size marker: λDNA/*Hind*III.

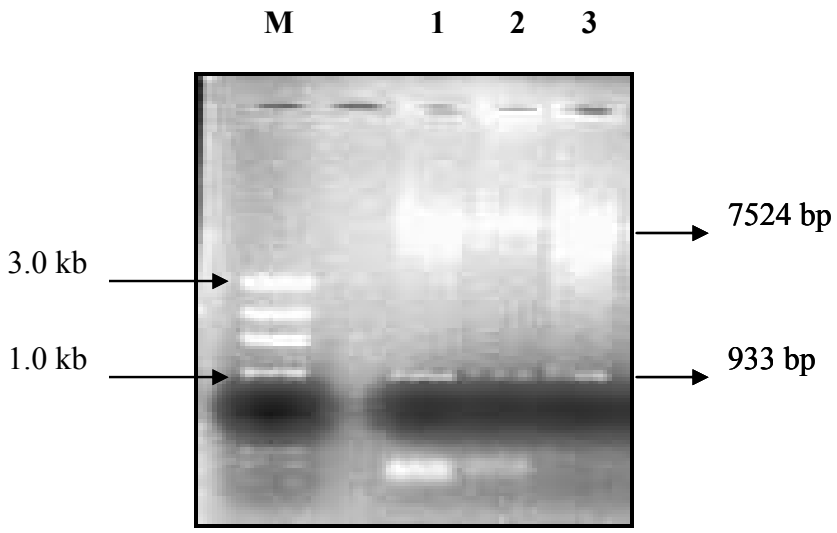


Figure 3.14 Restriction digestion analysis of recombinant plasmids. lane 1, *Nco*I digested pAN52-4CatS28; lane 2, *Nco*I digested pAN52-4CatS32; lane 3, *Nco*I digested pAN52-4CatS40; M, Gen100 DNA Ladder (100-3000bp).

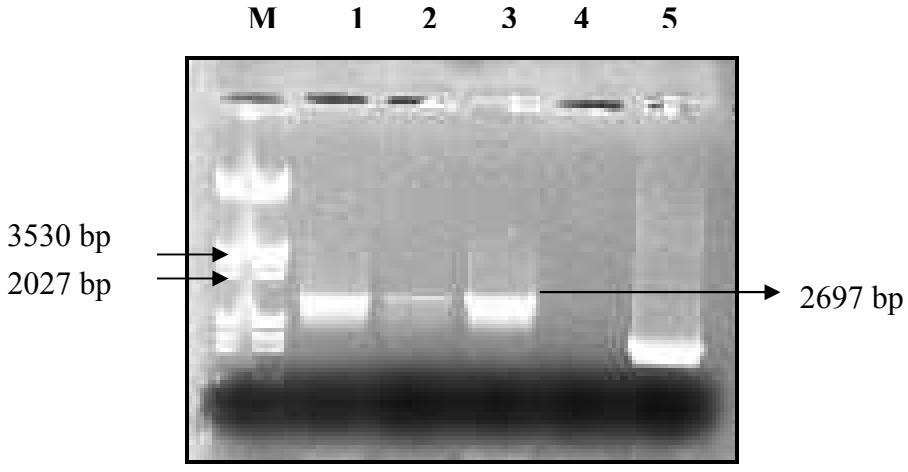


Figure 3.15 PCR fragments amplified by primers catS1 and catS2 from the recombinant plasmids pAN52-4CatS28 (lane1), pAN52-4CatS32 (lane2) and pAN52-4CatS40 (lane3); M, Molecular size marker: λDNA/*Eco*RI+*Hind*III; lane 4, negative PCR control; lane 5, positive PCR control.

3.4 Transformation of pAN52-4CatS28 into *Aspergillus sojae*

3.4.1 Protoplast Transformation

Aspergillus sojae expression system employs the *gpdA* promoter from *Aspergillus nidulans,* which is the promoter of the gene glyceraldehyde-3-phosphate dehydrogenase, an enzyme in glycolysis. Thereby, it is expected to attain high level of constitutive expression in *A. sojae*. Furthermore, as the signal sequence of the *catpo* gene was replaced by the *A. niger* glucoamylase signal and pro-peptide sequence (accession no. X00712 with a dibasic processing site (LYS-ARG), the efficiency of secretion is also expected to be better. On the other hand, the removal of the native propeptide of catalase may have undesirable effects such as incorrect in vivo folding of the protein and/or decrease in secretion of the active enzyme.

At the end of two transformations experiments, 76 transformants were obtained. Transformants were tested for catalase activity and 12 transformants showed relatively high catalase activities as compared to control *A.sojae* transformant with pAMDSPYRG. Therefore, first genomic DNA was isolated (Figure 3.16) and next, gene-specific PCR was performed using the genomic DNA of the 12 transformants in order to confirm the presence of the *catpo* gene of *Scytalidium thermophilum* on the genome of *Aspergillus sojae* (Figure 3.17).

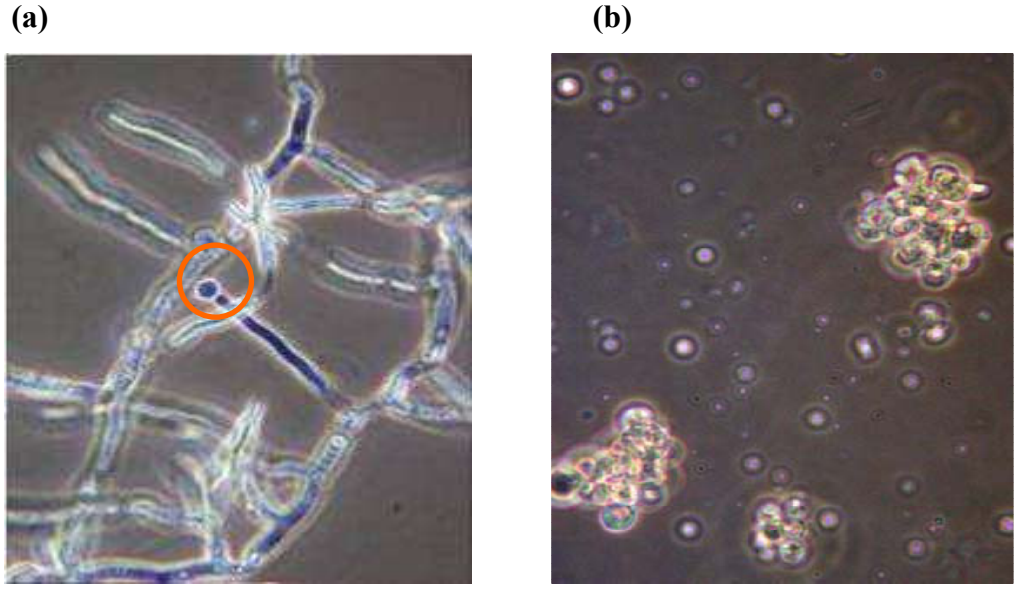


Figure 3.16 Protoplast formation from *A. sojae* mycelium. (a) Initial stages of protoplast formation. (b) After 2 hours of protoplasts formation.

3.4.2 Analysis of the Transformants

Amplification from the genomic DNA of 12 transformants using primers catS1 and catS2 was not successful by the enzyme *Taq* DNA polymerase, since the gene fragment was 2697 bp, which is rather large for amplification with this enzyme. Therefore, new primers, called Acat1 and Acat2, were designed to allow amplification of a PCR product of about 562 bp. In the PCR studies, genomic DNA of the pAMDSPYRG transformant was used as the negative control and genomic DNA of *Scytalidium thermophilum* was used as the positive control. PCR studies with 12 transformants showed that *Scytalidium catpo* gene was present in four of the transformants (45^{th}, 55^{th}, 57^{th} and 64^{th}) (Figure 3.17).

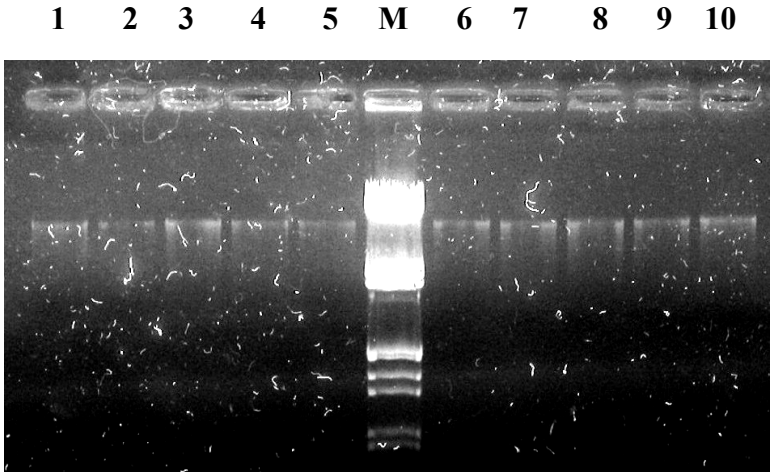
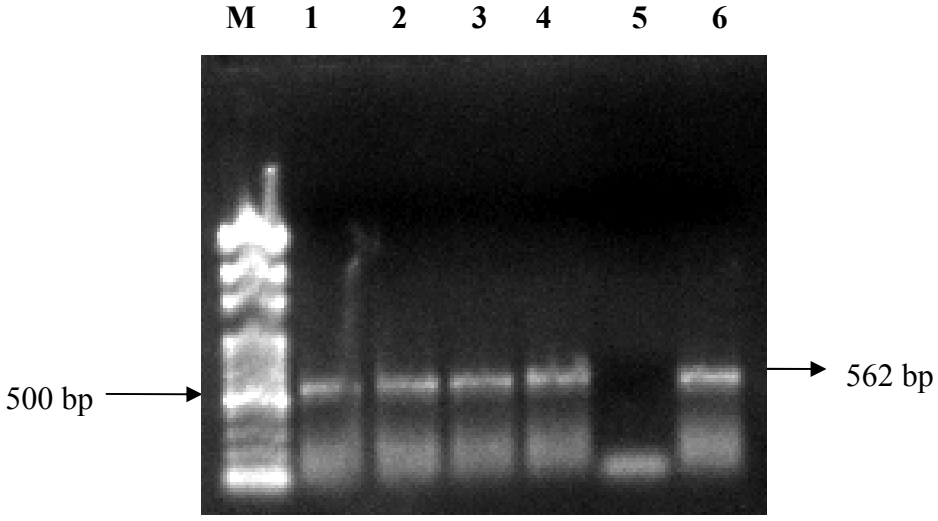


Figure 3.17 Genomic DNA isolation of the transformants, which had the highest catalase activities. M, Molecular size marker: λDNA/*Eco*RI+*Hind*III (0.5μg/μl); lane 1 - lane 9, genomic DNA of transformants 23, 24, 44, 45, 55, 57, 59, 64, 66, respectively; lane10; genomic DNA of pAMDSPYRG transformant.



