TRANSFORMATION OF *Aspergillus sojae* WITH *Vitreoscilla* HEMOGLOBIN GENE

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ABSTRACT

TRANSFORMATION OF *Aspergillus sojae* WITH *Vitreoscilla* HEMOGLOBIN GENE

Aspergillus sojae known as koji mold is a non-aflatoxigenic fungus, designated with a GRAS status by FDA. This study considers the transformation of *A. sojae* with *Vitreoscilla* hemoglobin gene. *Vitreoscilla* hemoglobin is the bacterial hemoglobin, which enhances the oxygen transfer under microaerophilic conditions. Since industrial fungal fermentation with the high demand for oxygen are accounted to face oxygen limitations during the production of value added products like enzymes, antibiotics and organic acids; oxygen supply becomes an enormous problem to be solved by the manufacturers. To overcome this problem, an alternative solution using the *vgb* gene of *Vitreoscilla* and recombinant DNA technology and taking the *A. sojae* organism as model organism was proposed. The product of interest in this study was the exo-polygalacturonase enzyme known to have wide applications in food, pharmaceutical, textile and paper industries. Here *vgb* gene was tried to be introduced to *A .sojae* via both protoplasting and electroporation methods. For transformation process *vgb* gene was cloned initially into plasmid ANIp4. For the selection of transformed *A. sojae* cells, uridine auxotrophic mutants were tried to be selected after UV mutagenesis. However, using a procedure based on selection of uridine supported growth did not result in *A. sojae* pyrG mutants. The success of transformation was initially observed by means of PCR analysis and agarose gel electrophoresis, later this was try to be confirmed by sequence analysis and agarose gel electrophoresis. However, due to the contamination problems accounted in the procedures the transformation with both methods could not be assured.

ÖZET

Vitreoscilla HEMOGLOBIN GENİYLE *Aspergillus sojae'*nin TRANSFORMASYONU

Koji küfü olarak bilinen *Aspergillus sojae*, FDA tarafından genel olarak güvenli kabul edilmiştir. Bu organizmayla ilgili sınırlı sayıda çalışma rapor edilmiş olup, bu çalışma, *A. sojae*'nin *Vitreoscilla* hemoglobin geni kullanılarak transformasyonuyla ilgili yapılan tek çalışmadır. Mikroaerofilik koşullarda oxygen transferini artıran *Vitreoscilla* hemoglobini bir bakteriyel hemoglobindir. Yüksek oksijen miktarına ihtiyaç duyan endüstriyel fungal fermentasyonları, enzim, antibiyotik ve organik asit gibi değerli ürünlerin üretimi sırasında oksijen sınırlamasıyla karşılaştığından, bu sistemlere oksijen sağlamak üreticiler tarafından çözülmesi gereken önemli bir sorundur. Bu problemin çözümü için *Vitreoscilla*'dan elde edilen *vgb* geni ve rekombinant DNA teknolojisi kullanılması ile ilk etapta örnek olarak, *A. sojae* organizması model olarak önerilmiştir. Bu çalışmada üretilen ürün, gıda, ilaç, tekstil ve kağıt endüstrisinde geniş kullanım alanına sahip ekzo-polygalakturonaz enzimidir. Transformasyon aşaması için *vgb* geni ilk olarak ANIp4 plasmidine klonlanmıştır. Transforme edilmiş *A. sojae* hücrelerinin seçimi için UV mutasyonundan sonra üridin okzotrofik mutantlar seçilmeye çalışılmış, ancak, üridin destekli büyümeye dayalı seçim metodu ile *A. sojae* pyrG mutantlarının seçimi sonuç vermemiştir. Transformasyonun başarılı oluşu ilk olarak PCR analizleri ve gel electroforezi yöntemiyle daha sonra ise sekans analizi yöntemleriyle doğrulanmaya çalışılmıştır. Ancak kontaminasyondan ileri gelen problemlerden dolayı transformasyon işleminin tam olark gerçekleşip geçekleşmediğinde emin olunamamıştır.

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CHAPTER 1

INTRODUCTION

Vitreoscilla is a filamentous bacterium belonging to the *Beggiatoa* family. It is a gram-negative aerobic or microaerophilic bacteria. The species *V. strecoraria* is strictly aerobic and is able to cope with the hypoxic conditions by synthesizing a soluble hemoglobin (VHb) (Zhang, et al., 2007). VHb is the first bacterial hemoglobin, which has 25% sequence homology with leghemoglobin (Kroneck, et al., 1991; Dikshit, et al., 1992). The synthesis of this hemoglobin is promoted under the hypoxic conditions. The rate constant for oxygen association (k_{on}) is relatively normal; however the rate constant for oxygen dissociation (k_{off}) is unusually large. The beneficial effect is based on the binding characteristics of the hemoglobin to oxygen and inducing the facillated diffusion into the cell under very low oxygen concentrations where normal diffusion does not take place with the concentration gradient. Overall the possible mechanism for *vgb* action can be summarized such as increasing the flux of oxygen to the respiratory apparatus, providing higher internal oxygen concentration, altering the internal redox state, functioning as an efficient terminal oxidase, or improving overall efficiency of oxygen limited ATP production (Sun, et al., 2002; Yubin, et al., 2009). Substantial improvements in the production of recombinant proteins such as chloramphenicol acetly transferase, beta galactosidase, catechol 2,3 dioxygenase, alpha amylase, protease and human tissue plasminogen activator, antibiotics such as actinorhodin, cephalosporin C, itaconic acid, erythromycin, ethanol and nicotine production (in *Nicotiana tabaccum* plant) using this gene have been reported (Dikshit, et al., 1990; Wu, et al., 2003)

Based on the given literature, introduction of this gene into corresponding organisms for the production of many other products would provide benefits to those fermentations which require high aeration and where the production of the products are very much oxygen dependent. It will enable such fermentations to work under much lower oxygen concentrations and therefore reduce the air supply and the operational cost. In large scales, such a strain improvement technique will not only save on air supply but also increase the product yields and promote cell growth. Fungal submerged fermentations are widely used in the production of enzymes, antibiotics and organic

acids which have lots of applications in the food, medicine, pharmaceutical, chemical and textile industry. Therefore any problem faced during their production will affect the final product significantly. One of the major accounted problems is the morphological change of the culture during the fermentation process. It is well observed that the fungal culture exhibits two major morphology which are very much determined by several environmental and genetic factors. These factors can be stated as pH, composition of the media, inoculation ratio, type of the inoculum, agitation speed, aeration rate, feeding rate and genetic factors of the culture.

The pellet morphology is desired in downstream processing due to the non-viscous (Newtonian) rheology of the broth. Since agitation and aeration is also much easier in such systems, the power input therefore the operating cost will also be reduced. Also, such morphology makes the downstream processing easier. However, the pellet formed, is subject to internal mass transfer resistances (oxygen and nutrient) which can result into low growth rate and productivity due to cell autolysis at the center of the pellets. Therefore, the presence of such a strain improvement technique will help to overcome these resistances.

Until 1974, strain improvement techniques were only based on mutation, screening and selection. These methods were time consuming, laborious and expensive. The first gene cloning of *Aspergillus* was reported in 1983 by Tilburn et al. (Lubertozzi and Keasling, 2009). The transformation techniques for *Aspergillus* and other filamentous fungi were more problematic with their multicellular morphology, thick chitinous cell walls, and lack of plasmids compared to *E. coli* and *S. cerevisia.* Methods for the genetic manipulation of *S. cerevisia* and *E. coli* were succeeded by the discovery of native plasmids; nevertheless, like most fungi, the *Aspergilli* lack natural extrachromosomaly replicating DNA elements. Consequently, researchers began to construct artificial plasmids for *Aspergillus* (Lubertozzi and Keasling, 2009).

In the transformation process of fungi, selection markers bearing vectors are essential for determination of transformed cells. The selection markers either depend on the complementation of a specific auxotrophy or resistance to a specific antibiotic. Antibiotic resistance markers are more easy to use because they do not require the availability of specific auxotrophic strains.

Transformation can be mediated either by the electroporation method or protoplast formation. Protoplast membrane is sufficiently competent for the uptake of the foreign DNA or the fusion process. To obtain protoplasts from fungal cells, cell wall

degradation is an important process. In protoplasting process rather than the non enzymatic methods, lytic enzymes are generally preferred (Peberdy, 1979).

Protoplasts need osmotic stabilizers in the suspending medium. Removing of the cell wall makes protoplasts undefended to the osmotic differences. Sorbitol is the most commonly used stabilizer and is sufficient for all fungi at the concentrations ranging from 0.8 to 1.2 M. Calcium ions are crucial for nearly all transformation procedures. Besides, up to 10 volumes of 40% PEG 4000 is used in the transformation mixture because in the presence of PEG, the treated cells do not clump and facilitate the trapping of DNA (Fincham, 1989).

As a result, in this study using the transformation techniques described above, an alternative solution to the important problem of the industrial fungal fermentations will be proposed.

CHAPTER 2

ASPERGILLUS

2.1. *Aspergillus*

Aspergillus is a genus of filamentous ascomycetes fungi which has medical, agricultural, industrial, and high pathological importance. *Aspergillus* species have an amazing nutritional flexibility since they grow and reproduce on many different carbon sources (Goldman and Osmani, 2008). There are some 250 species of *Aspergillus,* ubiquitous members of the air flora. Many *Aspergilli* contaminate food, produce toxic products like aflatoxin. Aflatoxin is synthesized by only a few *Aspergillus* species including *A. flavus* and A. *parasiticus*. Aflatoxin exposure is difficult to avoid because *A. flavus* and *A. parasiticus* grow in many food products particularly, under high moisture conditions. The main target organ in mammals is the liver that is why aflatoxicosis is primarily a hepatic disease.

About 40 to 250 species of *Aspergillus,* capable of growing at 37^{o}C are opportunistic human pathogens. *A. fumigatus* is the major organism associated with aspergillosis, although *A. terreus*, *A. flavus* and *A. niger* have also been implicated (Machida and Gomi, 2010; Wainwright, 1992).

Aspergillus species produce various primary and secondary metabolites and are important in the beverage, pharmaceutical, and enzyme industries. Commonly products produced by *Aspergillus* species include citric, gluconic, itaconic and kojic acids which are commodity products. Industrial enzymes, α-amylase, pectinases, acid proteases, and glucoamylases are examples produced from *Aspergillus* species (Wainwright, 1992).

2.2. The Taxonomy of *Aspergillus*

Fungal taxonomy is a complex issue because it continues to be a subject for change (Gellisen, 2005). The old taxonomy of *Aspergillus* focused on the characteristics of the whole colony (color, size, presence or absence of sclerotia and pigments) and also

relied on the morphology including spore bearing structure or conidiophore. It has a long stipe, in a swollen apex. Expanded apical region bears a series of spore bearing cells called phialides. In the phialide, repeated mitotic divisions take place and yield a chain of asexual spores called conidiaspores or conidia. Conidiaspores vary in shape ranging from spherical to elongate forms, which can be smooth or echinulade (Figure 2.1). Conidia are extremely hydrophobic and are easily dispersed by the air. The new taxonomies are based on both phenotypical characters and multigene DNA sequences (Goldman and Osmani, 2008; Samson, et al., 2006).

Aspergillus can be subdivided into eight subgenera. Subgenus *Circumdati* is further subdivided into the sections *Circumdati, Nigri, Flavi,* and *Cremei.* The section *Flavi* currently contains 18 accepted species of which two are domesticated forms, these are *A. sojae* and *A. oryzae* (Table 2.1).

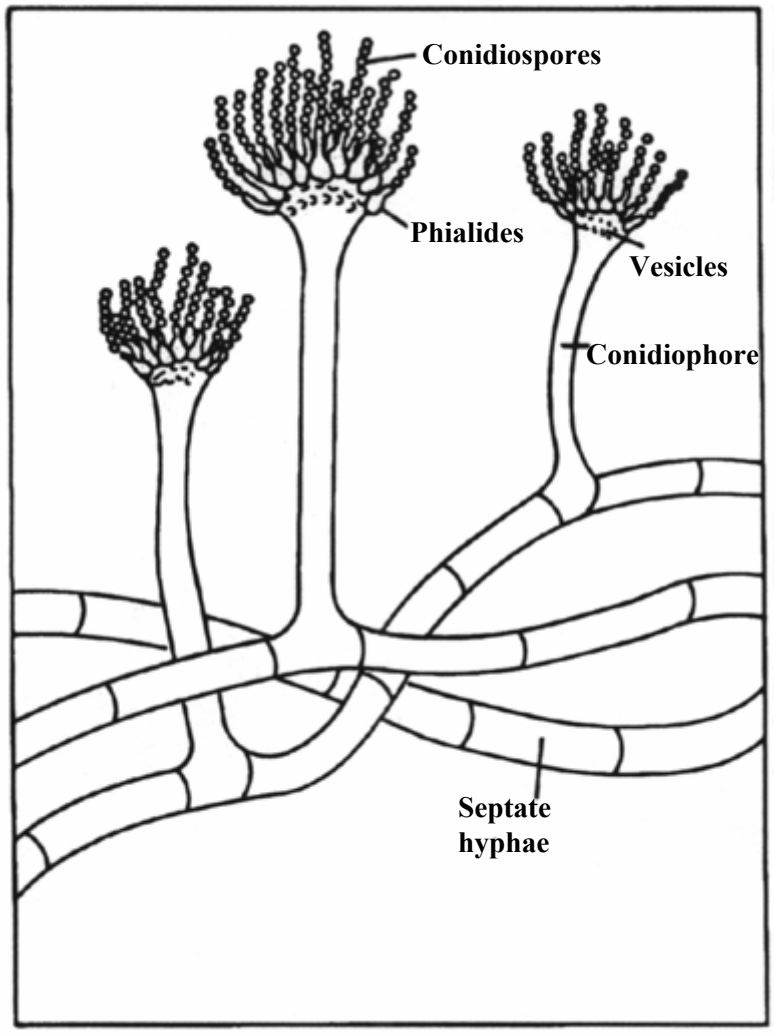


Figure 2.1. Asexual fruiting structure of *Aspergillus* species, illustrating septate hyphae, conidiophore, vesicle, phialides and conidiospores.
(Source: A. T. Still University, 2010)

Table 2.1.Characteristics of Aspergillus species

(Source: Samson, et al., 2006)

	Kojic Acid	Alfatoxin B type	Alfatoxin G type	Cyclopiazonic acid	Aspergillic acid	Ochratoxin A	Chrysogine	Parasi-ticolide
Aspergillus arachidicola	+	+	+	-	+	-	+	+
Aspergillus avenaceus	-	-	-	-	-	-	-	-
Aspergillus bombycis	+	+	+	-	+/-	-	-/+	-
Aspergillus caelatus	+	-	-	+	-	-	-	-
Aspergillus flavus	+	+/-	-	+	+	-	-	-
Aspergillus lanosus	+	-	-	-	-	-	-	-
Aspergillus leporis	+	-	-	-	-	-	-	-
Aspergillus minisclerotium	+	+	+	+	+	-	-	-
Aspergillus nomius	+	+	+	-	+	-	-/+	-
Aspergillus oryzae (domesticated from A. flavus)	+	-	-	+	-	-	-	-
Aspergillus parasiticus	+	+	+	-	+	-	-	+
Aspergillus parviselerotigenus	+	+	+	+	+	-	-	-
Aspergillus pseudotamarii	+	+	-	+	-	-	-	-
Aspergillus sojae (domesticated from A. parasiticus)	+	-	-	-	+	-	-	-
Aspergillus tamari	+	-	-	+/-	-	-	-	-
Aspergillus toxicarius	+	+	+	-	+	-	-	+
Petromyces albertensis	+	-	-	-	-	+	-	-
Petromyces aliaceus	+	-	-	-	-	+	-	-

2.3. *Aspergillus sojae*

Section *Flavi* includes both aflatoxigenic species such as *A. flavus* and *A. parasiticus*, and non-aflatoxigenic species such as *A. sojae* and *A. oryzae* (Figure 2.2). It was proposed that *A. sojae* is a domesticated variant of *A. parasiticus* and also *A. sojae* is virtually never found outside of food fermentation processes. However, these two species can be distinguished from each other in some ways such as *A. parasiticus* grows readily on media containing the antibiotic bleomycin while *A. sojae* does not; in addition, *A. sojae* never produces aflatoxins whereas *A. parasiticus* produces (Klich, et al., 1997). The reason why *A. sojae* does not produce aflatoxins was explained by the presence of a defect in the aflatoxin pathway regulatory gene homologue aflR, where the genes are not transcripted (Takahashi, et al., 2002). *A. sojae* is designated as GRAS (generally recognized as safe) by FDA (Takahashi, et al., 2008). These features make this organism a valuable tool for food fermentation and for the production of various enzymes. Besides, for over 1500 years, *A. sojae* with *A. oryzae* and other closely related species have been used for koji food and beverage processes (Goldman and Osmani, 2008).