F**igure 3.18** PCR analysis of the *A. sojae* transformants for the *catpo* gene. M, Gen100 DNA Ladder (100-3000bp); lane 1–lane 4, PCR from genomic DNA of transformants 45, 55, 57, 64; lane 5, PCR from genomic DNA of pAMDSPYRG transformant (negative control); lane 6, PCR from genomic DNA of *Scytalidium thermophilum* (positive control)

3.4.2.1 Catalase Activities of the Transformants

Transformants 45, 55, 57 and 64, shown to contain the *catpo* gene, were further grown in complete medium (without uridine and uracil) including 2 % glucose (w/v) with an initial pH of 6.5. Catalase activity was assayed at 25 °C by the method described in section 2.3.10; Maximum activities of the transformants were observed at the fourth day of incubation, which are compared in Figure 3.18. Accordingly, transformant 64th (AsT64) showed the highest catalase activity among the transformants, yielding; 13205.7 U/g. However, this was still lower than the catalase activity of the gene donor strain of *Scytalidium thermophilum*.

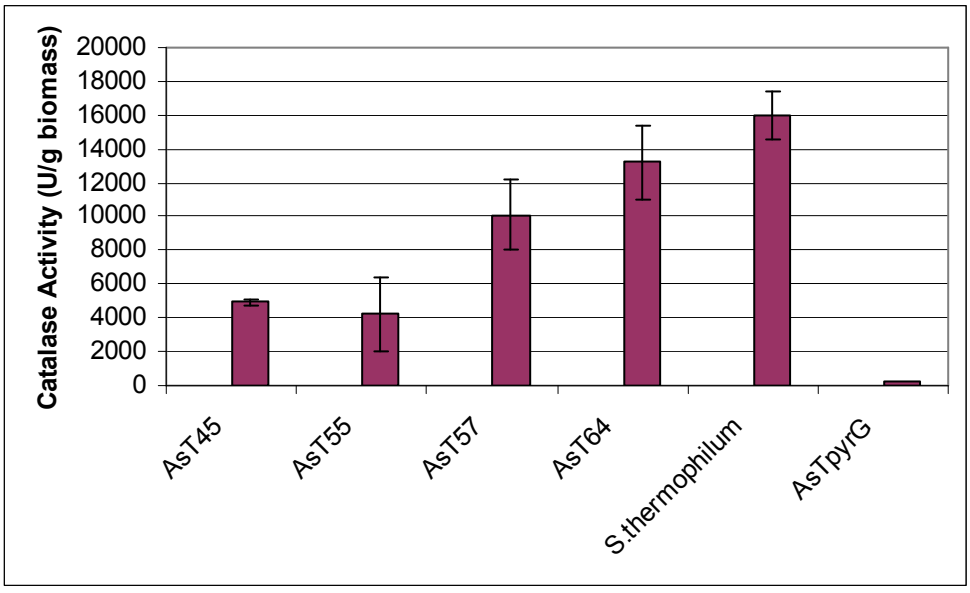


Figure 3.19 Comparison of maximum catalase activities of transformants and *Scytalidium thermophilum* in culture supernatants at day 4. *A.sojae* transfomants were grown at 30 °C and *S.thermophilum* was grown at 45 °C. Enzyme assay temperature was 25 °C. *As*T indicates *A. sojae* transformants; *As*TpyrG indicates *A. sojae* transformant with only marker vector (negative control).

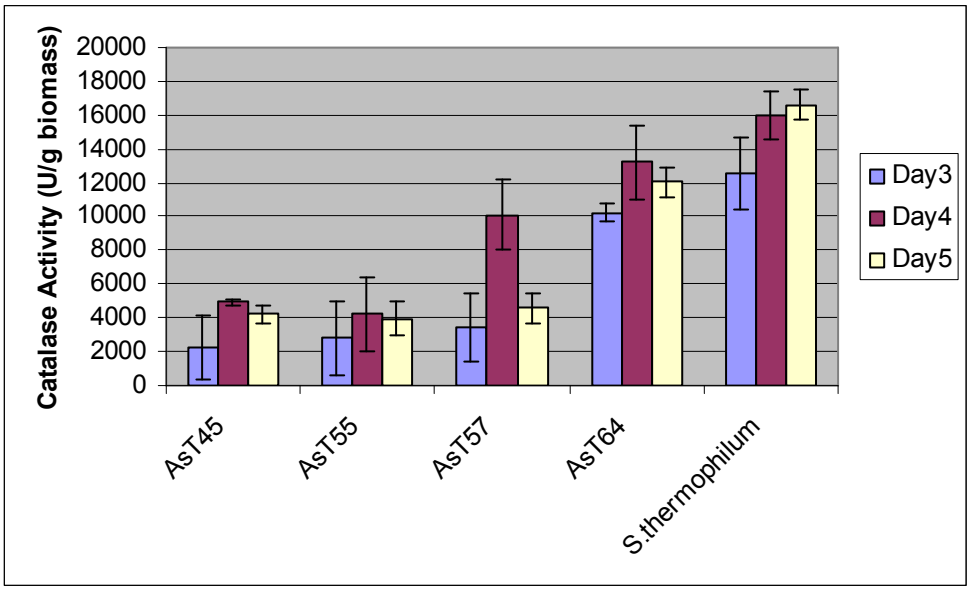


Figure 3.20 Comparison of catalase activities of transformants and *Scytalidium thermophilum* in culture supernatants at day 3, day 4 and day 5. *A.sojae* transformants were grown at 30 °C and *S.thermophilum* was grown at 45 °C. Enzyme assay temperature was 25 °C.

As mentioned earlier, *catpo* gene was placed under the control of the constituvely expressed *gpd*A promoter of *A. nidulans* to obtain high level of constitutive expression in *A. sojae*. Also, *A.niger* signal and propeptide seqeuence was used instead of the native signal and propeptide sequence of *Scytalidium* catalase in order to target the secretion. Although heterologous expression was achieved in *Aspergillus sojae*, as opposed to expectations, addition of the glucoamylase prosequence at the N-terminus of *catpo* gene did not provide a high level expression system. Limitation of the catalase activity may takes place due to problems during translation of mRNA and/or during subsequent translocation, folding, processing and transport of the protein through the secretory pathway.

Low mRNA stability or incorrect processing of mRNA during the translation process could be the reason for the lower production levels of heterologous proteins (Gouka *et al*., 1997).

Limitations at the (post) translational level, which is required to produce functional proteins, may also determine the efficiency of secretion of heterologous protein (Gouka *et al*., 1997). Translocation across the endoplasmic reticulum (ER) occurs in two ways: by the signal recognition particle (SRP) dependant pathway, in which the complex consisting of mRNA/nascent peptide chain/ribosome and SRP is targeted to the ER membrane, and by the SRP-independent pathway. Although both targeting routes may be universal, the specifity of the system and the proteins that follow either route may not be interchangeable among organisms. This could be important in heterologous gene expression. The secretory proteins enter the endoplasmic reticulum in an extended conformation; however proteins have to fold and mature into their native forms to be functional. This process is assisted *in vivo* by helper proteins, namely chaperones and foldases (Conesa *et al.,* 2001). Quality control of protein structure again involves chaperones. After leaving the ER, the proteins are transported in vesicles to the Golgi compartment in which the processing such as glycosylation and processing (e.g.: processing of the propeptides in the mature protein) takes place. The capacity of the glycosylation machinery and the specific glycosylation requirements that heterologous proteins may have also effect the production of heterologous proteins. After the modifications in the Golgi compartment, the protein is secreted into the extracellular medium (Gouka *et al*., 1997 and Conesa *et al.,* 2001).

The role of the N-terminal signal peptides is to introduce secretory proteins into the secretion pathway; therefore it is believed that signal sequences do not account for major differences in the production yields of the recombinant proteins. However, prosequences may play a role in secretion efficiency.

Prosequences are removed at a late stage in the secretion pathway and perform functions such as organelle targeting and protein folding (Conesa *et al.,* 2001). Propeptide sequence of the *Scytalidium* catalase gene may play a role as an intramolecular chaperone, thus affecting the correct folding of protein in vivo and secretion of the active catalase. As a result, removal of the native propeptide sequence of *Scytalidium* catalase may be the reason for the low level of catalatic activity.

3.5 Bioinformatic Studies

Scytalidium thermophilum catalase gene was absent in the NCBI database; however, the gene was found to be patented (United States Patent, No. 5646025) by Novo Nordisk in 1997. *Scytalidium* catalase is encoded by a 2791 bp gene, containing 7 introns. The predicted amino acid sequence of 717 residues appears to encode a pro-catalase with a 19 amino acid signal peptide, and a 17 amino acid propeptide (Appendix D).

Comparison of this bifunctional catalase of *Scytalidium thermophilum* with other catalase proteins was performed by BLAST (Basic Local Alignment Search Tool) search on NCBI (National Center for Biotechnology Information). BLAST search results are evaluated by two numbers; score and expect (E) value. Score is the value calculated from the number of gaps and substitutions associated with each aligned sequence. The higher the score value means the more significant the alignment. E value describes the likelihood that a sequence with a similar score will occur in the database by chance; therefore the smaller the E value means the more significant the alignment. Other factors are identities, positives and gaps. Identities indicate that amino acids are identical in both sequences. Positives mean that amino acids are different but their physico-chemical properties are conserved. Gaps represent the non-aligned pairs of residues. Protein-protein blast search among all organisms was carried out and organisms with zero E value

were chosen to draw the taxonomic tree (Figure 3.20). Comparisons of some of the organisms which gave the highest score after protein-protein blast search using the *Scytalidium* catalase amino acid sequence, revealed by Novo Nordisk are shown in Table 3.2. Protein-protein blast search using the amino acid sequence of *catpo* gene, determined in this study results in the same taxonomic tree (Figure 3.20); however as can be seen from Table 3.3, identities and positives increase 1% or 2% except for the comparison with *Penicillium vitale* amino acid sequence. Phylogenetic tree analysis and multiple sequence alignment of *Scytalidium thermophilum* bifunctional catalase with other catalases are given in Figure 3.20 and Figure 3.21.

As mentioned in section 1.1.1., catalases are classified into three groups according to structural and functional similarities, which are monofunctional catalases, catalase-peroxidases and minor catalases. Monofunctional catalases are also subgrouped based on subunit size with an accompanying attention to heme content. Unrooted phylogenetic tree based on the amino acid sequences of 32 monofunctional catalases are given in Figure 3.23. Fungal catalase sequences are found in two groups; which are clade II and clade III. Clade II enzymes include large subunit size catalases with fungal and bacterial origin, while clade III enzymes include small subunit catalases, consisting of fungal and animal catalases. Clade I enzymes contain the small subunit catalases with bacterial and plant origin (Nicholls *et al*., 2001 and Klotz *et al.,* 1997). According to the unrooted phylogenetic tree (Figure 3.23), *Scytalidium thermophilum* bifunctional catalase was classified as a monofunctional catalase in the subgroup of clade II enzymes with large subunit catalases. Fungi catalases may have more than one monofunctional catalases. *Neurospora crassa* has two large subunit monofunctional catalases; cat1 and cat3 and cat2 is in the group of catalase-peroxidases (Pereza *et al*., 2002). *Aspergillus oryzae* has two large subunit monofunctional catalases; namely catA and catB (Hisada et al., 2005). *Aspergillus fumigatus* has three catalases; CatAp and Cat1p are monofuınctional

catalases, while cat2p is a bifunctional catalase-peroxidase (Shibuya *et al*., 2006). *Aspergillus nidulans* has four catalases: CatA and CatB are large subunit monofunctional catalases; catC is a small subunit monofunctinonal catalase and CatD is in the group of catalase-peroxidases (Kawasaki *et al.,* 2001).

Catalase peroxidases have been characterized in both fungi and bacteria and resemble certain plant and fungal peroxidases in sequence. Nonheme catalases are the smallest group and all enzymes characterized so far in this group are bacterial catalases (Nicholls *et al*., 2001).

Table 3.2 Comparison of *S. thermophilum* catalase gene characterized by Novo Nordisk with other fungal catalases with a zero E value

	Length	Score	Identities (%)	Positivies (%)	Gaps (%)
Chaetomium globosum	705	1124	75	84	2
Podospora anserina	726	1065	72	82	1
Claviceps purpurea	716	1043	70	81	0
Neurospora crassa	719	1030	69	80	0
Botryotinia fuckeliana	714	1011	68	79	0
Gibberella zeae	716	1010	68	79	1
Aspergillus oryzae	725	976	66	78	2
Aspergillus fumigatus	728	965	66	78	2
Aspergillus niger	727	962	66	77	2
Aspergillus nidulans	722	954	65	77	2
Penicillium vitale	688	892	63	77	2

Table 3.3 Comparison of *S. thermophilum catpo* gene characterized in this study with other fungal catalases with a zero E value

	Length	Score	Identities (%)	Positivies (%)	Gaps (%)
Chaetomium globosum	705	1085	76	85	2
Podospora anserina	726	1045	75	84	0
Claviceps purpurea	716	1019	71	82	0
Neurospora crassa	719	1004	71	82	0
Botryotinia fuckeliana	714	1001	70	81	0
Gibberella zeae	716	989	69	80	1
Aspergillus oryzae	725	964	68	79	1
Aspergillus fumigatus	728	953	67	80	2
Aspergillus niger	727	946	68	78	2
Aspergillus nidulans	722	937	67	78	2
Penicillium vitale	688	888	63	77	2

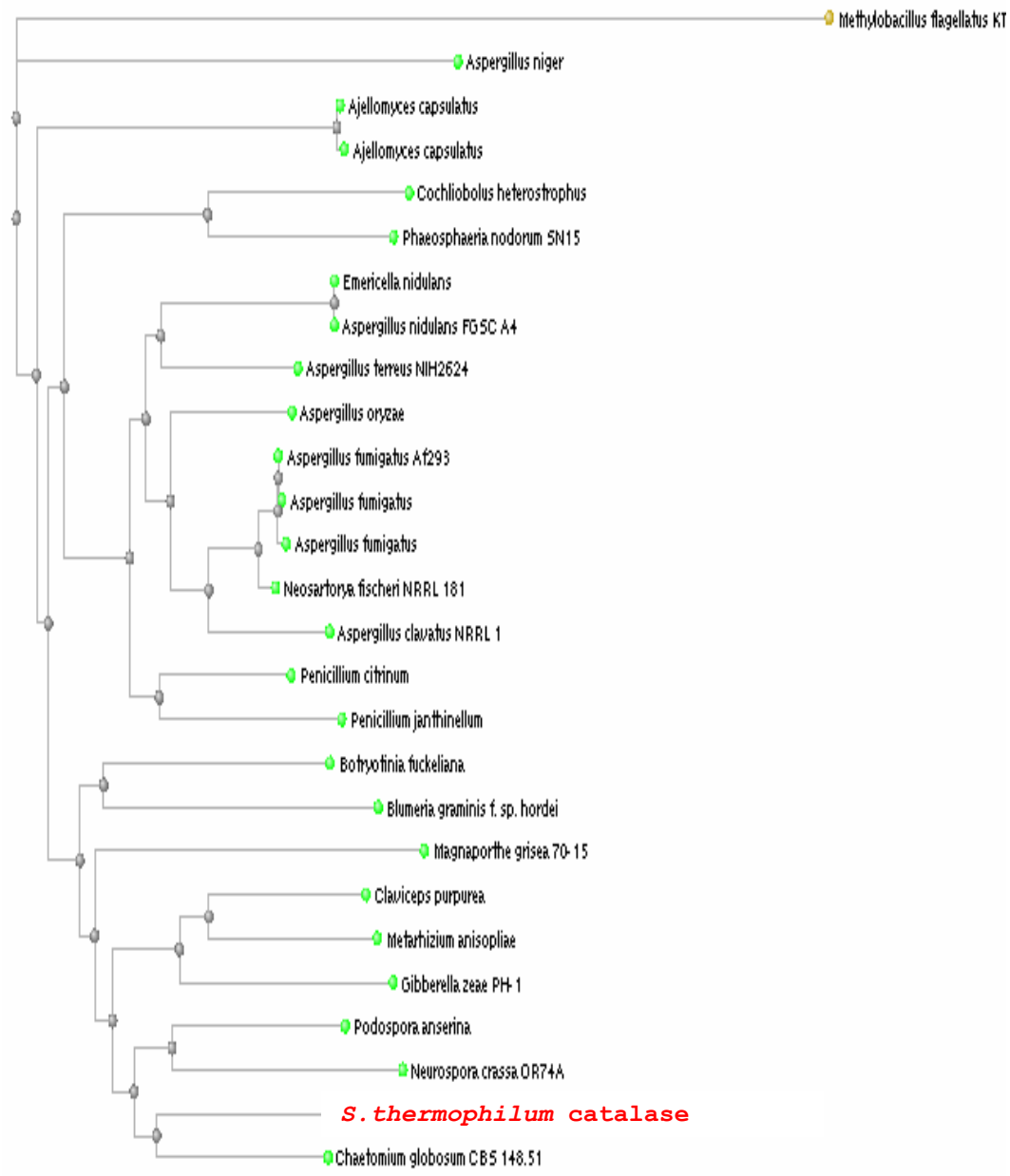


Figure 3.21 Distance tree of fungal catalases with zero E value based on protein-protein blast analysis