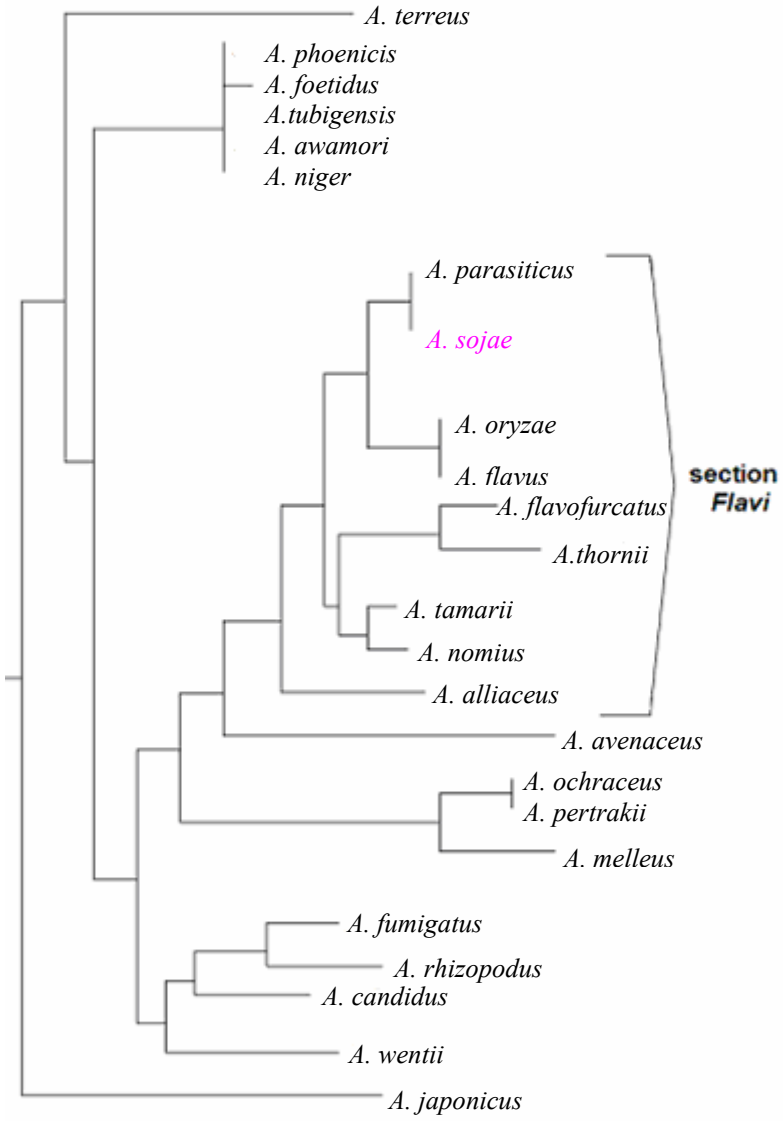


Figure 2.2. Molecular phylogeny of selected *Aspergillus* species, including species belonging to the section Flavi, based on ITS sequence data.
(Source: Gellisen, 2005)

CHAPTER 3

PECTINOLYTIC ENZYMES

In the industrial process of fruits and vegetables, pectinases are widely used enzymes. Among, *Aspergillus* species, especially *A. niger* are used commercially for pectinase production, since they produce several enzymes including endo- and exo-polygalacturonase (PG), pectin lyase (PL), pectin esterase (PE), and oligogalacturonase (OG) (Solís and Flores, 1997). These enzymes are primarly responsible for the degradation of the long and complex molecules called pectin which occurs as structural polysaccharides in the middle lamella and the primary cell walls of young plant cells. They break down pectin into simpler molecules of galacturonic acid units.

Pectinases are classified under three headings according to whether pectin, pectic acid or oligo-D-galacturonate is the preferred substrate, whether pectinases act by trans-elimination or hydrolysis and whether the cleavage is random (endo-, liquefying of depolymerizing enzymes) or endwise (exo- or saccharifying enzymes). The three major types of pectinases are as followings: Pectinesterases (PE), depolymerizing enzymes, and protopectinase. Depolymerizing enzymes contain polygalacturonases (PG) that catalyze hydrolysis of α-1,4-glycosidic linkages in pectic acid (polygalacturonic acid). These are also of two types: endo- polygalacturonase and exo-polygalacturonase. Endo-PG also known as poly (1,4-α-D-galacturonide) glycanohydrolase, catalyzes the random hydrolysis of α-1,4-glycosidic linkages in pectic acid. Exo-PG: also known as poly (1,4-α-D-galacturonide) galacturonohydrolase, catalyzes the hydrolysis in a sequential fashion of α-1,4-glycosidic linkages on pectic acid in sequencial fashion (Runco, et al., 2001).

3.1. Application of Pectinases

Pectinases mainly serve to prepare fruit and vegetable juices, such as: sparkling clear juices (apple, pear, grape), juices with clouds (citrus, prune, tomato juice and nectars), and juices, in which the intent is to preserve the integrity of the plant cells by selectively hydrolyzing the polysaccharides of the middle lamella. While in the case of

the production of clear juices the pectinolytic enzymes are added to increase the yield during pressing and for clarification. The stabilisation of clouds in orange juices is achieved by the use of pectic enzymes with high levels of polygalacturonase activity. Other applications of pectinases can be listed as, coffee and tea fermentation, paper making, oil extraction, isolation of protoplasts from plants for genetic manipulations and in clarification of grape juice and wine. The main micro-organism exploited for the production of commercial pectinase preparations is *A. niger*. A list of commercially produced pectinolytic enzymes are presented in Table 3.1. (Runco, et al., 2001) Furthermore, some of the characteristics of pectinases are given in Table 3.2.

Table 3.1. Commercial pectinases

(Source: Runco, et al., 2001)

Supplier	**Location**	**Brand name**
C.H. Boehringer Sohn	Ingelheim, West Germany	Panzym
Ciba-Geigy, A.G.	Basel, Switzerland	Ultrazyme
Grinsteelvaeket	Aarthus, Denmark	Pectolase
Kikkoman Shoyu, Co.	Tokyo, Japan	Sclase
Schweizarische Ferment, A.G.	Basel, Switzerland	Pectinex
Societe Rapidase, S.A.	Seclin, France	Rapidase, Clarizyme
Wallerstein, Co.	Des Plaines, USA	Klerzyme
Rohm, GmbH	Darmstadt, West Germany	Pectinol, Rohament

Table 3.2. Characterization of microbial pectinases

(Source: Kashyap, et al., 2001)

Producer	Type of pectinase	Opti. pH for activity	Opti. Temp. for activity (^{0}C)	Reference
Acidic pectinases				
Aspergillus niger CH4	Endo-pectinase, Exo-pectinase	4.5-6.0 3.5-5.0	Below 50	Acuna-Arguelles et al., 1995
Penicillium frequentans	Endopolygalacturonase (Endo-PG)	4.5-4.7	50	Borin et al., 1996
Selerotium rolfsii	Endo-PG	3.5	55	Channe and Shewal, 1995
Rhizoctonia solani	Endo-PG	4.8	50	Marcus et al., 1986
Mucor pusilus	PG	5.0	40	Al-Obaidi et al., 1987
Cloctridium thermosac-charolyticum	Polygalacturonate hydrolase	5.5-7.0	30-40	Rijssel et al., 1993
Alkaline pectinases				
Bacillus sp. RK9	PGL	10.0	-	Fogarty and Kelly, 1983
Bacillus sp. NT-33	PG	10.5	75	Cao et al., 1992
Bacillus polymyxa	PG	8.4-9.4	45	Nagel and Vaughn, 1961
Bacillus pumilis	PATE	8.0-8.5	60	Dave and Vaughn, 1971
Amucola sp.	Pectate lyase (PAL)	10.25	70	Bruhlmann et al., 1994
Xanthomonas compestris	PATE	9.5	25-30	Nasumo and Starr, 1967
Bacillus No. P-4-N	PG	10-10.5	65	Horikoshi, 1990
Bacillus stearothermophilus	PATE	9.0	70	Karbassi and Vaughn, 1980
Penicillium italicum CECT 22941	Pectin lyase	8.0	50	Alana et al., 1990
Basillus sp. DT 7	Pectin lyase	8.0	60	Kashyap et al., 2000
Basillus suptilis	PAL	8.5	60-65	Chesson and Codner, 1978
Pseudomonas syringae pv. Glycinea	PAL	8.0	30-40	Magro et al., 1994

CHAPTER 4

GENETIC MANIPULATIONS

4.1. Plasmids

Plasmids are DNA molecules that can replicate independently of the chromosome. Plasmids are associated with several traits that can pass down to its daughter cells like antibiotic resistance, toxin and bacteriocin production, modification of bacterial virulence and an over-increasing number of metabolic capabilities. These extra chromosomally determined properties can be taken up from an organism by natural transfer mechanisms, such as transformation, transduction and conjugation (Sgorbati, et al., 1982). Plasmids are found in prokaryotes like bacteria, but are sometimes found in eukaryotic organisms and conduced to the genetic and evolutionary potential of these microorganisms. Plasmids were first discovered in bacteria (1950s) (Molbak, et al., 2003), and later analogous molecules were found in eukaryotes (Griffiths, 1995). In 1970, a major discovery was reported that the yeast *Saccharomyces cerevisiae* bears a plasmid. The importance of this discovery relies on the possibility that plasmids could be transferred by the transformation process. In transformation process, the plasmid is used as a vector which bears desired gene and can be transmitted from one cell to another. By using the yeast protoplasts, plasmids can be taken up through the cell membrane. For this purpose, usually *Escherichia coli* is used as host organism to enable sufficient plasmid amount before transformation process. Transformation in yeast was facilitated by the discovery of yeast plasmids and after the discovery of the plasmids of filamentous fungi the genetic manipulation of these organisms could be performed. Today, many plasmids of filamentous fungi have been recognized (Wainwright, 1992). Double stranded DNA plasmids have been reported in a number of true fungi; including plant pathogens (Table 4.1). Most fungal plasmids are found within mitochondria, however, in some yeast species, plasmids are found within nuclei or in the cytosol. Plasmids are either circular or linear, but the majority of fungal plasmids are linear. Circular plasmids have been described in only limited number of

fungal genera, for example, in the genera *Saccharomyces, Cochliobolus pythium* and *Neurospora* (Katsuya, et al., 1997).

Table 4.1.True mitochondrial plasmids of filamentous fungi

(Source: Samac and Leong, 1989)

Species	Plasmid	Size (kbp)	Structure	Terminal proteins	Terminal repeats	Reference
Agaricus bitorquis	pEM	7.4	Linear	Unknown	Yes	Mohan et al., 1984
	pMPJ	3.7	Linear	Unknown	Unknown	
Ceratocystis fimbriata	pCF637	8.2	Linear	Yes	Unknown	Gaisson and Lalonde, 1987
	pFQ501	6.0	Linear	Unknown	Yes	Normand et al., 1987
Claviceps purpurea	pClK1	6.7	Linear	Unknown	Yes	Tudzynski and Esser, 1986
	pClK2	5.5	Linear	Unknown	Unknown	
	pClK3	1.1	Linear	Unknown	Unknown	
Fusarium merismoides		2.1	Unknown	Unknown	Unknown	Rubidge, 1986
		1.8	Unknown	Unknown	Unknown	
F. oxysporum						
f. sp. conglutinans	pFOXC1	1.9	Linear	Yes	Unknown	Kistler et al., 1987
f. sp. raphani	pFOXC2	1.9	Linear	Unknown	Unknown	
f. sp. matthioli	pFOXC3	1.9	Linear	Unknown	Unknown	
F. solani f. sp. cucurbitae	pFSC1	9.2	Linear	Yes	Yes	Samac and Leong, 1998
	pFSC2	8.3	Linear	Yes	Yes	
Gaeumannomyces graminis	E1	8.4	Linear	Unknown	Unknown	Honeyman and Currier, 1986
f. sp. tritici	E2	7.2	Linear	Unknown	Unknown	
Neurospora crassa	Mauriceville	3.6	Circular	NA*	NA	Colins et al., 1981
N. intermedia	Fiji	5.2	Circular	NA	NA	Stohl et al., 1982
	LaBelle	4.1	Circular	NA	NA	
	Varkud	3.8	Circular	NA	NA	Akins et al.,1988
N. tetrasperma	Hawaiian	5.0	Circular	NA	NA	Taylor et al., 1985
	Surinam	5.0	Circular	NA	NA	

*Not applicable

4.2. Transformation

The first transformation of fungal species *Neurospora crassa* was reported in 1973. The method used for transformation process depeded on the inositol-requiring mutant *(inl).* (The inl mutant was thought to crave for inositol, opening the pores of the cell membranes.) However; this method was not practical since transformants were revertible to the wild type with a remarkable frequency. *Saccharomyces cerevisia* was the first organism whose protoplast was prepared. These protoplasts were generated by using an enzyme called glucanase to remove the cell wall. However, these protoplasts were used to study on macromolecular synthesis, only (Fincham, 1989). The first gene cloning *Aspergilllus* was reported in 1983 by Tilburn et al. (Lubertozzi and Keasling, 2009). Several laboratory methods for *Aspergillus* transformation are currently in use. The most popular technique, adapted from that originally developed for yeast, is the

chemical treatment of nucleated protoplasts produced by enzymatic digestion of the fungal cell wall.

4.3. Shuttle Vectors

The transformation techniques for *Aspergillus* and other filamentous fungi were more problematic compared to *E. coli* and *S. cerevisia* because of their multicellular morphology, thick chitinous cell walls, and lack of plasmids. Methods for the genetic manipulation of *S. cerevisia* and *E. coli* were succeeded through the discovery of native plasmids; nevertheless, like most fungi, the *Aspergilli* lack natural extrachromosomaly replicating DNA elements. Consequently, researchers began to construct artificial plasmids for *Aspergillus* (Lubertozzi and Keasling, 2009).

The fungal molecular genetic was first applied with the development of *E. coli* shuttle vectors for *S. cerevisia* transformation using auxotropic markers and this was followed by the model filamentous fungi *Neurospora crassa* and *Aspergillus nidulans* transformation (Ruiz-Díez, 2002). In cloning process, a plasmid is constructed containing both part of recipient organism and bacterial plasmid. Bacterial plasmid and recipient organism plasmid must contain replication origin. The recipient organism origin is recognized by its replication system and the bacterial origin by the bacterium. These kinds of plasmids are called shuttle vectors. The bacterial plasmid also bears an antibiotic resistance gene that normally bacterial cells lack. Similarly, a marker gene cassette was inserted from recipient organism to the plasmid in order to detect the presence of the plasmid in a recipient organism. For yeast or fungi, usually this marker has the ability to synthesize a specific amino acid (auxotrophic), but in order to use this marker the recipient organism has to be unabled initially to synthesize this amino acid. After DNA insertion the strain will be cured and can grow in a media lacking this amino acid. Thus selection of plasmid bearing recipient organism can be screened (Wainwright, 1992). Shuttle vectors, which can multiply in two different organisms are needed to be designed. A host organism like *E.coli* is very advantageous since it can be cloned, propagated and maintained in the laboratory very easily.

4.4. Selection Markers for the *Aspergilli*

In the transformation process of fungi, selection markers bearing vectors are essential for determination of transformed cells. The selection markers can either depend on the complementation of a specific auxotrophy or resistance to a specific antibiotic. Antibiotic resistance markers are easier to use, because they do not require the availability of specific auxotrophic strains. (Gellisen, 2005)

4.4.1. Auxotrophic Markers

A. nidulans was used as model organism for *Aspergillus* transformation in which selection for the transforming DNA depended on auxotrophic mutants or some other related nutritional markers. A media that lack the required nutrient was used to plate the transformants. Strains complemented with the nutritional marker encoded by the DNA construct were able to grow. One of the most used selectable markers is orotidine -5-phosphate decarboxylase (pyrG). The transformation of *A. oryzae* using *A. niger* pyrG gene was reported (Mattern, et al., 1987). The orotidine-5'-phosphate decarboxylase or orotidine-5'-monophosphate (OMP) decarboxylase coded by *pyrG* gene plays an important role in uridine synthesis. The *pyrG* deficient strain can only grow on medium containing uridine or uracil. The selection is based on uridine supplementation. Besides, 5-fluoroorotic acid (5-FOA) can be used in addition in the selective media as it will be converted into a toxic intermediate 5-fluoro-UMP in prototrophic strains making the selection easier (Long, et al., 2008).

In order to understand how pyrG deficient strains can be used as selection for transformation experiments, first the pyrimidine synthese pathway should be considered. The biosynthesis of purine and pyrimidine nucleotides is an essential pathway in living organisms. The pathway consists of a small number (six) of enzymes. It is one of the oldest metabolic pathways and the six enzymatic steps are identical in eubacteria, archaebacteria, and eukaryotes (Gao, et al., 1999; Nara, et al., 2000). All six enzymes and their corresponding pyrimidine biosynthetic (pyr) genes and abbreviations are listed in Table 4.2. Some of the other markers are tryptophan utilization- trpC gene, arginine auxotrophy- argB gene and acetamidase utilization-acetamidase (Wainwright, 1992).

Table 4.2. List of pyr genes and corresponding enzymes involved in de novo pyrimidine nucleotide biosynthesis in prokaryotes and *Trypanosoma cruzi*

Prokaryote	***T. cruzi***	**Enzyme (abbreviated)**	**Enzyme code (EC) number**
pyrAa[a]	pyr1a[e]	Glutamine amidotransferase (GATase)	6.3.5.5
pyrAb[b]	pyr1b[e]	Carbamoyl-phosphate synthetase (CPSase)	6.3.5.5 (same)
pryB[c]	pyr2	Aspartate carbamoyltransferase (ACTase)	2.1.3.2
PyrC	pyr3	Dihydroorotase (DHOase)	3.5.2.3
pyrD	pyr4	Dihydroorotate dehydrogenase (DHODase)	1.3.3.1
pyrE	pyr5[f]	Orotate phosphoribosyltransferase (OPRTase)	2.4.2.10
pyrF	pyr6[f]	Orotidine-50-phosphate decarboxylase (OMPDCase)	4.1.1.23

a) Equivalent to carA in *E. coli*. b) Equivalent to carB in *E. coli*. c) The *E. coli* ACTase is an allosteric enzyme consisting of regulatory and catalytic subunits encoded by pyrI and pyrB, respectively, while the *Bacillus* ACTase is an unregulated enzyme encoded by pyrB. d) In Dictyostelium discoideum, pyr genes were defined as PYR1 for GATase-CPSase, PYR2 for ACTase, and so on (Faure, et al., 1989; Henriksson et al., 1990) slightly modified the original definition and expanded it to eukaryotes in general, including trypanosomatids and mammals. e) The pyr1a-1b gene encodes a single translation product, glutamine-dependent carbamoyl-phosphate synthetase, with GATase and CPSase domains in *T. cruzi*. f) The pyr6-5 gene encodes a single protein with OMPDCase and OPRTase domains in *T. cruzi*; the domain order is opposite to that of mammalian OPRTase-OMPDCase encoded by pyr5-6.
Source: (Gao, et al., 1999)

4.4.2. Dominant Selectable Markers

Selection against auxotrophic markers sometimes cannot provide a good solution. An alternative to auxotrophic selection is to use a dominant selectable marker, which confers mostly resistance to drugs. A list of dominant selectable markers is given in Table 4.3.