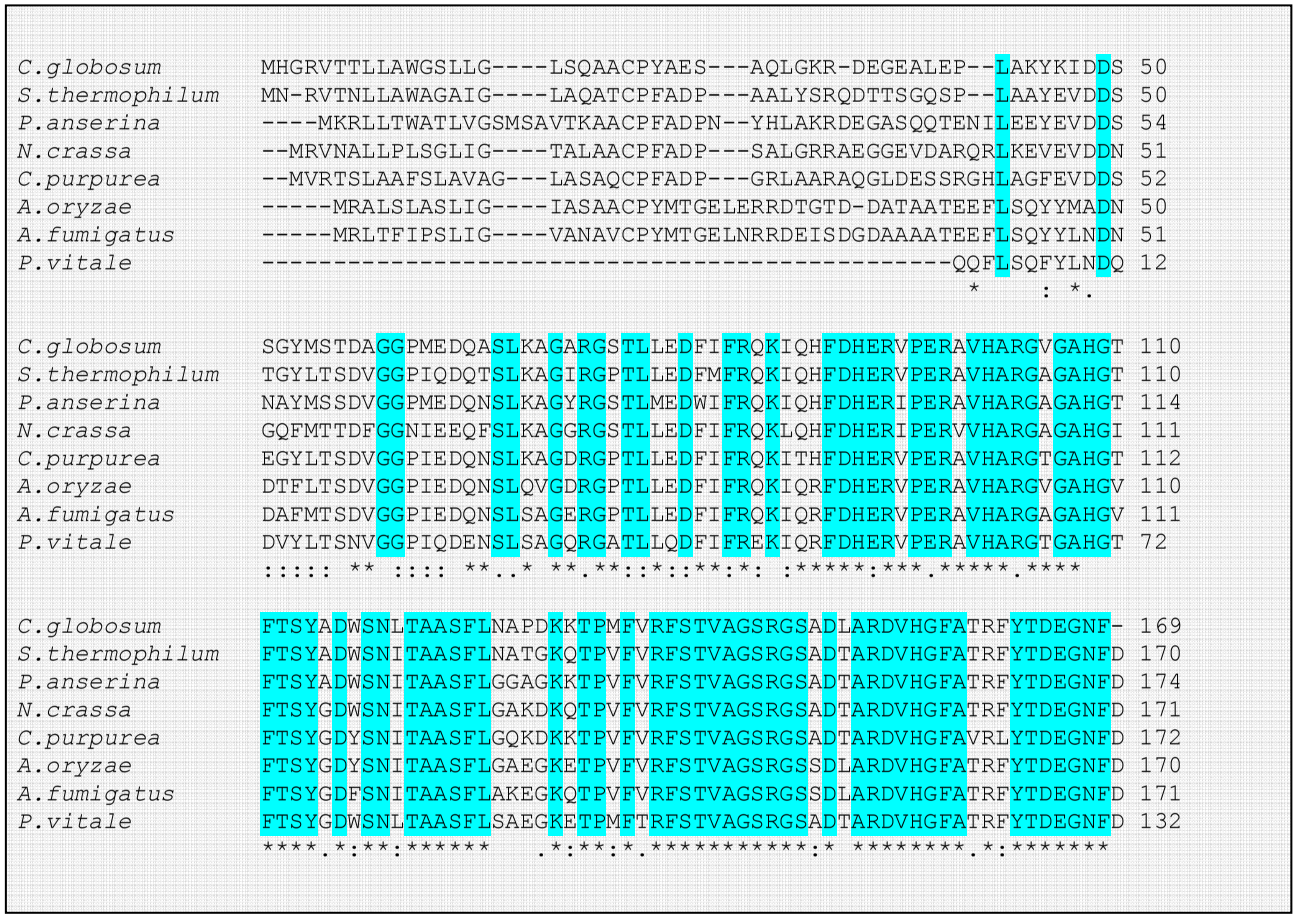


Figure 3.22 Multiple sequence alignment of *S.thermophilum* bifunctional catalase and fungal catalases with zero E value. (residues conserved in all sequences are highlighted in blue)

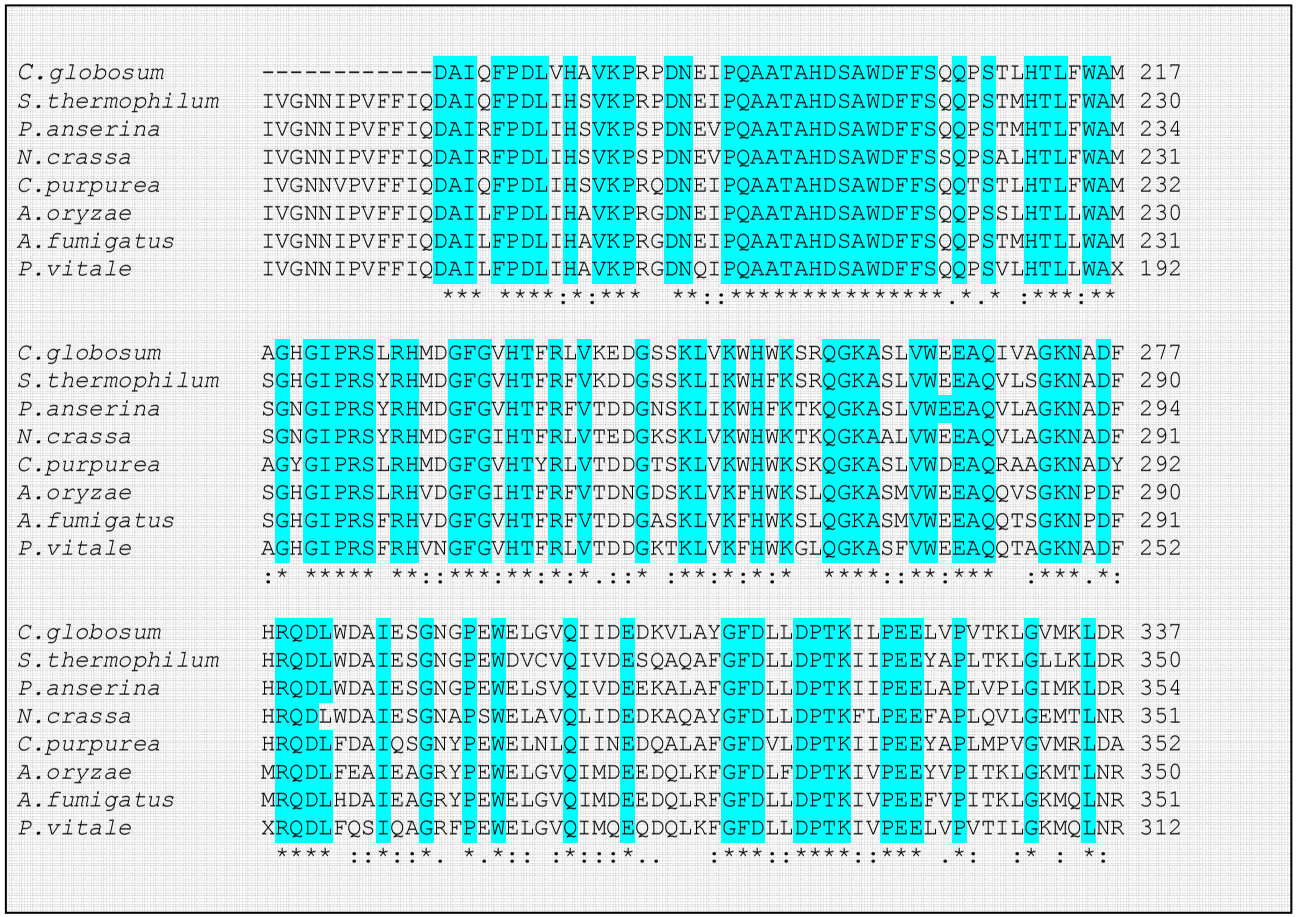


Figure 3.22 (continued).

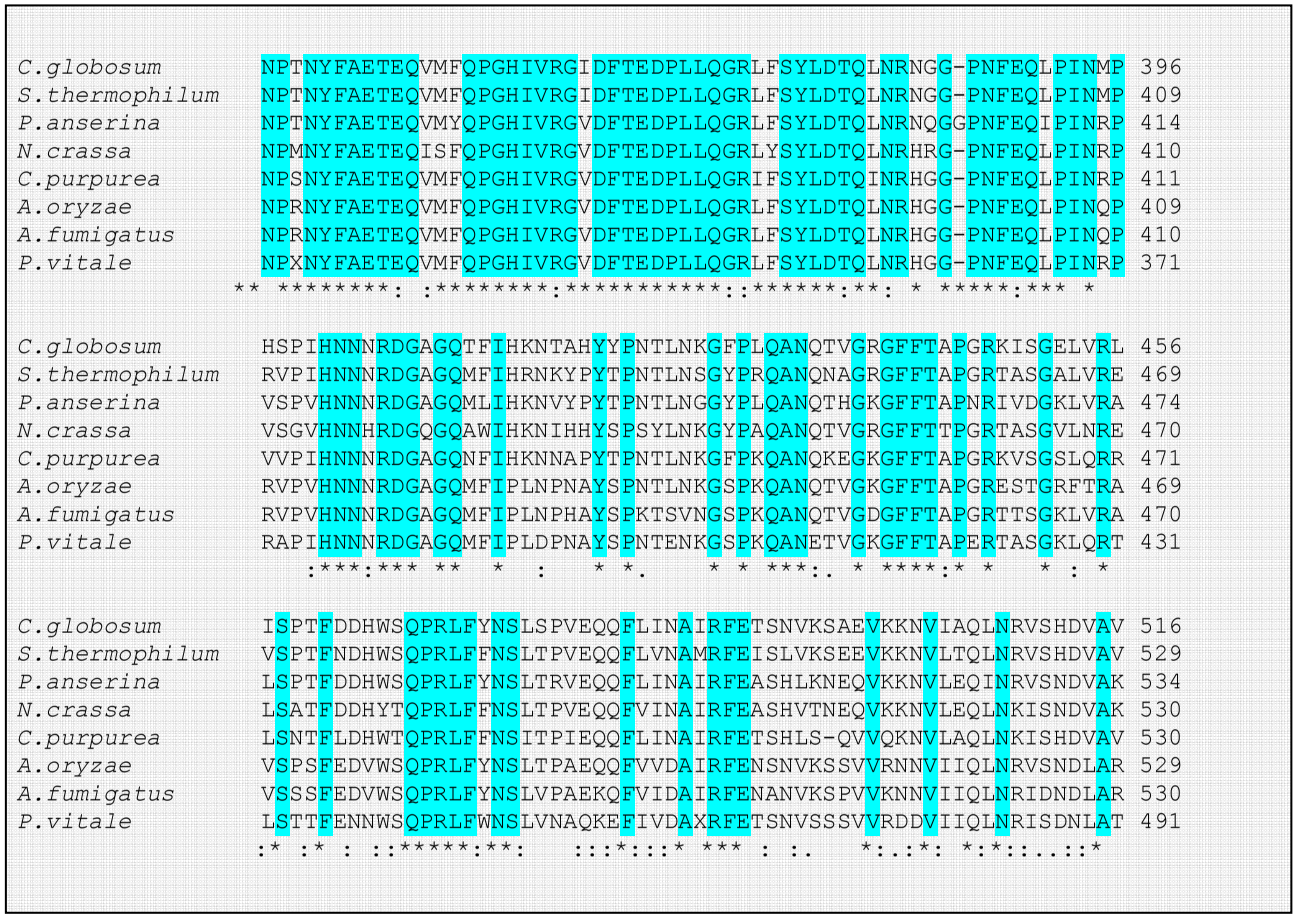


Figure 3.22 (continued).

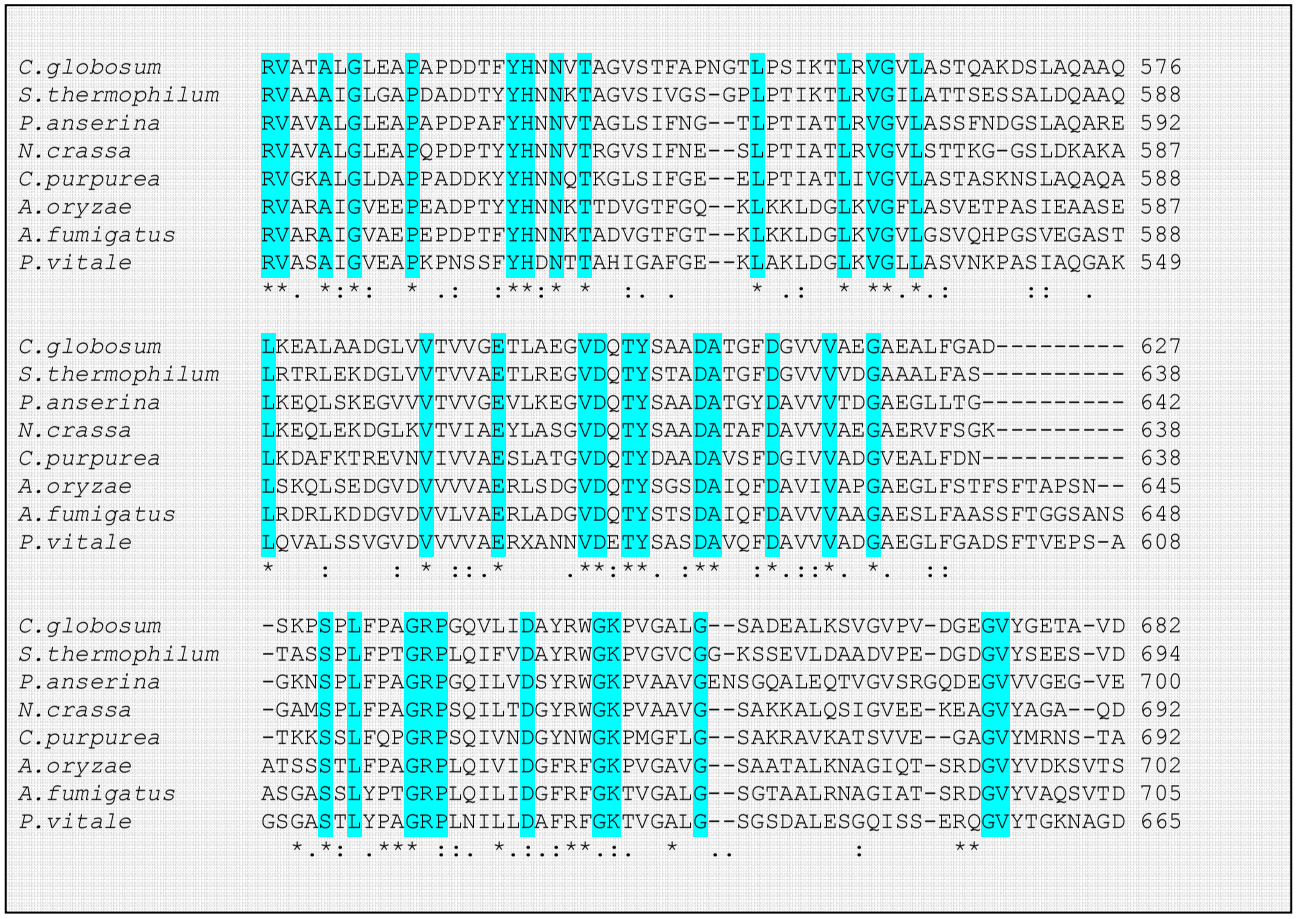


Figure 3.22 (continued).

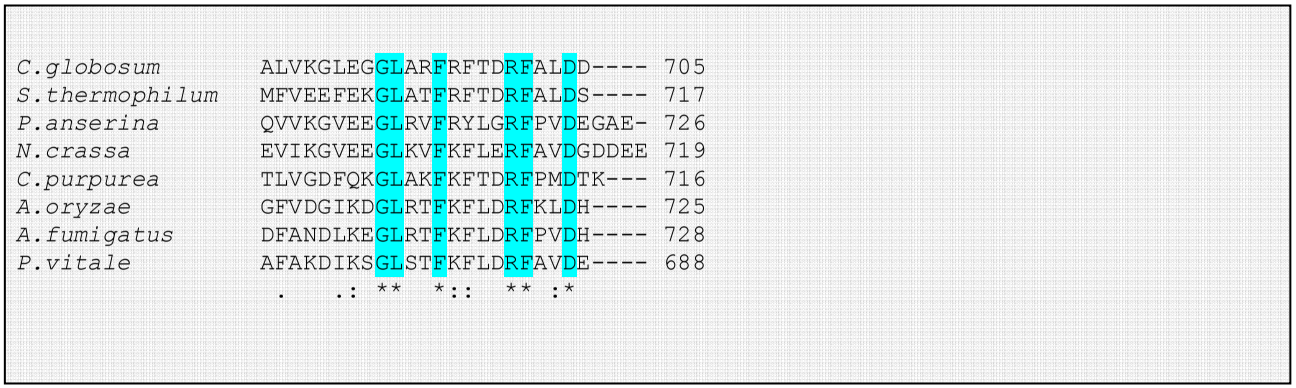


Figure 3.22 (continued).

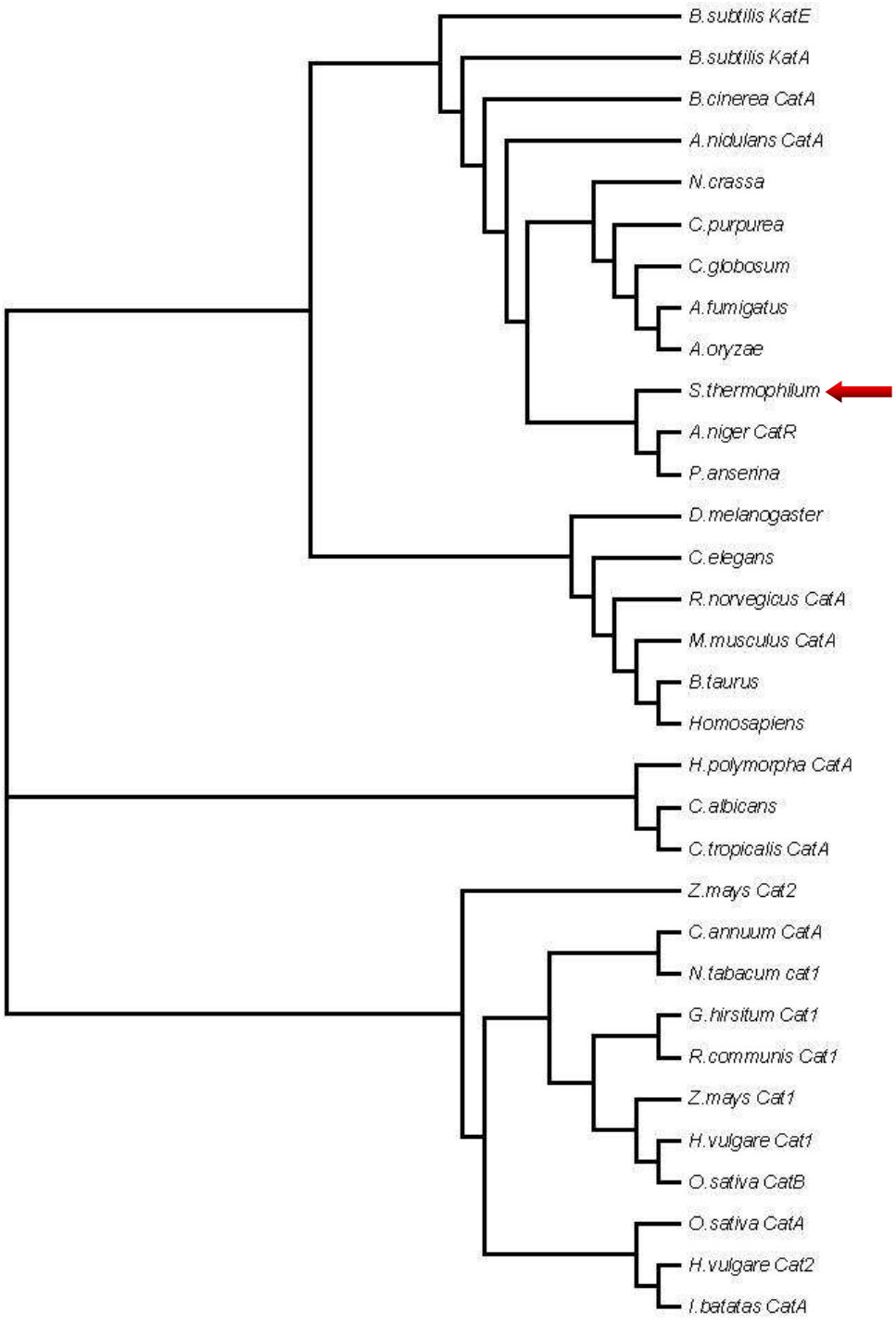


Figure 3.23 Phylogenetic tree based on the amino acid sequences of 32 monofunctional catalases. (*Scytalidium* catalase is indicated with a red arrow)

CHAPTER 4

CONCLUSIONS

- *Scytalidium thermophilum* catalase gene was first ligated onto pCR®2.1 vector and sub-cloned into *E. coli* XL1 Blue MRF' excluding the signal and pro-peptide regions. The gene cloned in this study was named as *catpo*.