Table 4.3. Dominant selectable markers used for different organisms

(Source: Fincham, 1989)

Marker	Species of Origin	Phenotype(s)	Genera in which marker was used
HygB^r	*E. coli*	Hygromycin B resistance	*Saccharomyces* *Cephalosporium* *Cochliobolus* *Colletotrichum* *Fulvia* *Septoria* *Ustilago*
Neo^r	*E. coli*	Kaynamycin G418 resistance	*Schizophyllum* *Ustilago* *Phycomyces*
Ben^r	*N. crassa*	Benomyl resistance	*Colletotrichum* *Gaeumannomyces*
oliC	*Aspergillus niger*	Oligomycin resistance	*Aspergillus*[a]
amdS$^+$	*Aspergillus nidulans*	Acetamide utilization	*Penicillium* *Cochliobolus* *Colletotrichum*
pyr-4$^+$	*N. crassa*	Pyrimidine synthesis	*Aspergillus* *Penicillium*
argB$^+$	*Aspergillus nidulans*	Arginine synthesis	*Aspergillus*[b] *Magnaporthe*
bla[c]	*E. coli*	B-Lactamase	*Saccharomyces*
lacZ[c]	*E. coli*	B-Galactosidase	*Aspergillus*

[a] *Aspergillus nidulans.*
[b] *Aspergillus niger*.
[c] For visual selection.

4.5. Protoplast Transformation

Protoplast membrane is sufficiently competent to uptake the foreign DNA or to undergo the fusion process. To obtain protoplasts from fungal cells, cell wall degradation is an important process. In protoplasting process lytic enzymes are generally preferred to the non enzymatic methods (Peberdy, 1979). In the preparation of the protoplasts capable of transformation, it is important to choose a proper enzyme or enzyme cocktail. A summary of enzymes used in protoplasting is given in Table 4.4.

In filamentous fungi, different cell types are used for protoplast preparation. In *Neurospora* species germinating macroconidia which are polynucleated, are commonly used. The uninucleate microconidia can also be used, but it is difficult to obtain. On the other hand young mycelium is an alternative to macroconidia, which are easily separated from hyphal debris (Fincham, 1989). Both conidia and mycelia are used in different fungi. In addition, the choice of cell type does not make significant difference (Ruiz-Díez, 2002). On the other hand, the physiological age of the culture and the

culture medium are effective on protoplasting rate. Fungal cell walls from cultures in the early and mid-exponential phase of growth are more suitable than the cell walls from older cultures (Peberdy, 1979).

Protoplasts need osmotic stabilizers in the suspending medium, because the removal of the cell wall makes the protoplasts undefended to osmotic differences. Sorbitol is the most commonly used stabilizer, which is sufficient at concentrations of 0.8 to 1.2 M for most fungi. Alternatives are sodium chloride, magnesium sulphate, mannitol and sucrose; these can be stored at -70^{o}C for long term storage (Ruiz-Díez, 2002).

Table 4.4. Enzymes used in Protoplasting

Organism	Enzyme	Reference
Aspergillus nidulans	Novozyme 234	Cullen, 1987
A. niger	hemicellulase β-glucuronidase (Sigma) chitinase	De Bekker, et al., 2009
A. nidulans	Novozyme 234	Oza and Kafer, 1990
A. nidulans	Novozyme 234	Osmani, et al., 1987
A. oryzae	Yatalase (Takara Shuzo) Lysing Enzymes (Sigma,St. Louis, MO,USA)	Shin Kanamasa, 2001
A.oryzae	Oerskovia's enzyme	Yuzuru et al., 1986
A. parasiticus	Novozyme 234 (Sigma) β-glucuronidase (Sigma)	Skory, et al., 1990
A. nidulans	Novozyme 234 (Sigma) β-glucuronidase (Sigma)	Yelton, et al., 1983
Penicillium expansum *P. griseoroseum*	Trichoderma harzianum lytic enzyme (Glucanex)	Varavallo et al., 2007
A. nidulans	Glucanex (Novozymes)	Koukaki, et al., 2003
A. nidulans	Novozyme 234 Helicase	Tilburn, et al., 1983
A. fumigatus	Novozyme 234 (Interspex,USA)	De Lucas et al., 2001
A. niger	Novozyme 234 (NOVO) β-glucuronidase (Sigma)	van Hartingsveldt, et al., 1987

4.6. Uptake of DNA

Calcium ions are crucial for nearly all transformation procedures. The only exception is the non-protoplasting transformation procedure in which lithium is used. In the presence of lithium, there is no need to both calcium ions and mechanical disruption

as described above. Calcium chloride in the amount of 10 mM to 50 mM is used in the protoplast transformation. The concentration of protoplast in the transformation mixture is about 10^8 to 10^9/ml, whereas the DNA concentration is usually 5 $\mu g/ml^{-1}$. Incubation time for 15-30 min at room temperature is generally sufficient for DNA uptake (Fincham, 1989).Up to 10 volumes of 40% PEG 4000 is used in the transformation mixture because in the presence of PEG, the treated cells do not clump which facilitates the trapping of DNA (Fincham, 1989). The chemical transformation of *Aspergillus* is presented in Figure 4.1.

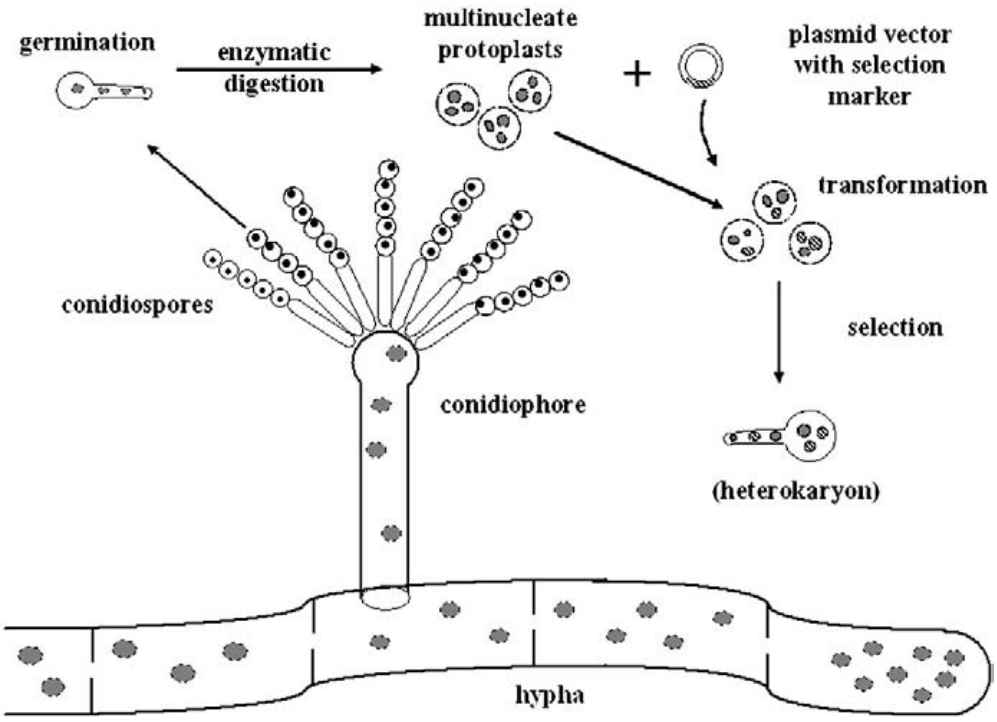


Figure 4.1. Chemical transformation of *Aspergillus*. Conidiospores subjected to an Enzymatic treatment to lyse the cell walls, in order to generate protoplasts, which are incubated with transforming DNA in a medium containing $CaCl_2$ and other additives. Plating on selective medium allows the regrowth of heterokaryotic transformants; since the conidia are uninucleate in most *Aspergillus* species, a homokaryotic strain is readily obtained by reselection of progeny. (Source: Lubertozzi and Keasling, 2009)

4.7. Transformation with Electroporation

Electroporation technique relies on the reversible permeabilization of the membranes by short duration of high amplitude electric fields. During the electric pulse,

the membrane lets the uptake of the DNA. This technique can be either used after pretreatment of a cell wall weakening agent in the case of the protoplasts or used directly to the recipient cell (Ruiz-Díez, 2002).

In literature, this method is widely used. Sanches and Aguirre in 1996 described a method for electroporation of *A. nidulans* germinated conidia in order to avoid protoplast preparation. Some other examples of electroporation mediated transformation of filamentous fungi are; *N. crassa, Penicillium urticae, Leptosphaeria maculans, A. oryzae, Scedosporium prolificans,* and *A. niger* (Ruiz-Díez, 2002). However, electroporation and biolistics have been shown to be inefficient in *Aspergillus* (De Bekker, et al., 2009).

CHAPTER 5

Vitreoscilla HEMOGLOBIN

Vitreoscilla is a filamentous bacterium belonging to the Beggiatoa family. It is a gram-negative aerobic or microaerophilic genus. The species *V. strecoraria* is strictly aerobic and able to cope with the hypoxic conditions by synthesizing soluble hemoglobin (VHb) (Zhang, et al. 2007). The early studies on *Vitreoscilla* hemoglobin indicated that VHb is a soluble cytochrome *o* until it was identified as the first bacterial hemoglobin which has a sequence (25%) homology with leghemoglobin (Kroneck, et al. 1991; Dikshit, et al., 1992).

VHb is a dimeric protein with two identical subunits each with a molecular weight of 15,775Da. The synthesis of the hemoglobin is promoted under the hypoxic conditions. The expression of the *vgb* gene in *Vitreoscilla* has shown that it is transcriptionally regulated by oxygen. *Vgb* specific transcriptional is increased in *E. coli* when oxygen level shifted from 20% to 5% (Dikshit, et al., 1990). VHb has an unusual property of oxygene association/dissociation rate constant when compared with other hemoglobins. The rate constant for oxygen association (k_{on}) is relatively normal; however the rate constant for oxygen dissociation (k_{off}) is unusually large (Table 5.1.). The reason for this large oxygen disassociation rate is probably related to its function. The possible role of *Vitreoscilla* hemoglobin is that the presence of VHb binds to oxygen and transfers it to the terminal oxidases (Liang, et al., 2007) and increases the activity of one or both terminal oxidases (Kallio, et al. 1994). As a summary the possible action of VHb is; increasing the flux of oxygen to the respiratory apparatus, providing higher internal oxygen concentrations, altering the internal redox state, or functioning as an efficient terminal oxidase (Sun, et al. 2002; Liu, et al., 2009). Heterologous expression of VHb by genetic engineering hosts containing *vgb* is used for various reasons such as for growth improvement, production of proteins and secondary metabolites (Sun, et al. 2002).

The VHb gene was first cloned and expressed in recombinant *E. coli* and the consequences for oxygen-limited growth were reported as enhanced growth properties and protein synthesis by Khosla and co-workers (Chen, et al., 1994). VHb seems to be

beneficial for many organisms for strain improvement. The effect of hemoglobin expression in microorganisms is presented in Table 5.2. For example, VHb gene was introduced into recombinant Chinese hamster ovary cell lines engineered to express human tissue plasminogen activator (tPA). The expression of VHb exhibited improved tPA production (Pendse and Bailey, 1994). Another example of the expression of VHb was demonstrated in fungus *Aspergillus terreus* in the investigation of the effect of a short break in aeration during cultivation on the itaconic acid production. Also here a significant improvement was detected (Lin, et al. 2004). Another transformation example was in the improved of β-galactosidase production in the recombinant *Pichia pastoris* yeast (Wu, et al. 2003).

Table 5.1. Kinetic constants for reactions of various hemoglobins with oxygen.

(Source: Eichhorn and Marzill, 1987; Zhang, et al., 2007)

Protein	k_{on} (μM^{-1} s^{-1})	k_{off} (s^{-1})	References
Vitreoscilla hemoglobin (VHb)	78	5000	Orii and Webster 1986
Lupin leghaemoglobin (Lba)	120	5,6	Aplleby 1992
Oryza sativa hemoglobin 1	68	0,038	Arredondo-Peter et al. 1997
Arabidopsis hemoglobin 1 (AHB1)	75	0,12	Trevaskis et al. 1997
Arabidopsis hemoglobin 2 (AHB2)	1,07	0,14	Trevaskis et al. 1997
Escherichia coli flavohemoglobins (HMP)	38	0,44	Gardner et al. 2000
Candida Hb(yeast)	850	17	Eichhorn and Marzilli 1987
Horse myoglobin	14	11	Eichhorn and Marzilli 1987
Soybean Lb	116	5,55	Eichhorn and Marzilli 1987
Glycera Hb (bloodworm)	190	2800	Eichhorn and Marzilli 1987

Table 5.2. Effects of hemoglobin expression in various microorganisms.

Organism	Effects of *Vgb*	References
Aspergillus niger	Alteration in the metabolism	Hofmann, et al. 2009
A. terreus	Alletiviate the effect of short breakin gaeration	Lin, et al.,2004
Pichia pastoris	Improved β-galactosidase production	Wu, et al., 2003
Serretia morcescens	Alteration in fermentation pahways	Wei, et al., 1998
Enterobacter aerogenes	Tolerance during exposure to high oxygen tension	Khleifat and Abooud, 2003
Pseudomanads	Increased cell growth	Liu, et al., 1995a
Saccharopolyspora erythrae	Increased erythromycin production	Brünker, et al., 1998
Enterobacter aerogenes	Efficient oxygene uptake	Geckil and Gencer, 2003
Gordonia amarae	Enhanced biosurfactant production	Dogan, et al., 2006
Bacillus subtilis	Enchanced total protein secretion and improved the production of α-Amylase and neutral protease	Kallio and Bailey, 1996
Bacillus subtilis	Improved poly-γ-glutamic acid production	Su, et al., 2010
Acremonium chrysogenum	Improved production of Cephalosporin C	J.A. DeModena 1993
Saccharomyces cerevisia	Altered the aerobic metabolism	Chen, et al., 1998
Xanthomonas maltophilia	Enchanced degradation of Benzoic Acid	Liu, et al. 1995b
Escherichia coli	Altered energy metabolism	Kallio, et al., 1994
E. coli	Altered amylase production	Khosravi, et al., 1990
Bacillus thringiensis	Improved the cell density and incecticidal crystal proteins yield	Liang, et al., 2006
Chinese Hamster Ovary Cells	Improved tPA production	Pendse and Bailey, 1994
Cephalosporium acremorium	Higher internal oxygene concentration	Liu et al., 2009

CHAPTER 6

MATERIALS AND METHODS

6.1. Materials

6.1.1. Chemicals

The chemicals used in the study are listed in Appendix A.

6.1.2. Media

A detailed list of used media and their compositions are provided in Appendix B.

6.1.3. Solutions and Buffers

Solutions and buffers were listed in Appendix C.

6.2. Methods

6.2.1. Strains, and Growth Conditions

DH5 α *E. coli* cells, which were obtained from Chemical Physical and Biology Department of Illinois Institute of Technology (IIT), USA, were used as host strain for plasmid DNA.

The propagation of DH5 α *E. coli* cells were carried out on Luria-Bertani (LB) medium at 37^{o}C for 12h. Transformed DH5 α *E. coli* cells were selected on LB-Amp (50 µl/ml) medium at 37^{o}C for 12h.

Mutant *Aspergillus sojae* ATCC 20235 (M7) was used for mutation procedure in order to obtain pyrG negative mutant *A. sojae* strains (M7/2).

The propagation of M7 was performed on YME (Yeast Malt Extract) medium and was incubated at 30^{o}C until well sporulation (1 week). These stock spores were transferred on molasses agar slants and incubated at 30^{o}C until well sporulation (1 week). Spores from the slants were either used to prepare stock cultures prepared with 20% glycerol water and stored at -80^{o}C or harvested from the slants by using 5 ml of sterile Tween-80 water (0.02%). The spore suspension was collected in a sterile 50 ml falcon tube and was stored at 4^{o}C. After this procedure, spore counts were determined by using Thoma bright line hemacytometer (Marienfield, Germany). The sterility of strain M7 was checked on BHI (Brain Heart Infusion) agar. M7/2 strains were selected using Minimal Media (MM) and Minimal Media with Uridine supplementation (10 ml of 2,4% stock in 1L).

6.2.2. Plasmid Construction

The following steps in plasmid construction were performed at Illinois Institute of Technology Department of Chemical Physical and Biology Sciences, USA by Associate Professor Dr. Canan Tarı.

In order to increase the *vgb* amount before ligation to ANIp4 plasmid, *vgb* was cloned into plasmid pNKD1 and *vgb* inserted plasmid pNKD1 was transformed into DH5 α *E. coli* cells. (pNKD1 was constructed by inserting *vgb* into the SmalI site of plasmid vector Bluescript from the reference given below (Liu, et al., 1994) (Figure 6.1 and Figure 6.2). Afterwards, cells were incubated in LB broth, the *vgb* insert from *E. coli* pNKD1 was extracted and amplification of the *vgb* was performed using PCR.

Vitreoscilla hemoglobin gene (*vgb*) was supplied by the Chemical Physical and Biology department of Illinois Institute of Technology (IIT), USA. Plasmid ANIp4 was supplied from the reference given below (Storms, et al., 2004). Plasmid ANIp4 was used as a vector that shuttle DNA between *E. coli* and *Aspergillus sojae*. ANIp4 was constructed from plasmid ANIp1 by inserting the pyrG gene cassette, a selectable marker for transformation of *A. sojae* (Storms, et al., 2004). Previously, plasmid pUC18 with an ampicillin resistance marker (Bou, et al. 2000) was cloned into plasmid ANIp1 therefore ampicillin resistance marker was inserted while constructing ANIp4. Ampicillin resistance marker was necessary for selection of transformed DH5 α *E. coli* cells.

In order to perform the ligation of *vgb* gene with ANIp4 plasmid, plasmid ANIp4 was digested at a place where *vgb* gene can be cloned (Figure 6.3) using NheI and FseI restriction enzymes. Besides, *vgb* gene was also double digested with NheI and FseI restriction enzymes. Double digestion was performed at 37^{o}C for 2 hours and enzymes were inactivated right after at 65^{o}C for 20 minutes. For the digestion 1 µl of NheI enzyme, 1 µl FseI enzyme, 2 µl of buffer N4, 0,2 µl of BSA, 2 µl of undigested vector DNA sample and 13,8 µl of ultrapure water was mixed. (Buffer 4 and BSA were sent by the company together with the enzymes). After digestion, 2 µl of sample, 18 µl ultrapure water and 4 µl of loading dye were added for agarose gel electrophoresis run. The rest of the sample was kept at either -20^{o}C or used for ligation procedure right after. The ligation step was performed using the double digested vector and double digested *vgb* insert at certain ratios such as 8:1 to 15:1 (insert: vector ratio) and incubated at 16^{o}C for overnight (16 hours). This ratio was based on the initial base pair ratio of vector and *vgb* gene. In this study insert was approximately 500 base pair and the vector was 5574 base pair, therefore the ratio 1:10 was used. The concentration of both the vector and the insert was 115 ng/µl. Based on this, 0,3 µl of vector, 3 µl of insert, 2 µl of T4 ligase buffer, 1 µl of the T4 ligase enzyme and 13,7 µl of ultrapure water to make up 20 µl of total volume was mixed in order to perform the ligation.

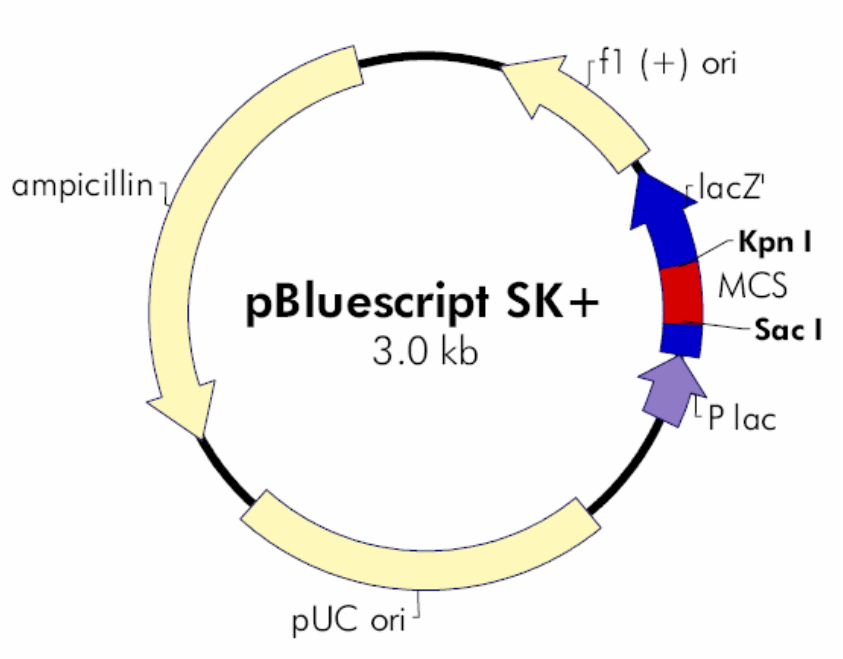


Figure 6.1. Plasmid vector Bluescript

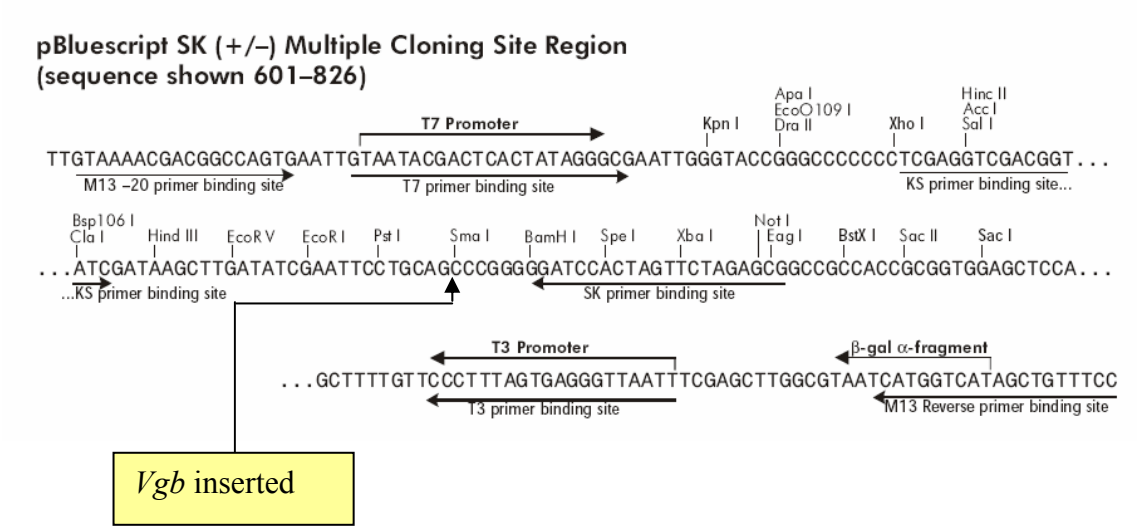


Figure 6.2. pBluescript SK (+/–) Multiple Cloning Site Region (sequence shown 601-826)

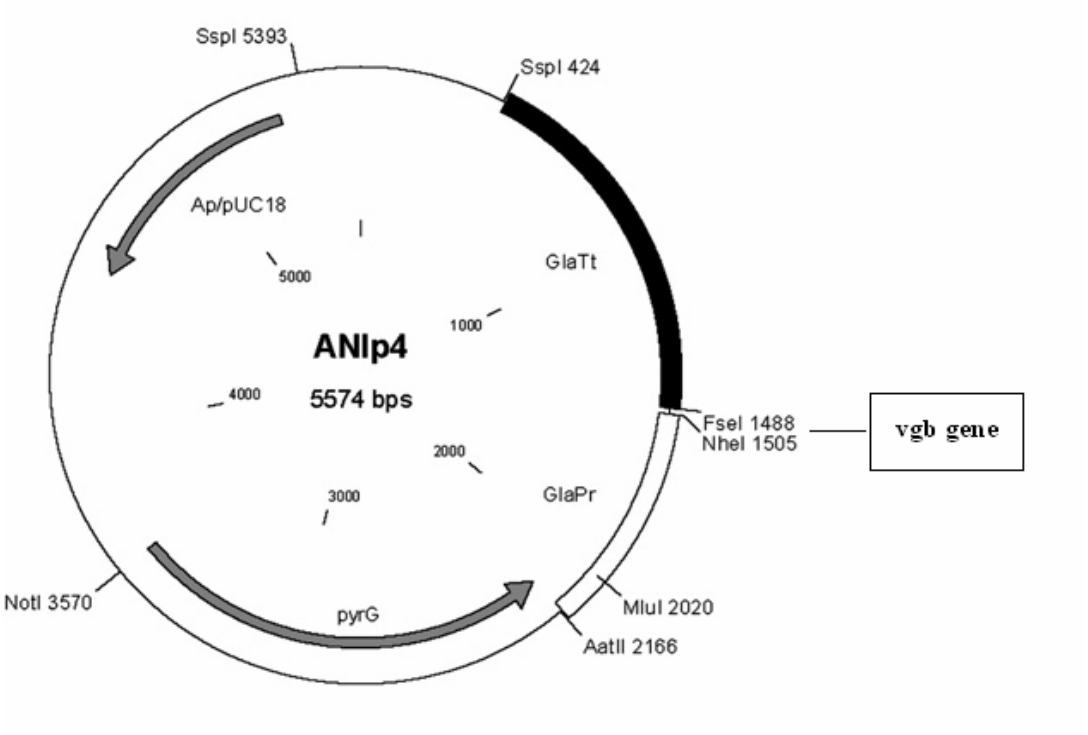


Figure 6.3. Shuttle vector (ANIp4). *vgb* was ligated from the restriction sites FseI and NheI of ANIp4. The various modules in the figure are indicating the pUC18 sequences (Ap/pUC18), glaA transcription termination region (GlaTt), *glaA* promoter region (*GlaPr*) and the selectable marker for *A. niger* (pyrG). (Source: Storms, et al., 2004).

6.2.3. Preparation of Competent *E. coli* using Calcium Chloride

Competency procedure is necessary for DH5 α *E. coli* cells to enable transformation procedure and it is fulfilled at the beginning of the transformation. Competency and transformation procedures were described by Sambrook et al. (Sambrook, et al., 1989). Single colony from a LB agar medium was picked after incubation for 16-20 hours at 37^{o}C and was used to inoculate 100 ml LB broth in 1 liter Erlenmeyer flask at 37^{o}C. For efficient transformation, less than 10^{8} cells/ml viable cells equivalent to an OD_{600} of ~ 0.4 was used. Culture was harvested when the OD_{600} reached 0,35. Ice-cold 50 ml falcon tubes were made ready before the bacterial cells were transferred and the cells were stored in ice for 10 minutes. After centrifugation for 10 minutes at 4100 rpm at 4^{o}C, supernatant was discarded from the cell pellets. 30 ml of ice-cold $MgCl_2$-$CaCl_2$ solution (80 mM $MgCl_2$, 20Mm $CaCl_2$) was used to resuspend cell pellets by gentle vortexing and afterwards cells were recovered by centrifugation at 4100 rpm for 10 minutes at 4^{o}C. For each 50 ml original culture, 2 ml of ice-cold 0.1M $CaCl_2$ was used for resuspension. These cells now competent, could be used for the transformation procedure, and rest was dispensed into aliquots and frozen at -80^{o}C (750 µl competent cell solution, 250 µl sterile 20% glycerol solution).

Transformation procedure was described previously (Sambrook, et al., 1989). According to this procedure, 200 µl of $CaCl_2$ treated competent cells were transferred into sterile ice-cold falcon tubes. DNA (no more than 50 ng/10 µl) was added to each tube and gently mixed with the cells and stored in ice for 30 minutes. Afterwards, these were transferred to 42^{o}C preheated water and bathed for 90 seconds before they were transferred to an ice bath without shaking, for 1-2 minutes. After adding 800 µl of LB-amp agar medium to each tube, culture was incubated at 37^{o}C for 45 minutes in a water bath for recovery. Following this, 100 µl of transformed competent cells were plated on LB-amp agar plates and incubated at 37^{o}C for 12-16 hours.

6.2.4. Plasmid Isolation from Transformed *E. coli* cells with Alkaline Lysis (Mini Prep)

This method can be used either to obtain high amount of plasmid or to check if the cells have the plasmid.

Mini prep protocol was performed by using Qiagen miniprep kit as described. A single colony of transformed and competent cells were scrapped from over night LB-amp agar plates and transferred to 1 ml of LB-amp medium in a 10 ml tube and incubated at 37^{o}C for 12-16 hours. In order to retrieve the plasmid DNA, different buffers were used. Buffer P1 was the resuspension buffer, buffer P2, the buffer used to break down the cells, and buffer N3 is the neutralization buffer (It precipitates everything but the DNA.). Buffer EB was used to rehydrate plasmid DNA (Qiagen miniprep kit included these buffers).

According to the procedure, 250 µl of P1 buffer was added to the pellet and resuspended gently until a nice suspension was formed. Afterwards 250 µl of P2 buffer was added to this whole suspension. A sticky layer was observed after inverting the tube 10-15 times. 250 µl of N3 buffer was added to this solution and the tube was inverted 10-15 times until debris precipitation and cloudy suspension was observed. After adding these 3 buffers, suspension was centrifugated at 12000 rpm for 10-12 minutes. 750 µl of isopropanol containing 1,5 ml Eppendorf tubes were prepared and supernatant was transferred into these tubes. Pellet was thrown away due to the fact that supernatant contained the plasmid DNA. The whole 1.5 ml bearing Eppendorf tubes were centrifugated at 12000 rpm for 10-12 minutes and supernatant was thrown away, while the pellet was kept. 200 µl of 70% ethanol was used to wash the pellet and afterwards centrifugated at 12000 rpm for 2 minutes. This step was repeated twice. After discarding the supernatant, the pellet was left for air drying where it turned transparent. 60 µl of EB was added to rehydrate the pellet and kept at 37^{o}C for 5 minutes and stored afterwards at -20^{o}C for long term storage.

6.2.5. Plasmid Isolation from Transformed *E. coli* Cells with Boiling Method

This method was applied instead of Qiagen miniprep kit. Lysozyme solution (10 mg/ml) with ultra pure water was prepared in 10 µl of aliquots in Eppendorf tubes. A single colony of transformed and competent cells were scrapped from over night LB-amp agar plates and transferred to 5 ml of LB-amp medium in a 10 ml tube and incubated at 37^{o}C for 12-16 hours. Afterwards this whole content was transferred into falcon tubes and centrifugated at 3620 rpm for 5 min at 4^{o}C and supernatant was discarded from the pellet. Pellets were transferred to 1,5 ml Eppendorf tubes and 200 µl of STET solution was added. The whole content was mixed gently and kept on ice and 10µl of lysozyme solution was added. After 5 seconds the mixture was immersed in the boiling water for another 40 seconds and centrifuged at 13000 rpm for 5 minutes. The pellet was removed and supernatant was kept since the supernatant contained the plasmid. 200 µl of isopropanol was added to the supernatant and mixed with shaking hands. After keeping the mixture on ice a couple of minutes, the mixture was centrifugated at 13000 rpm for 10 minutes. Then supernatant was drained and pellet was washed with 100 µl of 70 % ethanol. After mixing vigorously by vortexing, the sample was spinned at 13000 rpm for 2 minutes. This step was repeated twice. Then ethanol was discarded and pellet was left for air drying to remove any traces of ethanol. The pellet was the desired plasmid. After drying, 200 µl of ultrapure water was added in order to rehydrate the plasmid and stored at -20^{o}C.

6.2.6. Digestion of the Plasmid DNA with RNAase

This procedure was necessary in order to get rid of the RNA in the plasmid DNA sample. (In the procedure of Qiagen miniprep kit, this step was not necessary since the kit included RNAase). Initially RNAase enzyme solution (10 mg/ml) was immersed in boiling water for 15 minutes and cooled to room temperature. The plasmid and the 10 µl RNAase enzyme solution was mixed and incubated for 1 hour at room temperature. After incubation, 100 µl of 3 M Na-acetate at pH5.2 and 2.75 ml of 95% ethanol was added and mixed well in order to precipitate the plasmid. The whole suspension was centrifugated at 10000 rpm for 15 minutes and supernatant was drained,

pellet was kept. Afterwards pellet was left for air drying and 250 µl of ultrapure water was added and stored at -20^{o}C.

6.2.7. Digestion with EcoRI Restriction Enzyme

In order to digest the plasmid DNA and form it in the linear form, the restriction enzyme EcoRI was used. This enzyme cleaves the 5'…G↑AATTC…3' and 3'…CTTAA↑G…5' ends.

For the digestion procedure, reagents were thawed and placed on ice. 12 µl water, 1 µl EcoRI enzyme, 2 µl EcoRI buffer and 5 µl plasmid DNA were mixed together (total volume has to be 20 ul). The whole mixture was incubated at 37^{o}C for 2 hours. The whole procedure was repeated for the undigested DNA sample as negative control by adding water instead of EcoRI enzyme and was incubated also at 37^{o}C for 2 hours. After incubation at 37^{o}C for 2 hours, 4 µl of loading buffer was added to the whole mixture making up the total amount 24 µl. This mixture was ready for the run on the agarose gel electrophoresis.

6.2.8. Agarose Gel Electrophoresis

6.2.8.1. Gel Preparation

Agarose gel preparation for 50 ml of volume was as followings. 0.5 g of agarose gel (1%) and 50 ml of 1X TAE buffer (the stock solution of 50X TAE was used to prepare 1X TAE (Appendix C) were added to 250 ml Erlenmeyer flask and boiled while stirring. After cooling to 50-55^{o}C, 5 µl of gel stain was added and poured onto the gel cast. By putting appropriate comb, wells were prepared. After solidifying the gel, the comb was removed slowly and wells were formed to load the DNA samples.

6.2.8.2. Preparation of the Marker

In this study two markers were used: Gene ruler 100 bp DNA Ladder Plus (Fermentas) (ready to use) and Lambda DNA/HindIII Marker. Lambda DNA has to be

digested with HindIII enzyme before using. For this procedure 1 µl of Lambda DNA (500 ng/1 µl), 1 µl of HindIII enzyme, 2 µl of buffer N#2 was mixed and 16 µl of water was added to bring the whole content to 20 µl. In order to perform the digestion, the mixture was incubated at 37^{o}C for 2 hours where afterwards HindIII enzyme was inactivated by incubating at 65^{o}C for 15 min. This could be stored either for long term storage at -20^{o}C or used right after. For loading on the agarose gel, 5 µl of loading dye was added to this content.

6.2.8.3 Running the Gel

In order to run the agarose gel, the prepared gel as described in section 6.2.8.1 was loaded with all the samples (25 µl) and the markers. The tank was filled with 1X TAE buffer until the gel was completely immersed and then connected to the power unit. The power unit was adjusted to 80 V for approximately for 1 hour until the blue lines came to the other side of the gel. After the gel run was completed, the gel was carefully removed and the bands were observed under the UV light. Picture of the gel was captured by a camera and loaded onto the computer (Biolab Transilluminator).

6.2.9. Designing and Ordering Primers

Before any PCR process, the primers (reverse and forward) have to be custom designed. In this study, primers were ordered from Integrated Technologies in USA. In this study NheI recognition site was used (GCTAGC). After this, the actual transcriptional start primer region was used, which was made of the 15-19 bp units on the whole *vgb* sequence. These were provided from the NCBI nucleotide sequence for *V. stercoraria vgb* (GI: 155317, accession number: M27061) encoding hemoglobin with total of 745 bp. Based on this, the forward primer which was ordered was as 5'-GAT CAT GCT AGC ATG TTA GAC CAG CAA ACC ATT-3'

For designing reverse primer, once again control region GATCAT was used and was followed with FseI recognition site GGCCGGCCT. After that the stop transcriptional primer region obtained from the NCBI nucleotide sequence for *vgb* hemoglobin sequence was followed. Based on this the reverse primer, which was ordered was as 5'-GAT CAT GGC CGG CCT TCA ACC GCT TGA GCG TAC-3'.