- The gene *catpo,* having 7 introns in its nucleotide sequence, encodes a mature protein of 681 amino acids. According to the sequence analysis of the *catpo* gene, 10 amino acids of the mature protein encoded by the *catpo* gene were different from the amino acid sequence of the *Scytalidium* catalase gene, which was formerly cloned by Novo Nordisk. Theoretical pI value and the molecular weight of each subunit of the protein encoded by the *catpo* gene were calculated as 5.37 and approximately 75 kDa, respectively.

- The *catpo* gene was then ligated onto the fungal expression vector pAN52-4 which resulted in the recombinant plasmid, namely pAN52-4CatS, having the total size of 8457 bp. In this construct, the *catpo* gene was fused to the signal and pro-peptide region of the *Aspergillus niger* glucoamylase gene and put under the control of the constitutive glyceraldehyde-3-phosphate dehydrogenase (*gdp*A) promoter of *Aspergillus nidulans* to allow high-level expression.

- Transformation into *Aspergillus sojae* ATCC 11906 yielded 4 transformants expressing the *catpo* gene product.

- Heterologous catalase production in *Aspergillus sojae* was reached at a level of *c.* 13206 U/g (0.8 fold) after 4 days of cultivation without inducer, which is lower than the catalase activity of the gene donor strain of *Scytalidium thermophilum*.

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APPENDIX A

CHEMICALS, ENZYMES AND THEIR SUPPLIERS

Chemical or Enzyme	Supplier
λ.DNA/ *Hin*dIII	MBI Fermentas
λ.DNA/*Eco*RI+*Hin*dIII	MBI Fermentas
β-mercaptoethanol	Merck
ABTS	Sigma
Agar	Acamedia
Agarose	Sigma
Ampicilin	Mustafa Nevzat İlaç San.
ATP	MBI Fermentas
Calcium Chloride	Merck
Casein Hydrolysate	Fluka
Chloroform	Merck
$CuSO_4$	Sigma
dNTP mix	MBI Fermentas
EDTA	Merck
Ethanol	Gurup Deltalar
Ethidium Bromide	Sigma
GeneRuler^{TM} DNA Ladder Mix 1kb	MBI Fermentas
Gen100 DNA Ladder (100-3000 bp)	GeneMark
Glacial Acetic Acid	Merck
Glucose	Merck

Chemical or Enzyme	Supplier
*Hind*III	MBI Fermentas
Hydrocholoric Acid	Merck
IPTG	MBI Fermentas
KCL	Merck
K_2HPO_4	Merck
KH_2PO_4	Merck
KOH	Merck
Loading Dye	MBI Fermentas
$MgCl_2.7H_2O$	Merck
NaCl	Merck
NaOH	Merck
*Nco*I	MBI Fermentas
N,N'-dimethylformamide	Sigma
Phenol:Chloroform:Isoamylalcohol	AppliChem
Platinum *Pfx* DNA Polymerase	Invitrogen
RNase A	Roche
SDS	Merck
Sipermidine	Sigma
Sucrose	Merck
Sodium Acetate	Merck
Sodium Citrate	Merck
Soluble Starch	Sigma
T4 DNA Ligase	Sigma
Taq DNA Polymerase	MBI Fermentas

Chemical or Enzyme	Supplier
Tetracycline	Mustafa Nevzat İlaç San.
Trichoderma harzianum	Sigma
Tris	Merck
Triton X-100	Sigma
Tryptone	Difco
Tween 20	Sigma
Uracil	Sigma
Uridine	Sigma
X-gal	MBI Fermentas
Yeast Extract	Merck

APPENDIX B

PREPARATIONS OF GROWTH MEDIA, BUFFERS AND SOLUTIONS

1. Agarose Gel 0. 8 % (w/v)

0.8 g agarose is dissolved in 100 ml 1X TAE buffer by heating in microwave.

2. $CaCl_2$ (1 M, 50 ml)

5.55 g of $CaCl_2$ is dissolved in 40 ml H_2O. The volume is adjusted to 50 ml and sterilized by filtration.

3. Complete (transformation) Medium Broth

70 mM $NaNO_3$

7 mM KCl

11 mM KH_2PO_4

2 mM Mg_2SO_4

1 % (w/v) glucose

0.5 % (w/v) yeast extract

0.2 % (w/v) casamino acids

1X Trace elements

1X Vitamins

10 mM uridine

10 mM uracil

pH is adjusted to 6.5 with KOH pellets. Sterilized by autoclaving. $MgSO_4$, vitamin and glucose are added after autoclave.

4. Complete Medium Agar

Complete medium broth is supplemented with 1.5 % agar.

5. DNase free Rnase

RNase A is dissolved in 0.01 M Sodium acetate (pH 5.2) to give a final concentration of 10 mg/ml. The solution is heated to 100°C for 15 min in a boiling water bath for the inactivation of DNase. It is cooled slowly to room temperature. 0.1 volume of 1 M Tris-HCl (pH 7.4) is added until the pH of the solution is 7.0. The solution is dispensed into aliquots and stored at -20°C.

6. EDTA (0.5M, pH 8.0)

186.1 g of ethylenedinitrilotetraacetic acid disodium salt dihydrate added to 800 ml of distilled water. It is stirred vigorously on a magnetic stirrer while the pH is adjusted to 8.0 with NaOH pellets. The solution is dispensed into aliquots and sterilized by autoclaving.

7. Ethanol (70%, 100 ml)

70 ml absolute ethanol is mixed with 30 ml distilled sterile water.

8. Glucose (20% w/v, 100 ml)

20 g of glucose is dissolved in 80 ml H_2O. The volume is adjusted to 100 ml and filter sterilized.

9. IPTG (100 mM)

0.24 g IPTG is dissolved in 10 ml H_2O, filter sterilized, dispensed into aliquots and stored at -20°C.

10. $MgSO_4$ (10 mM, per Liter)

2.46 g $MgSO_4.7H_2O$ is dissolved in 1 liter distilled water. The solution is sterilized by autoclaving.

11. LB Broth (per Liter)

10 g tryptone

5 g yeast extract

10 g NaCl

Final volume is adjusted to 1 liter with distilled water and pH is adjusted to 7.0 with NaOH and autoclaved. 100 μg/ ml ampicilin is added whenever it is used. The medium is stored at 4 °C.

12. LB Ampicillin Agar (per Liter)

10 g tryptone

5 g yeast extract

10 g NaCl

20 g agar

Final volume is adjusted to 1 liter with distilled water and pH is adjusted to 7.0 with NaOH and autoclaved. When it cools to 55 °C, 100 μg/ml ampicillin is added and poured to petri dishes. The plates stored at 4 °C.

13. LB Tetracycline Agar (per Liter)

10 g NaCl

10 g tryptone

5 g yeast extract

20 g agar

Final volume is adjusted to 1 liter with distilled water. After adjusting pH to 7.0 with NaOH, the medium is autoclaved. 1.5 ml of 10 mg/ml tetracycline is added when it cools to 55°C, and poured to petri dishes. The plates are covered with parafilm and stored in dark at 4°C.

14. Lysis Buffer

40 mM Tris-HCl

10 mM EDTA

0.2 M NaCl

1.5 % (w/v) SDS

15. Lytic Solution

0.27 M $CaCl_2$

0.6 M NaCl

16. Minimal Medium Agar

70 mM $NaNO_3$

7 mM KCl

11 mM KH_2PO_4

2 mM Mg_2SO_4

1 % (w/v) glucose

1X Trace elements

1X Vitamins

325.2 g sucrose (0.95 M) (in case of top agar)

The pH is adjusted to 6.5 with KOH pellets. 1.2 % agar is added, in case of top agar 0.6 % is used. Sterilized by autoclave.

17. NaOH (10 N, 100 ml)

40g of NaOH pellets is added slowly to 80 ml of H_2O. When the pellets have dissolved completely, the volume is adjusted to 100 ml with H_2O. The solution is stored at room temperature.

18. NE Buffer

0.3 M NaAC (pH 7)

1 mM EDTA

19. PEG Buffer (25 % PEG-6000)

2.5 g PEG-6000 is weighted, 7.5 ml TC is added and dissolved by heating to approximately 60 ^{o}C in a microwave oven (when prepared fresh), or filter sterilized (0.2 μm membrane).

20. SDS (10% w/v, per Liter)

100 g of SDS is dissolved in 900 ml of H_2O. The solution is heated to 68 °C and stirred with a magnetic stirrer to assist dissolution. If necessary, pH is adjusted to 7.2 by adding a few drops of concentrated HCl. The volume of the solution is adjusted to 1 liter with H_2O. It is stored at room temperature.

21. Saline Tween (ST)

0.8 % NaCl

0.005 % Tween-80 (1:100 dilution from 0.5% (v/v) Tween-80 stock)

22. Sodium Acetate (3 M, pH = 5.2)

408.1 g of sodium acetate is dissolved in 800 ml distilled water. The pH of the solution is adjusted to 5.2 with glacial acetic acid. The volume is adjusted to 1 liter with distilled water. The solution is sterilized by autoclaving.