While ligating the *vgb* gene into ANIp4 plasmid, the amplified *vgb* nucleotide sequence (Figure 6.4.) excluded the promoter. According to this, prokaryotic *vgb* promoter was not cloned into ANIp4 plasmid, hence its expression was achieved by eukaryotic ANIp4 promoter region.

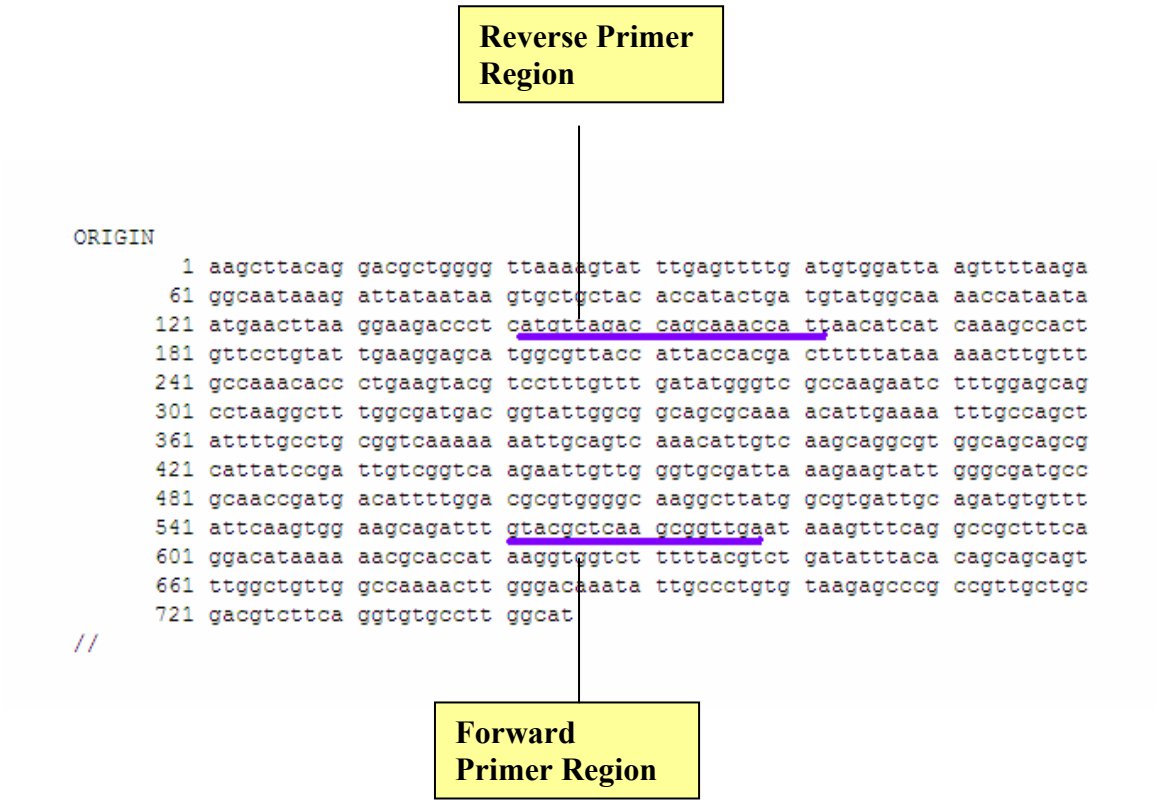


Figure 6.4. *V.stercoraria vgb* gene encoding hemoglobin, complete cds from NCBI web page (The accession number is M27061).

6.2.10. PCR (Polymerase Chain Reaction)

The Polymerase Chain Reactions (PCR) was used in order to amplify the *vgb* gene sequence for confirmation of the transformation. Therefore, the reagents used for this procedure were as followings.

a) Maxtermix: (Tag enzyme polymerase+nucleotides) 2X concentration diluted to 1X.

b) Template DNA (20-30 ng/µl): Concentration of the template DNA was measured by Nanodrop analysis.

c) Reverse primers: 10 nmol/ml of working stock.

d) Forward primers: 10 nmol/ml of working stock

For the PCR procedure, 12.5 µl of maxtermix, 2 µl of template DNA, 2 µl of forward primer, 2 µl of reverse primer and 6.5 µl of ultrapure water making up the total volume to 25 µl, was mixed in 200 µl in an Eppendorf tube. The operating step was summarized in Figure 6.5.

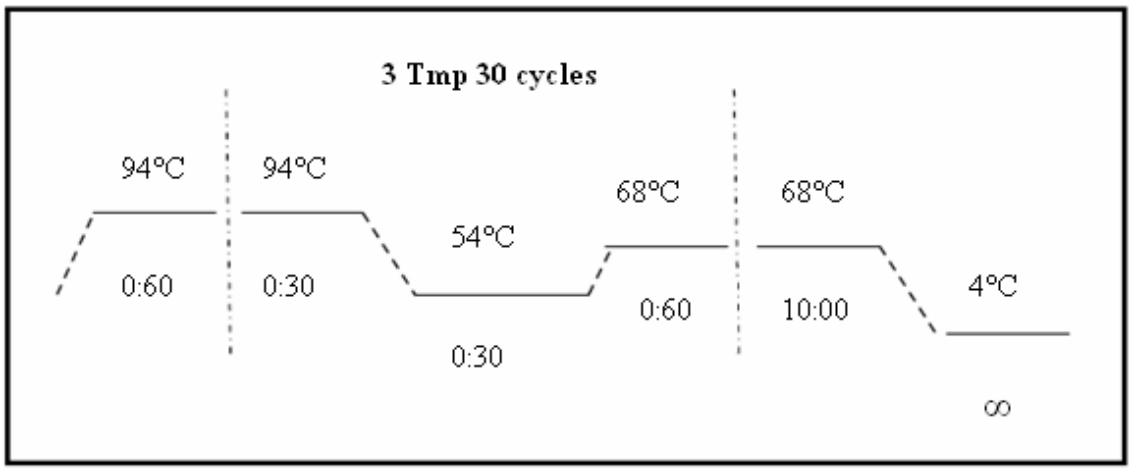


Figure 6.5. PCR run conditions

After the PCR run, 3 µl of PCR product, 17 µl of DI water and 4 µl loading buffer was mixed and run on the agarose gel.

6.2.11. Isolation of *A. sojae* pyrG Negative Mutants by UV Mutation

The pyrG gene encodes the orotidine 5'-phosphate carboxylase, the terminal enzyme in uridine 5'-phosphate biosynthesis. The pyrG negative mutants require uridine supplementation for growth. After transformation of these mutants with plasmid ANIp4, pyrG negative mutants will be cured and will no longer need uridine supplementation, since plasmid ANIp4 bears pyrG cassette. Thus to select the transformed ones, first pyrG deleted mutants have to be obtained by replica plating method. Selection depends on uridine auxotrophy.

Isolation of *A. sojae* pyrG deleted mutants by UV irradiation was as described by Van Hartingsveldt, et al. 1987. with slight modifications A suspension of freshly harvested strain M7 (initially UV mutated spores) spores in sterile Tween-80 water

(0.02%) was diluted to 10^7spores/ml with sterile PBS buffer. This solution was exposed to 600 J/m^2 UV light. During 10 minutes of exposure time, 100 µl of spore solution was taken for every one minute to obtain 10% survival rate. The irradiated spore solution (100 µl) samples (survival 10%) were added to 900 µl of PBS buffer and placed in ice bath for 5 minutes. Afterwards samples were kept in dark for 30 minutes and diluted to $1x10^2$ spores/ml with PBS buffer. For each minute, 100 µl of appropriate diluted samples were used to plate on YME agar and incubated at 30°C for 48 hours. The first sample before treating the spores by UV irradiation and also the last sample was plated on BHI for sterility check. After formation of colonies, these were tested for uridine auxotrophy by replica plating method. In this method colonies were replica plated on both minimal media and minimal media supplemented with uridine. Colonies surviving on uridine containing plates were selected.

In these experiments colonies acting like pyrG negative mutants were tested for the second time with replica plating method. Second verifications were unsuccessful; nevertheless, the colony acting like pyrG negative mutant that could not pass the second verification but passed the first verification step was chosen. This colony was propagated and named as Mutant7/2 (M7/2) and was used for the transformation procedure.

6.2.12. Agar Diffusion Method for Detection of Pectinase Production using Polygalacturonic Acid (PGA)

The use of agar diffusion method allows the rapid determination of production of pectinase from *Aspergillus sojae*. This method is very useful for the detection of pectinase production potential of wild type and the mutants of *A. sojae* before and after mutations and cloning with *vgb*. It also provides information about the effect of the mutations on the pectinase production.

The solid medium used for detection of pectinase, described by Hankin and Anagnostakis (Hankin and Anagnostakis, 1975) was modified. These media were inoculated with spore solutions of the strains used. Spores from spore solutions of harvested molasses agar slants were used. They were picked up by using automatic pipette and transferred into the plates by puncturing the medium. After incubation at 30°C for 2 days, plates were flooded with 5 N of HCl to show the clear zone formation

around a colony, which was measured and compared among the strains M5/6, M7, M7/2 TraGlux and TraElectro.

6.2.13. Protoplast Formation and Transformation with Glucanex Enzyme

Protoplast formation and transformation procedures are based on the methods described by the references (Koukaki, et al. 2003; Tilburn, et al. 1983). According to these, *A. sojae* pyrG negative mutant strain (M7/2) was grown at 30°C for 7h 30 min at 100 rpm in broth minimal media supplemented with uridine. Under these conditions, young mycelium was obtained after inoculation of 250 ml cultures, in 1l flasks, with $10^{10\text{-}11}$conidiospores. Mycelium was resuspended in 20 ml of isosmotic buffer (1.2M $MgSO_4$, 10Mm orthophosphate, pH 5.8) and protoplasting was carried out using cell wall lysing enzyme (Glucanex) at a final concentration of 100 mg per 0.5 mg of mycelium at 30°C for 90 min. Protoplasts were washed twice in 1M Sorbitol, 10mM Tris-HCI, 10mM CaCI2, pH 7.4 and resuspended with the same buffer at a concentration of 10^8 conidiospores/200 μl and distributed in sterile Eppendorf tubes. These were used for transformations. 0.1-1 μg of plasmid DNA was added to the osmotic medium. The protoplasts were incubated with DNA for 20 minutes at room temperature, and then 10 volumes of 25%w/v PEG 4000, 10mM $CaCl_2$, 10mM Tris-HCI, pH 7.5 solutions was added. The mixture was left at room temperature for 20 minutes, pelleted and washed with 1.2M sorbitol, 10mM $CaCl_2$, 10mM Tris-HCI pH 7.5 solution and plated on the YME medium in a 2 ml soft agar overlay (Tilburn, et al. 1983). Agar overlay technique allows producing a homogeneous lawn of organism within a thin layer of agar across the surface of a plate. Organisms are added to a soft top agar (0.75% agar, as opposed to the usual 1.5% for agar plates), which has been melted at 100°C and cooled to 45°C. This is warm enough to keep the agar liquid, and also organisms are not killed (for a period of time). The melted agar/organism suspension is mixed and poured evenly across the top of an agar plate and allowed to solidify and incubated at 30°C for 48 hours. After visible colonies formed, (Figure 6.6.) this new strain was named as transformant using glucanex (TraGlux).

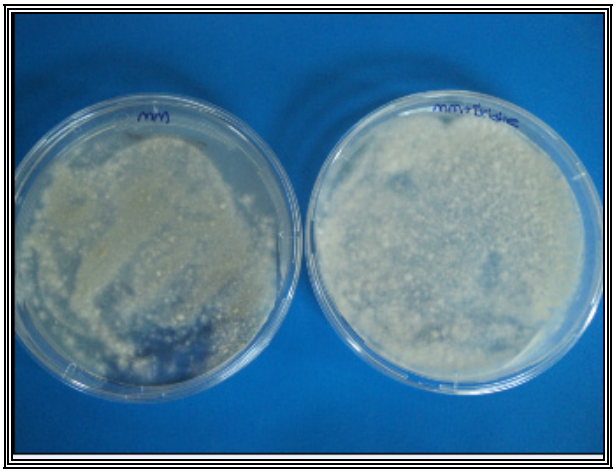


Figure 6.6. Picture of master plate of TraGlux with agar over lay technique after 48 hours, in minimal media and minimal media containing uridine. These plates were used for future propagation of the strain TraGlux.

6.2.14. Transformation of *Aspergillus sojae* with Electroporation Method

The electroporation procedure was described by the references (Sanchez, Aquirre, 1996; Weidner, et al., 1998). The *A. sojae* mutant strain M7/2 was harvested from the YME plates using Tween 80 solution. The total viable count obtained from this plate was around $1{,}25x10^7$ spore/ml which was used for the transformation. Total of 3 ml was collected from the plate corresponding to $3{,}75x10^7$ total spores used as inoculum size of 10^7 spore/ml into 125ml of YG media. After inoculation, the strain was left for growth at 30°C and 250 rpm. Spores were collected by centrifugation and resuspended in 200 ml ice-cold sterile water and centrigugated again. Afterwards, spores were resuspended in 12.5 ml YED and incubated for 60 min at 30°C and 100 rpm. After incubation, spores were collected by centrifugation and resuspended in 1 ml ice-cold EB with 10^9 conidia/ml and kept on ice. 50 µl of this conidial suspension was mixed with 1 or 2 µl DNA in eppendorf tubes and kept on ice for 15 min. For transformation process, 1000 V for 300 µsec with single pulse was used. 1 ml ice-cold YED was added and the whole suspension was transferred to 15 ml tube and kept on ice for 15 min. Afterwards, this was incubated at 30°C for 90 min at 100 rpm on rotary shaker. 100, 200 and 700 µl spore solutions were spread on minimal medium agar plates and incubated at 30°C, where transformats were observed after 36-48h.

6.2.15. Plasmid Isolation from Transformed *A. sojae*

After transformation of *A. sojae* with *vgb* gene bearing ANIp4 plasmid, verification of the transformation was necessary. In order to verify this, plasmid ısolation procedure was performed with slight modifications as described in the reference given below (Storms, et al., 2004). Freshly prepared spore solution (10^7spore/ml) was used to inoculate 150 ml of minimal media broth supplemented with uridine and incubated in 1L Erlenmeyer for 12-16 hours at 30°C and 100 rpm. After incubation, mycelium were filtered through buhner funnel. 100 µl lysing solution consist of 2,5M LiCI, 50 mM Tris-HCI pH 8,0, 4% Triton X-100, and 62.5 mM EDTA, an equal volume of phenol:chloroform and 0,2 g glass beads (0.45-0.5 mm) were added into microcentrifuge tubes and vortexed for 2 min, then centrifugated at top speed for 1 minute. Two volumes of 95% ethanol was added in order to precipitate the DNA from the cleared supernatant and pelleted for 15 min. at top speed. Later, this was washed twice with 70 % ethanol and dissolved in 20-60 µl water. After the PCR run of this extracted DNA, agarose gel electrophoresis method was used in order to observe the *vgb* bands.

CHAPTER 7

RESULTS AND DISCUSSION

Prior to the transformation of *Aspergillus sojae* with the *Vitreoscilla* hemoglobin gene (*vgb*) certain experiments were performed and their results are discussed below:

1) Transformation of the *DH5 α E. coli* cells with the shuttle vector (ANIp4).
2) Cell growth of the plasmid bearing cells in LB-Amp broth at 37^{o}C and 225 rpm in order to increase the plasmid amount.
3) Extraction of the plasmid and verification of the plasmid on the agarose gel.
4) Cell growth of *E.coli* pNKD1 containing the plasmid *vgb* in LB-Amp broth in order to increase its amount.
5) Extraction of the *vgb* insert from *E coli* pNKD1 and amplification using PCR.
6) Ligation of the insert *vgb* with ANIp4 shuttle vector.
7) Transformation of *DH5 α E. coli* with ligated plasmid (ANIp4 containing the insert *vgb*).
8) Cell growth of *DH5 α E. coli* with ligated plasmid in order to increase its amount for *A. sojae* transformation.
9) Extraction of the ligated plasmid and amplification using PCR and verification on the agarose gel.
10) UV mutation of *A. sojae* in order to obtain pyridine negative mutants
11) Preparation of the protoplasts of *A. sojae* pyridine negative mutants.
12) Transformation of *A. sojae* mutants using the ligated plasmid via protoplast method.
13) Transformation of *A. sojae* mutants using the ligated plasmid via electroporation method.
14) Verification of the transformed *A. sojae* if the transformation was successful by gene sequence analysis and agarose gel.

The first seven steps were performed at Illinois Institute of Technology Department of Biological Sciences, USA by Associate Professor Dr. Canan Tarı, therefore are not disccused within the scope of this thesis**.** Hence, the results and discussions will start from step 8 on.

7.1. Isolation of *A. sojae* pyrG Negative Mutants by UV Mutation

UV mutation procedure was performed. In Table 7.1. survival rates of the UV exposed spores are given. In Figure 7.1., master plate of UV mutated colonies showed. In Figures 7.2., 7.3. and 7.4. plates were replica plated from the master plates and colonies that did not grow on minimal media, but grew on minimal media supplemented with uridine were selected. These selected colonies were used as master plates and subjected to verification for second approval of pyrG negative mutation checking if these colonies could still grow on minimal media supplemented uridine, but could not grow on minimal media. In these experiments, none of the colonies could pass all verifications. A colony could pass second approval however, when a new test was performed, colony could not exhibit as pyrG negative mutant. Nevertheless, this colony was used for transformation and named as M7/2.

Another supplement, 5-fluoroorotic acid (5-FOA), which is converted into a toxic 5-fluoro-UMP in prototrophic strains, (Long, et al., 2008) was not used in these experiments. Usage of this second selective supplement could facilitate to obtain pyrG negative strains. It is reported that, if a modified selective FOA medium which contained uracil in addition to uridine is used, it could be possible to obtain FOA resistant mutants which will require uridine to grow (Gellissen, 2005).

In future studies, considering the pyrimidine synthese pathway, mutations on either the pyrF gene (Orotate phosphoribosyltransferase (OPRTase)) or on the pyrG gene (OMP decarboxylase) will result in a loss of enzymatic activity confering resistance to FOA by preventing the production of 5-fluoro- UMP. Colonies that are resistant to FOA will be replica plated onto MM with and without uridine. Since FOA selects for pyrF or pyrG mutant strains, enzyme analysis with radiolabeled substrates can be used to determine the ability of auxotrphic isolates to convert orotic acid to OMP (pyrF) and the ability of isolates to convert OMP to UMP (pyrG) (Table 4.1). According to this method both mutants can be identified. (Skory, et al., 1990). In addition to this method, a similar method using FOA, selecting the mutations on pyrF and pyrG genes can be used. In this method after obtaining FOA resistance mutants and selecting them on uridine positive and negative minimal media, will decrease the probability of the mutations observed on pyrG gene. These colonies can then be transformed in order to recover the uridine auxotrophy with a plasmid containing homolog pyrG gene. After confirming the

recovery with homolog pyrG gene, strains used for transformation can be named as pyrG mutant strain (Van Hartingsveldt, et al.,; Kanamasa, et al., 2003). This thesis study showed that, selection depending on only uridine auxotropy was inadequate to select the mutants which could be cured by homolog pyrG gene. According to this above mentioned literature, the selection will depend on the pyrG gene deficiency by UV mutation or chemical mutations that can be extended to be used in *Aspergillus sojae* transformation as well.

Table 7.1. The survival rate of spores after 10 minutes of UV exposure.

Minutes	**Survival Rate**
1	2%
2	2%
3	1%
4	0,4%
5	0,6%
6	0,02%
7	0,065%
8	0,035%
9	0,0004%
10	0,0002%

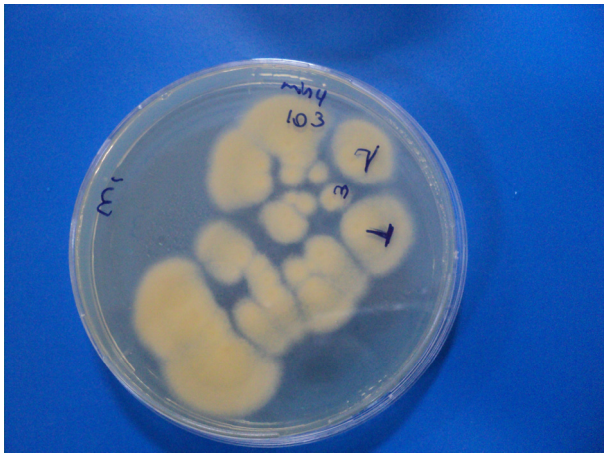


Figure 7.1. A selected plate of UV mutated *A. sojae* M 7/2 strain. This plate is inoculated with the UV treated spore solution at the 4th minute of treatment and diluted to 10^3 spore/ml. Colonies that were distinct from each other were chosen for pyrG negative assay.

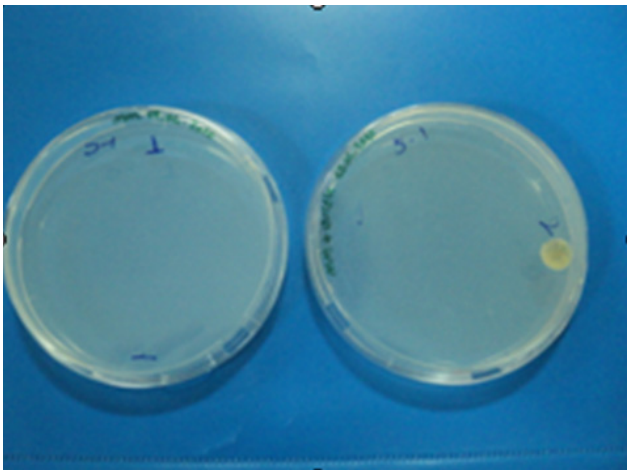


Figure 7.2. Colony number 2 could not grow without uridine.

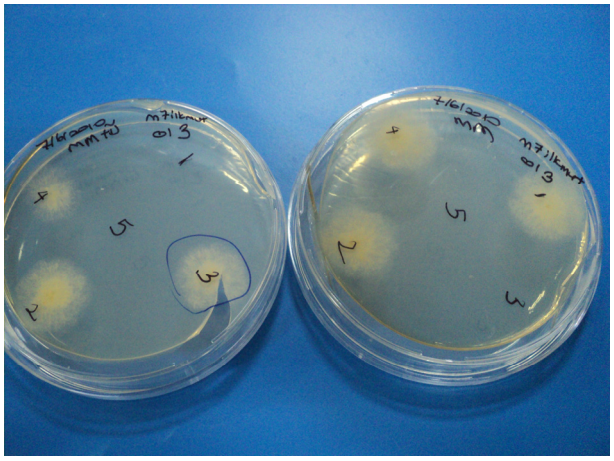


Figure 7.3.Colony number 3 could not grow without uridine.

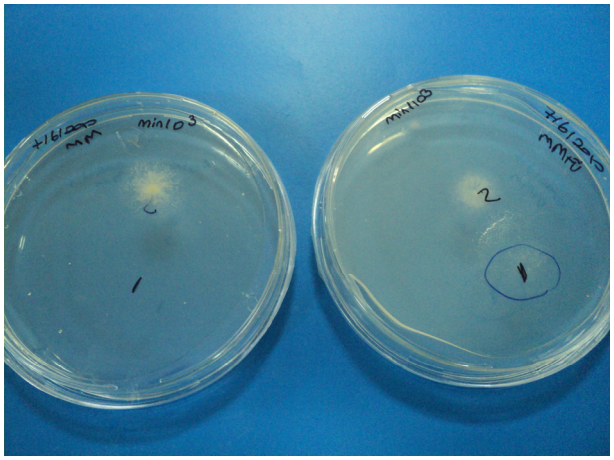


Figure 7.4. Colony number 1 could not grow without uridine.

7.2. Plasmid Isolation from Transformed *E. coli* Cells with Alkaline Lysis (Mini Prep)

DH5 α E. coli cells were transformed initially with shuttle vector (ANIp4) containing *vgb*, pyrG and amp resistant gene using $CaCI_2$ method described in section 6.2.3. In order to verify if the transformation was performed efficiently plasmid extraction via Mini-prep kit was carried out. The plasmid isolate was run on the agarose gel electrophoresis which is presented in Figure 7.5. According to the result (plasmid bands) observed it can be seen that the base pair of this plasmid was approximately 6170 bp confirming the successful transformation of *DH5 α E. coli* cells. After this step, these cells were grown in LB-Amp broth for overnight at 37^oC and 225 rpm in order to obtain sufficient amount of *vgb* carrying plasmid to be used in the transformation of *A. sojae* pryG negative mutants. Following this, plasmid extraction using the miniprep kit was carried out and the plasmid extract of *DH5 α E coli* was subjected to PCR in order to increase the *vgb* amount and also to observe the bands on the gels. For this reason the plasmid DNA was used as template and the approximately 500 bp of *vgb* fragment was amplified using gene-specific primers. The bands corresponding to the *vgb* base pairs are presented on Figure 7.6., 7.7., and 7.8. The experimental details are given on the legend of the figures. In Figure 7.7, transformed A .sojae was subjected to PCR and, the bands similar to *vgb* were observed. In Figure 7.8., *vgb* bands were observed, but in lanes 3 and 4, which are DNA samples extracted from M7/2 used as negative control, a contamination with *vgb* was observed. These lanes were false-negative. The reason of observing false- negative bands might depend on the PCR technique due to its extreme sensitivity and represents a potential source of false-positive results and extreme care has to be taken in order to avoid contamination during sample preparation (Vary, et al,, 1990). Because of the contamination, the transformation cannot be confirmed.

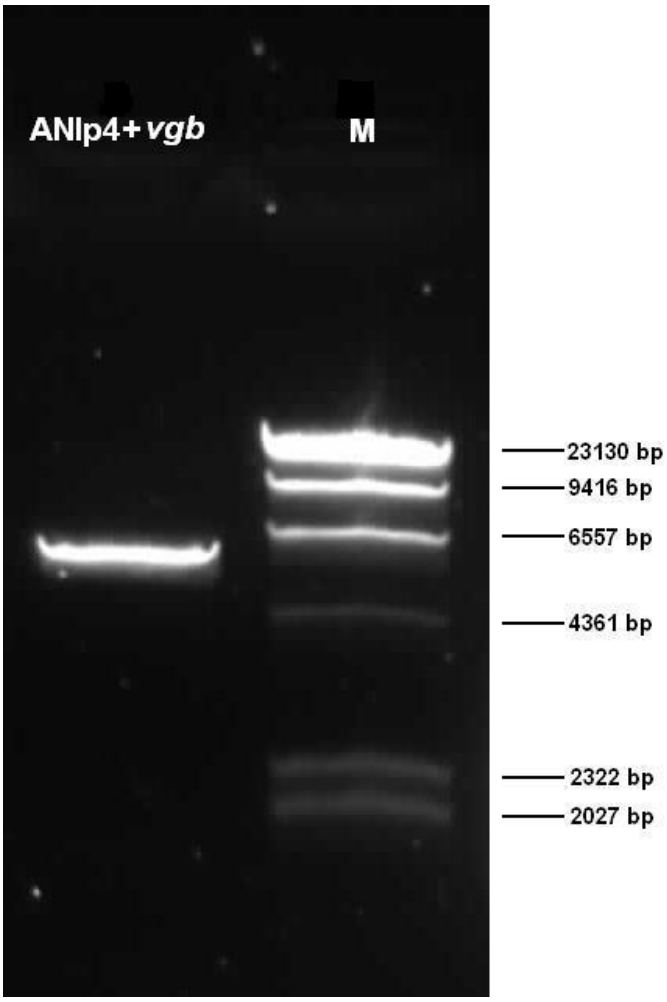


Figure 7.5. Agarose gel electrophrosis of transformant *E.coli*. Transformant *E.coli* cells were used to extract plasmid ANIp4+*vgb* by miniprep. Lane M indicates lambda DNA/HindIII marker.

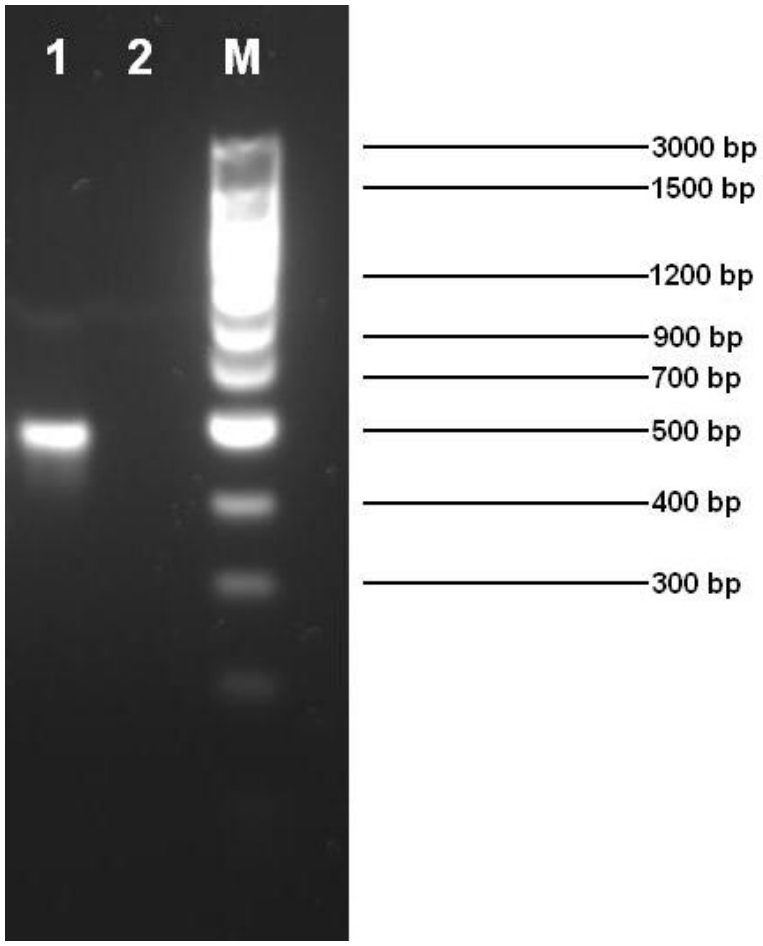


Figure 7.6. Agarose gel electrophrosis of PCR product from transformant *E.coli.* Plasmid samples extracted from *DH5 α E. coli* were used. FseI and NheI restriction enzymes were used to cut ANIp4+*vgb* plasmid to obtain *vgb* gene. After double digestion, samples were subjected to PCR and later agarose gel electrophoresis was performed. Lane 1 was the PCR product of double digested plasmid ANIp4+*vgb*. Lane1 indicates *vgb* gene band from transformed *E. coli*. Lane 2 indicates negative control. (In negative control water is used instead of DNA when PCR run is carried out.) Lane M indicates 100bp DNA ladder.

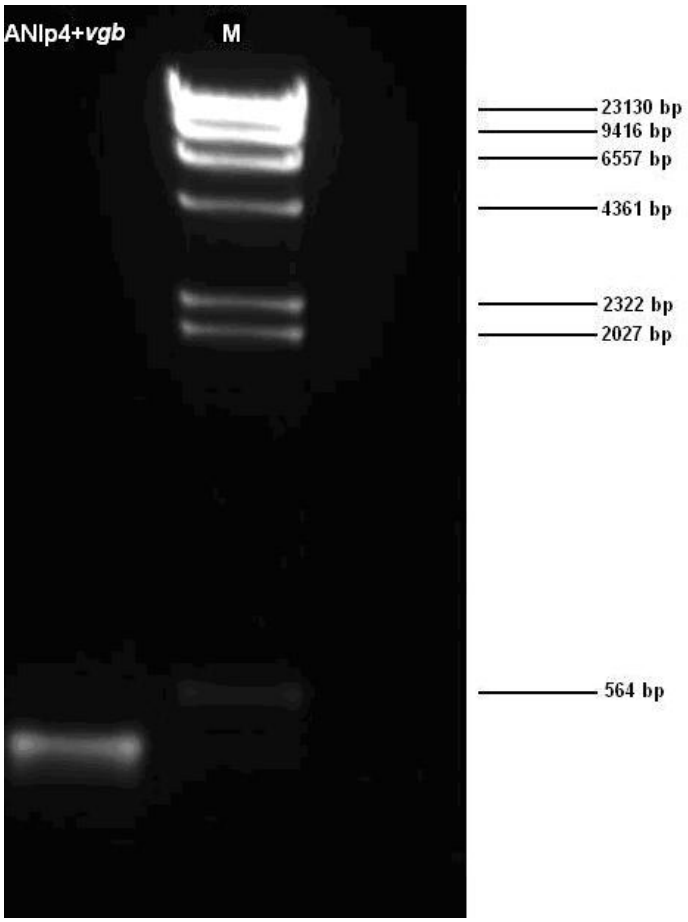


Figure 7.7. Agarose gel electrophrosis of PCR product from transformant *A.sojae*. After transformation of Mutant 7/2, the plasmid extraction was subjected to PCR procedure. Lane M is lambda DNA marker.

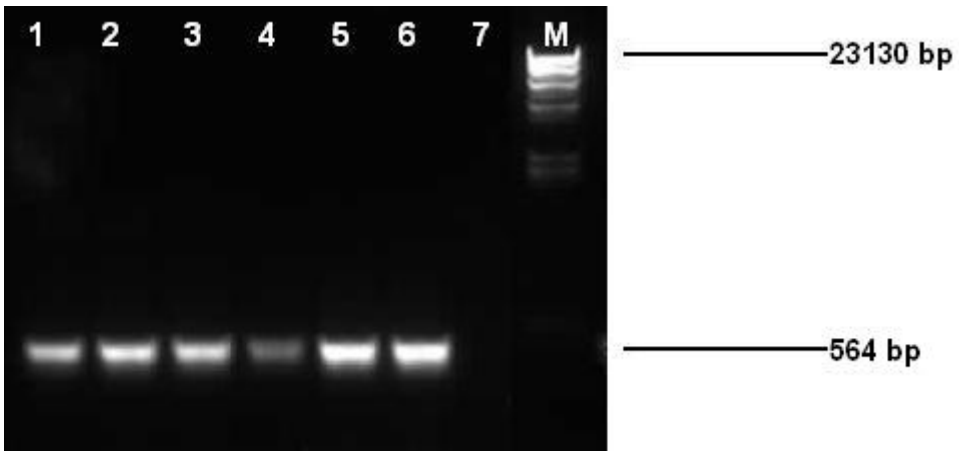


Figure 7.8. Agarose gel analysis of transformant *A. sojae* and M7/2. Lane 1 and 2 indicates the PCR product of TraGlux, lane 3 and 4 indicate PCR product of M7/2 (contamination observed), lane 5 and 6 PCR product of TraElectro.

7.3. Results of Sequence Analysis

In order to confirm and verify that both of the transformations performed by using Glucanex and electroporation technique were successful, sequence analysis of the transformed strains were performed. Sequence analysis was performed by Izmir Institute of Technology, Biotechnology and Bioengineering Central Research Laboratories with Applied Biosystems 3130xl DNA Sequencer with the extracted plasmid samples of TraGlux, TraElectro, M7/2 and *vgb* gene stock coming from Illinois Institute of Technology Department of Biological Sciences, USA. Plasmid samples were later subjected to PCR procedure and the PCR products were used as templates for sequence analysis.

Figures 7.9. the coding sequence for the PCR product of *vgb* stock coming from Illinois Institute of Technology Department of Biological Sciences, USA was given for comparison. The alignments of *vgb* sequence analysis results and output of the blast results from Finch TV program presented in Figures 7.10.and 7.11., respectively, confirmed that the stock *vgb* has an alignment of 99% with *Vitreoscilla* sp. hemoglobin gene (*vgb*), complete cds; and UvrA (uvrA) gene, partial cds. Similarly, in the figures 7.12. and 7.13. the transformation of *A. sojae* with *vgb* via glucanex has an alignment with *Vitreoscilla* sp. hemoglobin gene (*vgb*), complete cds; and UvrA (uvrA) gene,

partial cds of 100%. In figures 7.14.and 7.15. the alignment of TraElectro sequence result was 100%. However; when negative control was used with water and strain M 7/2 subjected to plasmid extraction procedure as a negative control and used for sequence analysis, the sequence results showed that the strain M7/2 and water sample had an alignment with *Vitreoscilla* hemoglobin gene (Figure 7.16., 7.17.). According to these results, a contamination with *vgb* was detected, and because of this contamination observed in strain M7/2, the transformation with *vgb* was not confirmed despite the fact that 100% alignment was observed for transformed strains. Further analysis showed that the contamination was born from the mastermix used in PCR procedure (Data not shown). Therefore, these analyses will be repeated using a new mastermix in future studies.