23. Solution A

50 mM CaCl2

10 mM Tris-HCl (pH 8.0)

24. Solution 1 (for Plasmid Isolation)

50 mM Glucose

25 mM Tris-HCl (pH 8)

10 mM EDTA

25. Solution 2 (for Plasmid Isolation)

0,2 N NaOH

1% SDS

26. Solution 3 (for Plasmid Isolation)

3M NaAc (pH 4.8)

27. STC Buffer

1.33 M sorbitol in TC

28. TAE Buffer (50X, per Liter)

242 g of Tris base is dissolved in 600 ml distilled waster. The pH is adjusted to 8.0 with approximately 57 ml glacial acetic acid. Then, 100 ml 0.5 M EDTA (pH 8.0) is added and the volume is adjusted to 1 liter.

29. TC Buffer

50 mM $CaCl_2$

10 mM Tris/HCl pH 7.5

30. TE Buffer

10 mM Tris- HCl (pH 8.0)

1 mM EDTA (pH 8.0)

31. Tris HCl Buffer

121.1 g Tris base is dissolved in 800 ml of distilled water. The pH is adjusted to the desired value with concentrated hydrochloric acid. The solution is cooled to room temperature before making final adjustment to pH. The volume of the solution is then adjusted to 1 liter with distilled water and sterilized by autoclaving.

32. Trace Elements (1000X)

76 mM $ZnSO_4$

178 mM H_3BO_3

25 mM $MnCl_2$

18 mM $FeSO_4$

7.1 mM $CoCl_2$

6.4 mM $CuSO_4$

6.2 mM $Na2MoO_4$

174 mM EDTA

Trace elements solution was dissolved in 80 mL and the volume brought to 100 ml. Sterilized by autoclave. Stored at 4-8 °C.

33. TTE Buffer

10 mM Tris –HCl (pH 8.5)

10 mM EDTA (pH 8)

4 mM spermidine

10 mM β-mercaptoethanol

0.5 M sucrose

36 mM KCl

0.25 % Triton X-100

34. Vitamins (1000X)

100 mg/Lthiamin

100 mg/L riboflavin

100 mg/L nicotinamide

50 mg/L pyridoxine

10 mg/L pantothenic acid

0.2 mg/L biotin

35. X-gal (2%)

20 mg X-gal was dissolved in 1 ml N,N'-dimethylformamide, covered with foil and stored at -20°C.

36. YpSs Agar

4.0 g/L Yeast extract

1.0 g/L K_2HPO_4

0.5 g/L $MgSO_4.7H_2O$

15.0 g/L Soluble starch

20.0 g/L Agar

37. YpSs Broth

4.0 g/L Yeast extract

1.0 g/L K_2HPO_4

0.5 g/L $MgSO_4.7H_2O$

10 g/L Glucose

38. YpSs Broth (Modified)

4.0 g/L Yeast extract

1.0 g/L K_2HPO_4

0.5 g/L $MgSO_4.7H_2O$

0.1 g/L $CuSO_4.5H_2O$

40 g/L Glucose

APPENDIX C

DNA SIZE MARKERS

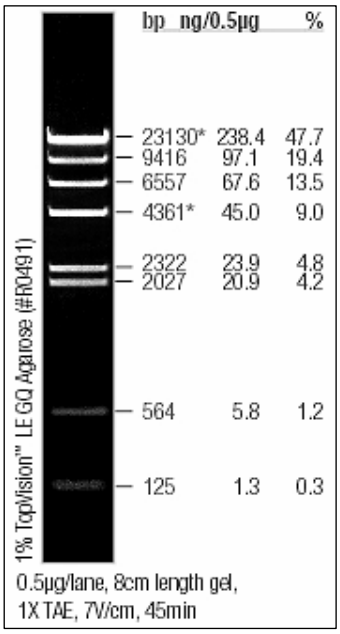

Figure C.1 Lambda DNA/ *Hin*dIII Marker

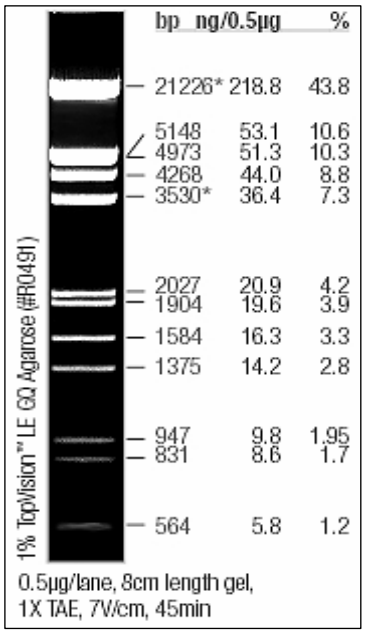

Figure C.2 Lambda DNA/*Eco*RI+*Hin*dIII Marker

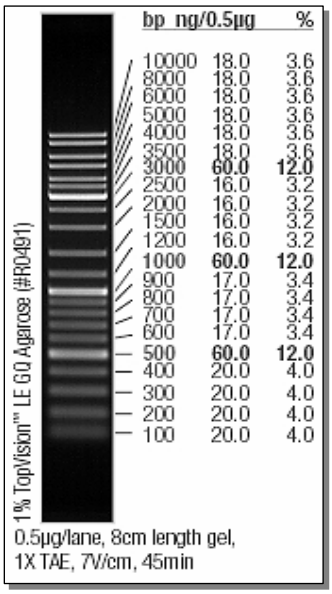

Figure C.3 GeneRuler^{TM} DNA Ladder Mix (Fermentas)

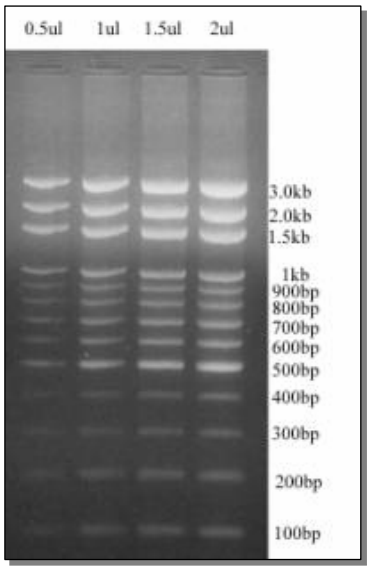

Figure C.4 Gen100 DNA Ladder (GeneMark)

APPENDIX D

NUCLEOTIDE AND AMINO ACID SEQUENCE OF THE *SCYTALIDIUM THERMOPHILUM* CATALASE

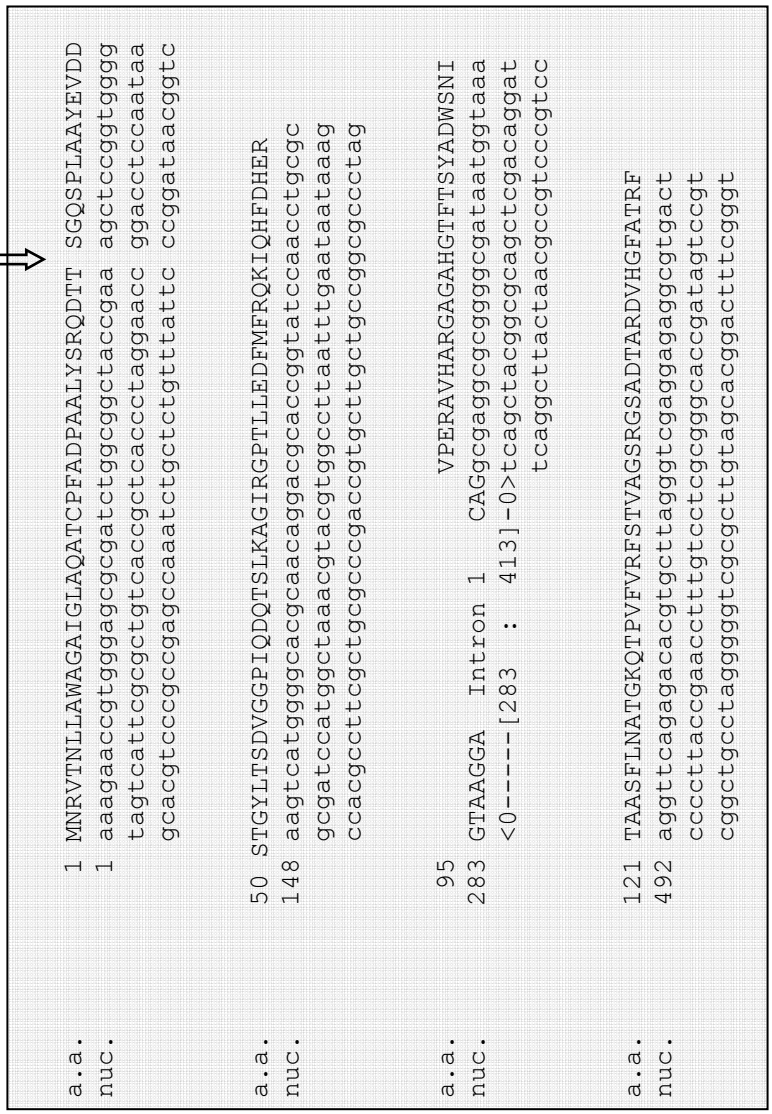

Figure D.1 Nucleotide and amino acid sequence of *Scytalidium thermophilum* catalase gene, determined by Novo Nordisk in 1997 (United States Patent, No. 5646025). Arrow indicates the matue protein start site.