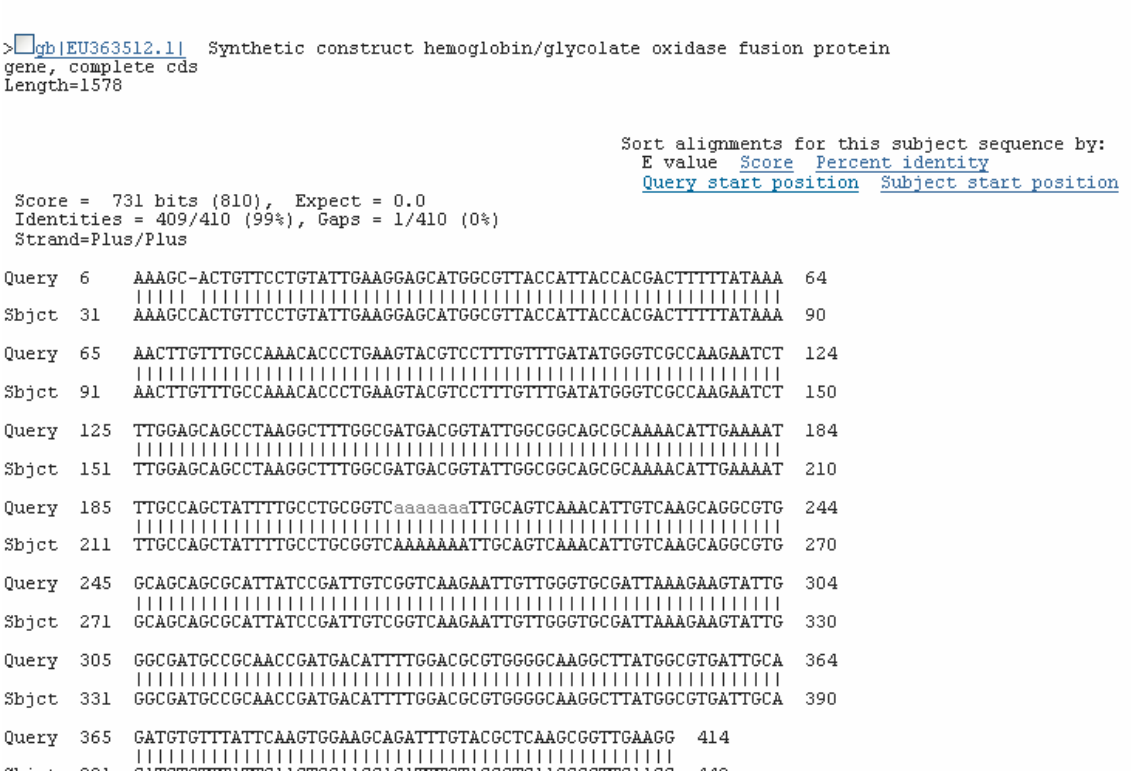
7.4. Sample 1: PCR Product *vgb* Gene Stock Coming from Illinois Institute of Technology Department of Biological Sciences, USA

AATCTAAAGCACTGTTCCTGTATTGAAGGAGCATGGCGTTACCATTA
CCACGACTTTTTATAAAAACTTGTTTGCCAAACACCCTGAAGTACGTC
CTTTGTTTGATATGGGTCGCCAAGAATCTTTGGAGCAGCCTAAGGCTT
TGGCGATGACGGTATTGGCGGCAGCGCAAAACATTGAAAATTTGCCA
GCTATTTTGCCTGCGGTCAAAAAAATTGCAGTCAAACATTGTCAAGC
AGGCGTGGCAGCAGCGCATTATCCGATTGTCGGTCAAGAATTGTTGG
GTGCGATTAAAGAAGTATTGGGCGATGCCGCAACCGATGACATTTTG
GACGCGTGGGGCAAGGCTTATGGCGTGATTGCAGATGTGTTTATTCA
AGTGGAAGCAGATTTGTACGCTCAAGCGGTTGAAGGCCGGCCATGAT
CAAGCCTGGTTGAAGGATCATGCGTTACCATTACCACGACTTTTTATA
AAAACTTGTTTGCCAAACACCCTGAAATACCGTCCTTTGTTTGATATG
GGTCGCCAAAGAATCCTTTGAAGCAGCCTAAG

Figure 7.9. Coding sequence for PCR product of *vgb* stock coming from Illinois Institute of Technology Department of Biological Sciences, USA

Table 7.2. Output of blast results from Finch TV program by using the sample of *vgb* gene stock. Alignment with Vitreoscilla sp. hemoglobin gene (*vgb*) , complete cds; and UvrA (uvrA) gene, partial cds is 99%.

Accession	Description	Max score	Total score	Query Coverage	E value	Max ident
EU363512.1	Synthetic construct hemoglobin/glycolate oxidase fusion protein gene	731	902	95%	0.0	99%
L21670.1	*Vitreoscilla sp.* hemoglobin (*vgb*) gene, complete cds; and UvrA (uvrA) gene, partial cds	728	898	95%	0.0	99%
AF292694.1	*Vitreoscilla sp.* HG1 hemoglobin (*vgb*) gene, complete cds	728	898	95%	0.0	99%



```
>gb|EU363512.1|  Synthetic construct hemoglobin/glycolate oxidase fusion protein
gene, complete cds
Length=1578

                                                    Sort alignments for this subject sequence by:
                                                      E value  Score  Percent identity
                                                      Query start position  Subject start position
 Score =  731 bits (810),  Expect = 0.0
 Identities = 409/410 (99%), Gaps = 1/410 (0%)
 Strand=Plus/Plus

Query  6    AAAGC-ACTGTTCCTGTATTGAAGGAGCATGGCGTTACCATTACCACGACTTTTTATAAA  64
            ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  31   AAAGCCACTGTTCCTGTATTGAAGGAGCATGGCGTTACCATTACCACGACTTTTTATAAA  90

Query  65   AACTTGTTTGCCAAACACCCTGAAGTACGTCCTTTGTTTGATATGGGTCGCCAAGAATCT  124
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  91   AACTTGTTTGCCAAACACCCTGAAGTACGTCCTTTGTTTGATATGGGTCGCCAAGAATCT  150

Query  125  TTGGAGCAGCCTAAGGCTTTGGCGATGACGGTATTGGCGGCAGCGCAAAACATTGAAAAT  184
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  151  TTGGAGCAGCCTAAGGCTTTGGCGATGACGGTATTGGCGGCAGCGCAAAACATTGAAAAT  210

Query  185  TTGCCAGCTATTTTGCCTGCGGTCaaaaaaaTTGCAGTCAAACATTGTCAAGCAGGCGTG  244
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  211  TTGCCAGCTATTTTGCCTGCGGTCAAAAAAATTGCAGTCAAACATTGTCAAGCAGGCGTG  270

Query  245  GCAGCAGCGCATTATCCGATTGTCGGTCAAGAATTGTTGGGTGCGATTAAAGAAGTATTG  304
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  271  GCAGCAGCGCATTATCCGATTGTCGGTCAAGAATTGTTGGGTGCGATTAAAGAAGTATTG  330

Query  305  GGCGATGCCGCAACCGATGACATTTTGGACGCGTGGGGCAAGGCTTATGGCGTGATTGCA  364
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  331  GGCGATGCCGCAACCGATGACATTTTGGACGCGTGGGGCAAGGCTTATGGCGTGATTGCA  390

Query  365  GATGTGTTTATTCAAGTGGAAGCAGATTTGTACGCTCAAGCGGTTGAAGG  414
            ||||||||||||||||||||||||||||||||||||||||||||||||||
```

Figure 7.10. Alignments of *vgb* gene sequence analysis result. The query sample sent for sequence analysis was for positive control since it is the original *vgb* gene stock coming from Illinois Institute of Technology Department of Biological Sciences, USA. Alignment with *Vitreoscilla sp.* hemoglobin gene (*vgb*) , complete cds; and UvrA (uvrA) gene, partial cds is 99%.

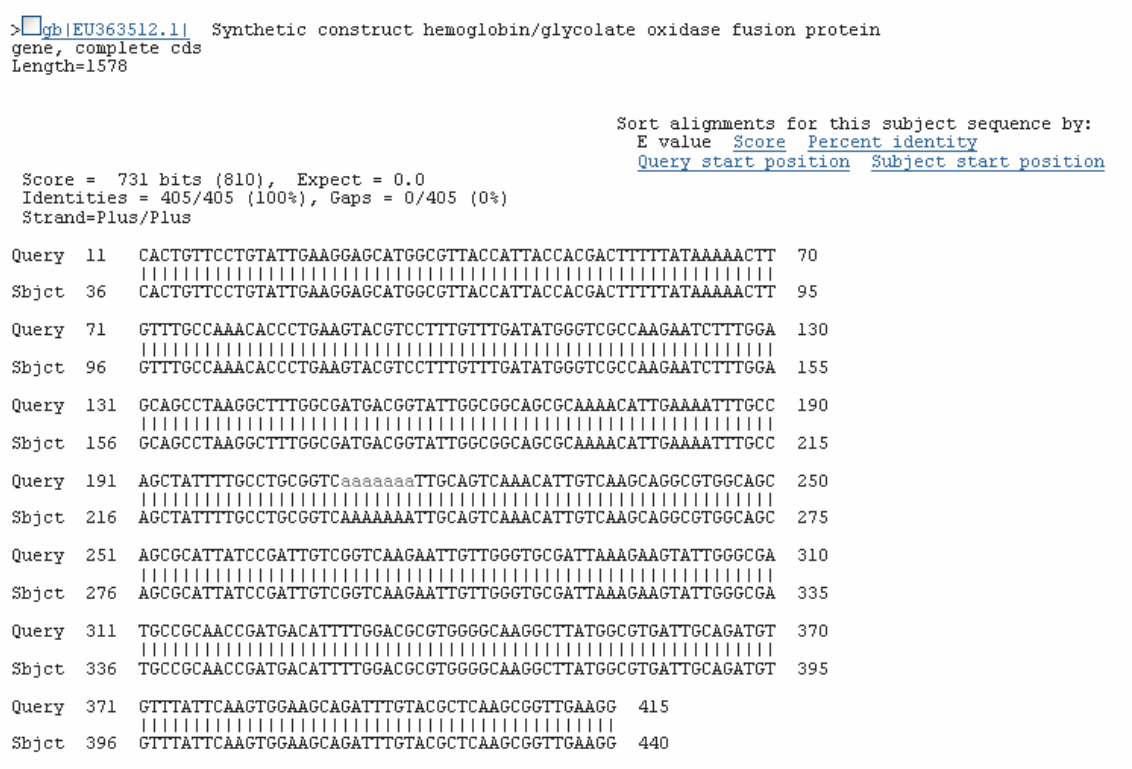
7.5. Sample 2: Transformed M 7/2 *A. sojae* Strain with Glucanex (TraGlux)

AACTCTCAAGCACTGTTCCTGTATTGAAGGAGCATGGCGTTACCATTAC
CACGACTTTTTATAAAAACTTGTTTGCCAAACACCCTGAAGTACGTCCT
TTGTTTGATATGGGTCGCCAAGAATCTTTGGAGCAGCCTAAGGCTTTGG
CGATGACGGTATTGGCGGCAGCGCAAAACATTGAAAATTTGCCAGCTA
TTTTGCCTGCGGTCAAAAAAATTGCAGTCAAACATTGTCAAGCAGGCG
TGGCAGCAGCGCATTATCCGATTGTCGGTCAAGAATTGTTGGGTGCGA
TTAAAGAAGTATTGGGCGATGCCGCAACCGATGACATTTTGGACGCGT
GGGGCAAGGCTTATGGCGTGATTGCAGATGTGTTTATTCAAGTGGAAG
CAGATTTGTACGCTCAAGCGGTTGAAGGCCGGCCATGATCAAGCCTGT
ATTGAAGGAGCATGGCGTTCCATTACCACCACTTTTTATAAAAACTTGT
TTGCCAAACCCCCTGAACTACCTCCTTTGTTTGATATGGGTCGCCCAAC
AATCTTTGGAGCACCTAAGGCTTTGGCGATGACGGTATTGGCGGCAGC
CCAAAACATTGAAAATTTTGCCAGTTATTTTTGCTGCGGTCAAAAAA

Figure 7.11.Coding sequence for strain TraGlux

Table 7.3. Alignments of strain TraGlux extracted plasmid sequence analysis result.

Accession	Description	Max score	Total score	Query Coverage	E value	Max ident
EU363512.1	Synthetic construct hemoglobin/glycolate oxidase fusion protein gene	731	1019	96%	0.0	100%
L21670.1	*Vitreoscilla* sp. hemoglobin (*vgb*) gene, complete cds; and UvrA (uvrA) gene, partial cds	728	1016	95%	0.0	100%
AF292694.1	*Vitreoscilla sp*. HG1 hemoglobin (*vgb*) gene, complete cds	728	1016	95%	0.0	100%



```
>gb|EU363512.1|  Synthetic construct hemoglobin/glycolate oxidase fusion protein
gene, complete cds
Length=1578

                                                    Sort alignments for this subject sequence by:
                                                      E value  Score  Percent identity
                                                      Query start position  Subject start position
 Score =  731 bits (810),  Expect = 0.0
 Identities = 405/405 (100%), Gaps = 0/405 (0%)
 Strand=Plus/Plus

Query  11   CACTGTTCCTGTATTGAAGGAGCATGGCGTTACCATTACCACGACTTTTTATAAAAACTT  70
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  36   CACTGTTCCTGTATTGAAGGAGCATGGCGTTACCATTACCACGACTTTTTATAAAAACTT  95

Query  71   GTTTGCCAAACACCCTGAAGTACGTCCTTTGTTTGATATGGGTCGCCAAGAATCTTTGGA  130
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  96   GTTTGCCAAACACCCTGAAGTACGTCCTTTGTTTGATATGGGTCGCCAAGAATCTTTGGA  155

Query  131  GCAGCCTAAGGCTTTGGCGATGACGGTATTGGCGGCAGCGCAAAACATTGAAAATTTGCC  190
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  156  GCAGCCTAAGGCTTTGGCGATGACGGTATTGGCGGCAGCGCAAAACATTGAAAATTTGCC  215

Query  191  AGCTATTTTGCCTGCGGTCaaaaaaaTTGCAGTCAAACATTGTCAAGCAGGCGTGGCAGC  250
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  216  AGCTATTTTGCCTGCGGTCAAAAAAATTGCAGTCAAACATTGTCAAGCAGGCGTGGCAGC  275

Query  251  AGCGCATTATCCGATTGTCGGTCAAGAATTGTTGGGTGCGATTAAAGAAGTATTGGGCGA  310
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  276  AGCGCATTATCCGATTGTCGGTCAAGAATTGTTGGGTGCGATTAAAGAAGTATTGGGCGA  335

Query  311  TGCCGCAACCGATGACATTTTGGACGCGTGGGGCAAGGCTTATGGCGTGATTGCAGATGT  370
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  336  TGCCGCAACCGATGACATTTTGGACGCGTGGGGCAAGGCTTATGGCGTGATTGCAGATGT  395

Query  371  GTTTATTCAAGTGGAAGCAGATTTGTACGCTCAAGCGGTTGAAGG  415
            |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  396  GTTTATTCAAGTGGAAGCAGATTTGTACGCTCAAGCGGTTGAAGG  440
```

Figure 7.12. Out put of blast results from Finch TV program by using the sample of strain TraGlux. Alignment with *Vitreoscilla sp*. hemoglobin gene (*vgb*), complete cds; and UvrA (uvrA) gene, partial cds is 100%.

7.6. Sample3: Transformed M 7/2 *A. sojae* Strain with Electroporation (TraElectro)

AATACTCAAGCACTGTTCCTGTATTGAAGGAGCATGGCGTTACCATTAC
CACGACTTTTTATAAAAACTTGTTTGCCAAACACCCTGAAGTACGTCCT
TTGTTTGATATGGGTCGCCAAGAATCTTTGGAGCAGCCTAAGGCTTTGG
CGATGACGGTATTGGCGGCAGCGCAAAACATTGAAAATTTGCCAGCTA
TTTTGCCTGCGGTCAAAAAAATTGCAGTCAAACATTGTCAAGCAGGCG
TGGCAGCAGCGCATTATCCGATTGTCGGTCAAGAATTGTTGGGTGCGA
TTAAAGAAGTATTGGGCGATGCCGCAACCGATGACATTTTGGACGCGT
GGGGCAAGGCTTATGGCGTGATTGCAGATGTGTTTATTCAAGTGGAAG
CAGATTTGTACGCTCAAGCGGTTGAAGGCCGGCCATGATCA

Figure 7.13 Alignments of strain TraElectro extracted plasmid sequence analysis result.

Table 7.4. Coding sequence for the sample of transformed *A. sojae* with electroporation (TraElectro).