Figure D.1 (continued).

```
a.a.          296 WDAIESGNGPEWDVCVQIVDESQAQAFGFDLLDPTKIIPEEYAPLTKLG
nuc.         1378 tggagtgagcgtggtgcagggtcgcgtgtgtcgcaaaacggtgctaacg
                  gactacgagcagatgtattaacacactgtattaccattcaaacctcatg
                  gtttgcgcaaagtcccgtctgcggactcccggcgagcccggcccggggc

a.a.          345 LLKLDRNPTNYFAETEQVMFQPGHIVRGIDFTEDPLLQGRLFSYLDTQL
nuc.         1525 ctacgcacaattggagcgatccgcagcgagtaggccccgcctttcgacc
                  ttatagaccaatcacaatttacgattggtatcaacttaggttcatacat
                  cgggtctgccccccggggcgcacttcccccccggtcgagacctgctcggg

a.a.          394 NRNGGPNFEQLPINMPRVPIHNNNRDGAGQMFIHRNKYP
nuc.         1672 acaggcatgcccaaaccgcacaaacggggcatacaaatc
                  agaggcataatctatcgtctaaaagagcgatttagaaac
                  cgtcgcctgggcccgggggtccttcccccggcccgcgtt

a.a.          433                        YTPNTLNSGYPRQANQNAGRGFFTAP
nuc.         1789 GTAAGTG  Intron 6   CAGtacaacaagtcccgacaggcgttagc
                  <0-----[1789 : 1842]-0>accactaggacgacaaacgggttccc
                                        ctcccgctttggaccatcacaccagt

a.a.          459 GRTASGALVREVSPTFNDHWSQPRLFFNSLTPVEQQFLVNAMRFEISLV
nuc.         1921 gcagaggcgcggtcatagcttccccttatcacggcctcgagactgaacg
                  ggccggcttgatccctaaagcacgtttactcctaaatttactgtatgtt
                  ctccctccctgggaaccccgggctccccctccaagcccccgccacctg
```

Figure D.1 (continued).

```
a.a.          508 KSEEVKKNVLTQLNRVSHDVAVRVAAAIGLGAPDADDTYYHNNKTAGVS
nuc.         2068 atgggaaagcaccacgacggggcggggagcggcggggattcaaaagggt
                  acaataaattcatagtgaatctgtccctgtgccacaacaaaaaaccgtc
                  ggaacggcgccgccccctcgcgcgcctccccgccgccacccccggtcca

a.a.          557 IVGSGPLPTIKTLRVGILATTSESSALDQAAQLRTRLEKDGLVVTVVAE
nuc.         2215 aggagctcaaaaccggacgaaagtagcgcggcccaccgaggcggagggg
                  ttgggctcctactgtgttcccgacgctaaccatgcgtaaagtttcttca
                  ctacgcgtccgtcccccgtcgcggcggtggcgccctgagcgtgcgtgta

a.a.          606 TLREGVDQTYSTADATGFDGVVVVDGAAALFASTASSPLFPTGRPLQIF
nuc.         2362 accggggcattagggagtgggggggggggctgaagttcttcagactcat
                  ctgagtaacacccaccgtagttttagcccttcgccccccttccggctatt
                  ggcggacggcgggtcgtccctttgcgggggtcccgggggcggcggggct

a.a.          655 VDAYRWGKPVGVCGGKSSEVLDAADVPEDGDGVYSEESVDMFVEEFEKG
nuc.         2509 gggtctgacgggtggataggtgggggcgggggggttggtggatgggtgag
                  tacagggactgtgggacgattaccatcaagagtacaactatttaataag
                  gcgtggaggctgttgggcgggtggttgacgcggtgggggcgtgggtggg

a.a.          704 LATFR                       FTDRFALDS
nuc.         2656 tgataGTGAGTC  Intron 7   TAGtagctgcgt
                  tcctg<0-----[2671 : 2764]-0>tcagtctac
                  gttcg                       tctgttcct
```

Figure D.1 (continued).

APPENDIX E

VECTOR MAPS

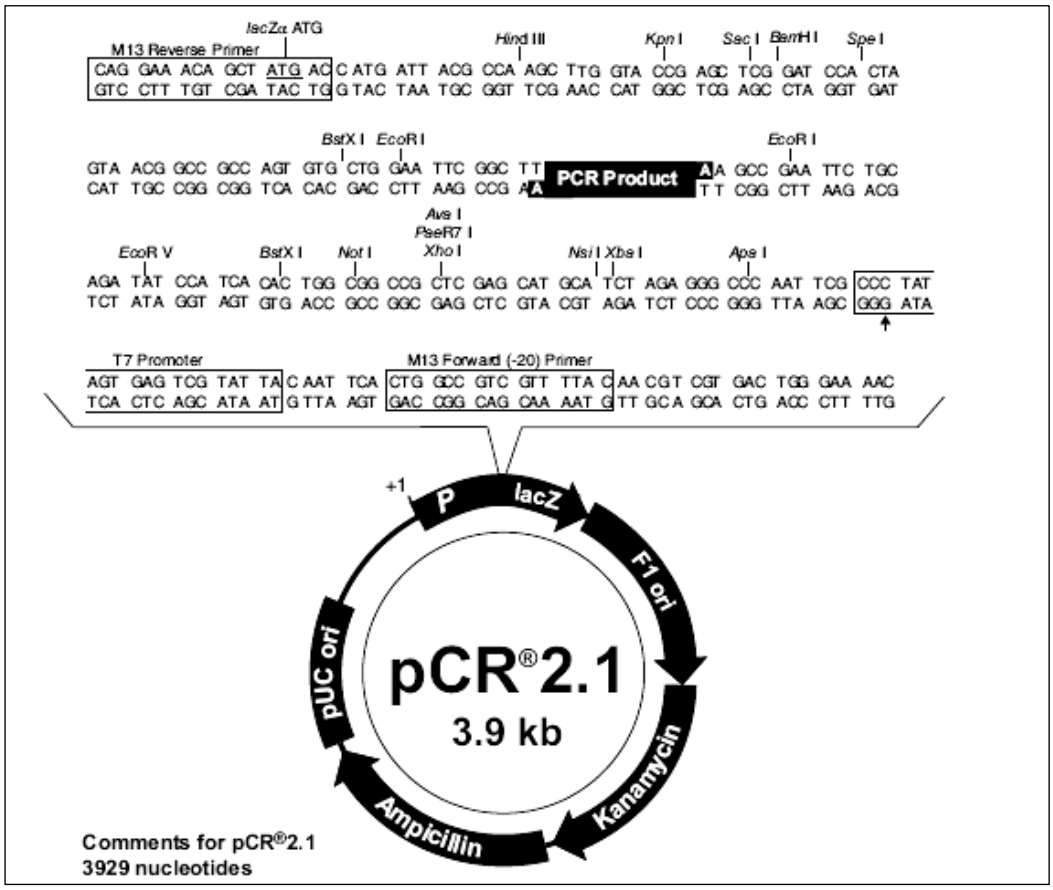

Figure E.1 Map of sub-cloning vector, pCR®2.1 (Invitrogen)

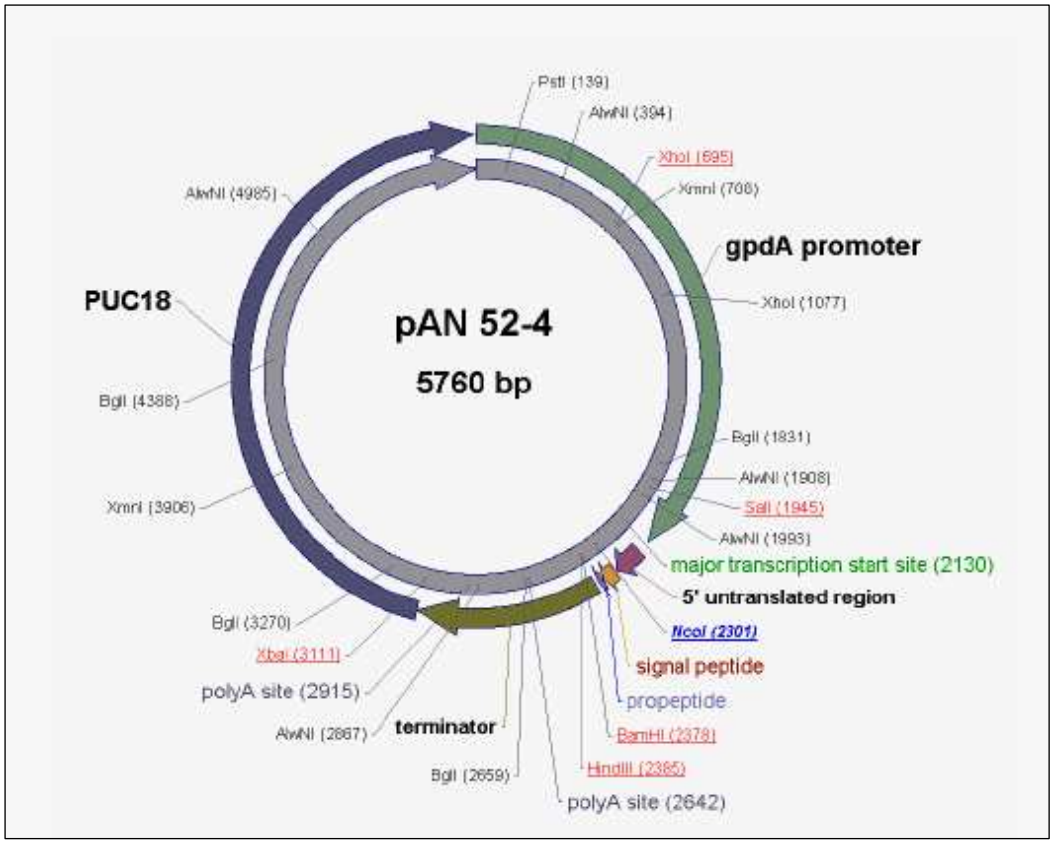

Figure E.2 Map of *Aspergillus sojae* expression vector, pAN52-4.
(Enzymes with unique restriction sites are shown in red color)