Accession	Description	Max score	Total score	Query Coverage	E value	Max ident
EU363512.1	Synthetic construct hemoglobin/glycolate oxidase fusion protein gene	731	731	94%	0.0	100%
L21670.1	*Vitreoscilla sp*. hemoglobin (*vgb*) gene, complete cds; and UvrA (uvrA) gene, partial cds	728	728	94%	0.0	100%
AF292694.1	*Vitreoscilla sp*. HG1 hemoglobin (*vgb*) gene, complete cds	728	728	94%	0.0	100%

```
>gb|EU363512.1|  Synthetic construct hemoglobin/glycolate oxidase fusion protein
gene, complete cds
Length=1578

 Score =  731 bits (810),  Expect = 0.0
 Identities = 405/405 (100%), Gaps = 0/405 (0%)
 Strand=Plus/Plus

Query  11   CACTGTTCCTGTATTGAAGGAGCATGGCGTTACCATTACCACGACTTTTTATAAAAACTT  70
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  36   CACTGTTCCTGTATTGAAGGAGCATGGCGTTACCATTACCACGACTTTTTATAAAAACTT  95

Query  71   GTTTGCCAAACACCCTGAAGTACGTCCTTTGTTTGATATGGGTCGCCAAGAATCTTTGGA  130
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  96   GTTTGCCAAACACCCTGAAGTACGTCCTTTGTTTGATATGGGTCGCCAAGAATCTTTGGA  155

Query  131  GCAGCCTAAGGCTTTGGCGATGACGGTATTGGCGGCAGCGCAAAACATTGAAAATTTGCC  190
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  156  GCAGCCTAAGGCTTTGGCGATGACGGTATTGGCGGCAGCGCAAAACATTGAAAATTTGCC  215

Query  191  AGCTATTTTGCCTGCGGTCaaaaaaaTTGCAGTCAAACATTGTCAAGCAGGCGTGGCAGC  250
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  216  AGCTATTTTGCCTGCGGTCAAAAAAATTGCAGTCAAACATTGTCAAGCAGGCGTGGCAGC  275

Query  251  AGCGCATTATCCGATTGTCGGTCAAGAATTGTTGGGTGCGATTAAAGAAGTATTGGGCGA  310
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  276  AGCGCATTATCCGATTGTCGGTCAAGAATTGTTGGGTGCGATTAAAGAAGTATTGGGCGA  335

Query  311  TGCCGCAACCGATGACATTTTGGACGCGTGGGGCAAGGCTTATGGCGTGATTGCAGATGT  370
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  336  TGCCGCAACCGATGACATTTTGGACGCGTGGGGCAAGGCTTATGGCGTGATTGCAGATGT  395

Query  371  GTTTATTCAAGTGGAAGCAGATTTGTACGCTCAAGCGGTTGAAGG  415
            |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  396  GTTTATTCAAGTGGAAGCAGATTTGTACGCTCAAGCGGTTGAAGG  440
```

Figure 7.14 Out put of blast results from Finch TV program by using TraElectro. Alignment with *Vitreoscilla sp*. hemoglobin (*vgb*) gene, complete cds; and UvrA (uvrA) gene, partial cds is 100%.

7.7. Sample 4: Mutant 7/2 *Aspergillus sojae*

AATCTAAAGCACTGTTCCTGTATTGAAGGAGCATGGCGTTACCATTACCA
CGACTTTTTATAAAAACTTGTTTGCCAAACACCCTGAAGTACGTCCTTTG
TTTGATATGGGTCGCCAAGAATCTTTGGAGCAGCCTAAGGCTTTGGCGAT
GACGGTATTGGCGGCAGCGCAAAACATTGAAAATTTGCCAGCTATTTTG
CCTGCGGTCAAAAAAATTGCAGTCAAACATTGTCAAGCAGGCGTGGCAG
CAGCGCATTATCCGATTGTCGGTCAAGAATTGTTGGGTGCGATTAAAGA
AGTATTGGGCGATGCCGCAACCGATGACATTTTGGACGCGTGGGGCAAG
GCTTATGGCGTGATTGCAGATGTGTTTATTCAAGTGGAAGCAGATTTGTA
CGCTCAAGCGGTTGAAGGCCGGCCATGATCAAGCCTGGTTGAAGGATCA
TGCGTTACCATTACCACGACTTTTTATAAAAACTTGTTTGCCAAACACCC
TGAAATACCGTCCTTTGTTTGATATGGGTCGCCAAAGAATCCTTTGAAGC
AGCCTAAG

Figure 7.15.Coding sequence for Mutant 7/2 *Aspergillus sojae.*

Table 7.5. Alignments of Mutant M7/2 *Aspergillus sojae*

Accession	**Description**	**Max score**	**Total score**	**Query Coverage**	**E value**	**Max ident**
EU363512.1	Synthetic construct hemoglobin/glycolate oxidase fusion protein gene	731	902	95%	0.0	99%
L21670.1	*Vitreoscilla sp*. hemoglobin (*vgb*) gene, complete cds; and UvrA (uvrA) gene, partial cds	728	898	95%	0.0	99%
AF292694.1	*Vitreoscilla sp*. HG1 hemoglobin (*vgb*) gene, complete cds	728	898	95%	0.0	99%

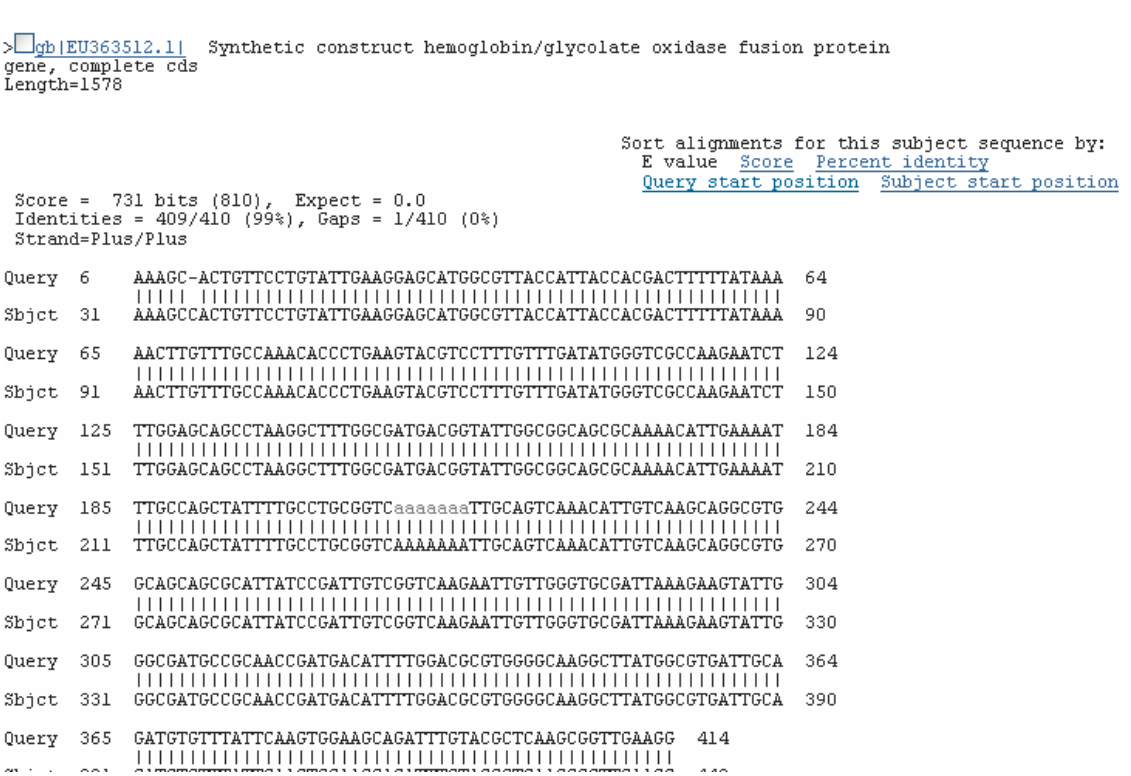


Figure 7.16. Output of blast results from Finch TV program by using the sample of Mutant 7/2 *Aspergillus sojae*. There is alignment with *Vitreoscilla* hemoglobin gene (indicating contamination).

7.8. Sample 5: Negative Control with Water

ATCTCAAGCACTGTTCCTGTATTGAAGGAGCATGGCGTTACCATTA
CCACGACTTTTTATAAAAACTTGTTTGCCAAACACCCTGAAGTACGT
CCTTTGTTTGATATGGGTCGCCAAGAATCTTTGGAGCAGCCTAAGG
CTTTGGCGATGACGGTATTGGCGGCAGCGCAAAACATTGAAAATTT
GCCAGCTATTTTGCCTGCGGTCAAAAAAATTGCAGTCAAACATTGT
CAAGCAGGCGTGGCAGCAGCGCATTATCCGATTGTCGGTCAAGAAT
TGTTGGGTGCGATTAAAGAAGTATTGGGCGATGCCGCAACCGATGA
CATTTTGGACGCGTGGGGCAAGGCTTATGGCGTGATTGCAGATGTG
TTTATTCAAGTGGAAGCAGATTTGTACGCTCAAGCGGTTGAAGGCC
GGCCATGATCAAACCTGTATTGAAGGAGCATGGCGTTACCATTACC
ACGACTTTTTATAAAAACTTGTTTGCCAAACACCCTGAAGTACGTCC
TTTGTTTT

Figure 7.17.Coding sequence for negative control with water.

Table 7.6. Alignments of negative control with water.

Accession	Description	Max score	Total score	Query Coverage	E value	Max ident
EU363512.1	Synthetic construct hemoglobin/glycolate oxidase fusion protein gene	731	889	95%	0.0	100%
L21670.1	*Vitreoscilla s*p. hemoglobin (*vgb*) gene, complete cds; and UvrA (uvrA) gene, partial cds	728	886	94%	0.0	100%
AF292694.1	*Vitreoscilla sp.* HG1 hemoglobin (*vgb*) gene, complete cds	728	886	94%	0.0	100%

```
>gb|EU363512.1|  Synthetic construct hemoglobin/glycolate oxidase fusion protein
gene, complete cds
Length=1578

 Score =  731 bits (810),  Expect = 0.0
 Identities = 409/410 (99%), Gaps = 1/410 (0%)
 Strand=Plus/Plus

Query  7    AAAGC-ACTGTTCCTGTATTGAAGGAGCATGGCGTTACCATTACCACGACTTTTTATAAA  65
            ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  31   AAAGCCACTGTTCCTGTATTGAAGGAGCATGGCGTTACCATTACCACGACTTTTTATAAA  90

Query  66   AACTTGTTTGCCAAACACCCTGAAGTACGTCCTTTGTTTGATATGGGTCGCCAAGAATCT  125
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  91   AACTTGTTTGCCAAACACCCTGAAGTACGTCCTTTGTTTGATATGGGTCGCCAAGAATCT  150

Query  126  TTGGAGCAGCCTAAGGCTTTGGCGATGACGGTATTGGCGGCAGCGCAAAACATTGAAAAT  185
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  151  TTGGAGCAGCCTAAGGCTTTGGCGATGACGGTATTGGCGGCAGCGCAAAACATTGAAAAT  210

Query  186  TTGCCAGCTATTTTGCCTGCGGTCaaaaaaaTTGCAGTCAAACATTGTCAAGCAGGCGTG  245
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  211  TTGCCAGCTATTTTGCCTGCGGTCAAAAAAATTGCAGTCAAACATTGTCAAGCAGGCGTG  270

Query  246  GCAGCAGCGCATTATCCGATTGTCGGTCAAGAATTGTTGGGTGCGATTAAAGAAGTATTG  305
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  271  GCAGCAGCGCATTATCCGATTGTCGGTCAAGAATTGTTGGGTGCGATTAAAGAAGTATTG  330

Query  306  GGCGATGCCGCAACCGATGACATTTTGGACGCGTGGGGCAAGGCTTATGGCGTGATTGCA  365
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  331  GGCGATGCCGCAACCGATGACATTTTGGACGCGTGGGGCAAGGCTTATGGCGTGATTGCA  390

Query  366  GATGTGTTTATTCAAGTGGAAGCAGATTTGTACGCTCAAGCGGTTGAAGG  415
            ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  391  GATGTGTTTATTCAAGTGGAAGCAGATTTGTACGCTCAAGCGGTTGAAGG  440
```

Figure 7.18. Output of blast results from Finch TV program by using the sample of negative control with water.

7.9. Agar Diffusion Method for Detection of Pectinase Production Using Polygalacturonic Acid

The reason in performing the agar diffusion test was to determine the effect of the mutation on the PG production ability. The idea was either not to adversely effect or to enhance the PG production caused by the mutations, which were necessary in order

to select the uridine negative mutants that could be used in the future transformation of *A. sojae* with the *vgb.* Therefore, the best possible potent isolate was selected among the mutants and coded as M7/2 and used as host train for the transformation. This strain was compared with the other strains (control M5/6, wild type M7 and the transformed strains TraGlux and TraElectro) used in the study. The PG production ability of the strains was compared based on the average ratio of the diameters of clearance zones to the colony diameters measured. According to the results, control strains M 5/6 and M 7 showed higher ratio (Table 7.7 and 7.8; Figure 7.19 and 7.20). It can be concluded, that the mutation caused, resulted into a slight decrease in PG activity. Therefore, the actual comparison among the strains should be done among M7 and M7/2 in order to observe the effect of mutation and among M7/2, TraGlux and TraElectro in order to observe the effect of transformation. Similarly, PG activity based on the clear zones indicated, that strain TraElectro exhibited higher PG activity compared to *vgb* host strain M7/2 and TraGlux strain.

In general agar diffusion method is considered as a fast, cheap and an easy technique compared to analytical methods, in spite of the fact that it doesn't give exact activity results. As a result this technique is a simple technique, which should be only used as prelimary method for the selection of isolates, where other techniques are difficult to conduct under the same conditions. The individual pictures and their clear zone measurements are given in Table 7.7.-7.11. and Figure 7.19-7.23., respectively.

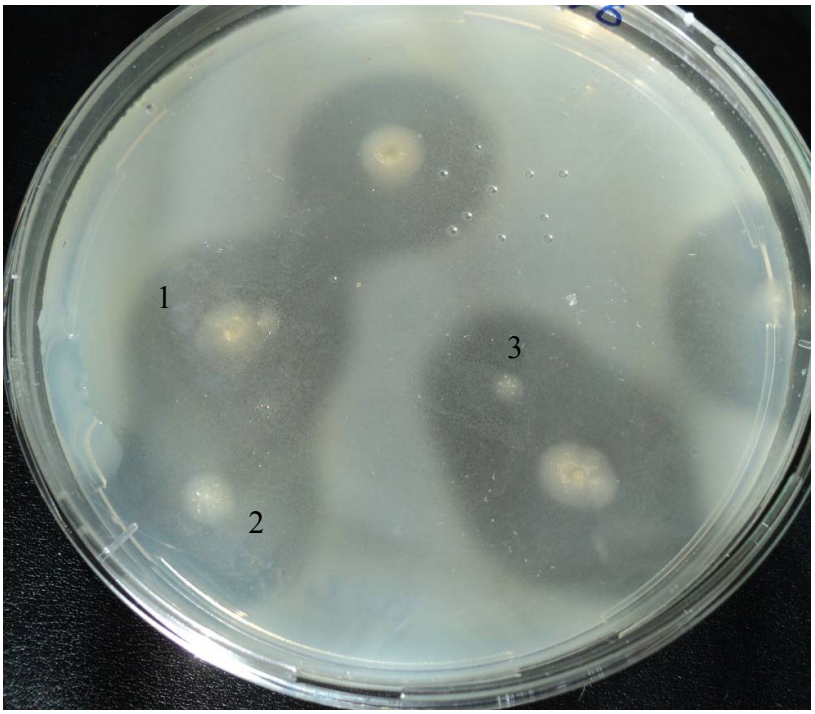


Figure 7.19. Solid media containing polygalacturonic acid (PGA) for detection of enzyme production of the control strain M5/6.

Table 7.7. Ratio of the diameters of clearance zones of the control strain M 5/6.

Colony	Clearance Zone/Colony (cm)	Ratio (cm)	Average Ratio (cm)
1	3,23/0,924	3,49	4,05
2	3,20/0,62	5.13	
3	3,35/0,95	3,53	

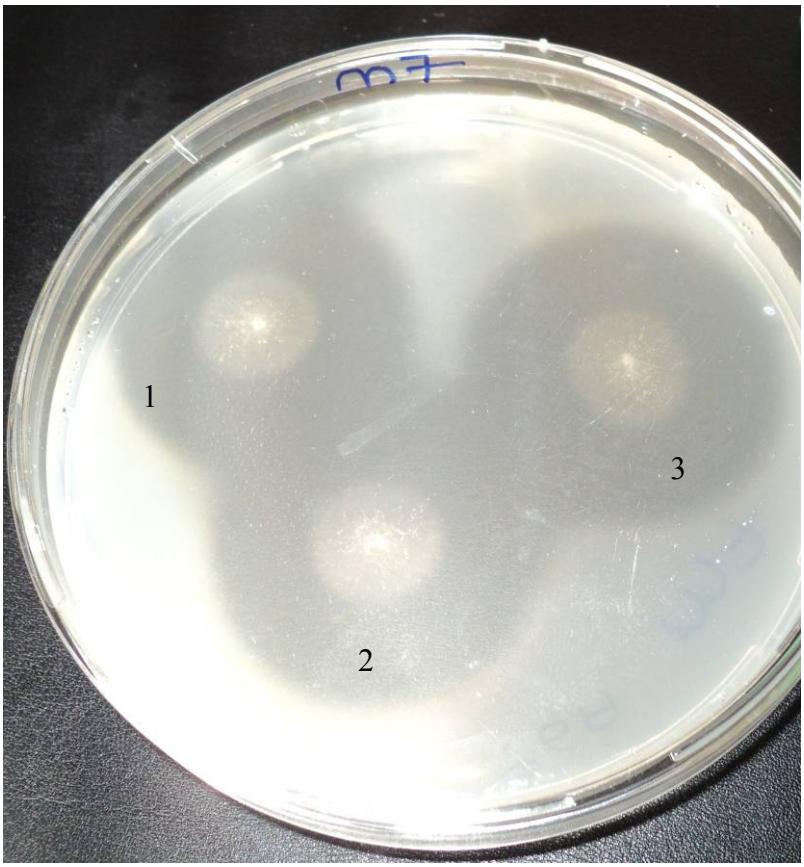


Figure 7.20. Solid media containing polygalacturonic acid (PGA) for detection of enzyme production of the wild type strain M7.

Table 7.8. Ratio of the diameters of clearance zones of the wild type strain M7

Colony	Clearance Zone/Colony (cm)	Ratio (cm)	Average Ratio (cm)
1	4,092/1,53	2,66	2,626
2	4,54/1,74	2,614	
3	4,18/1,60	2,612	

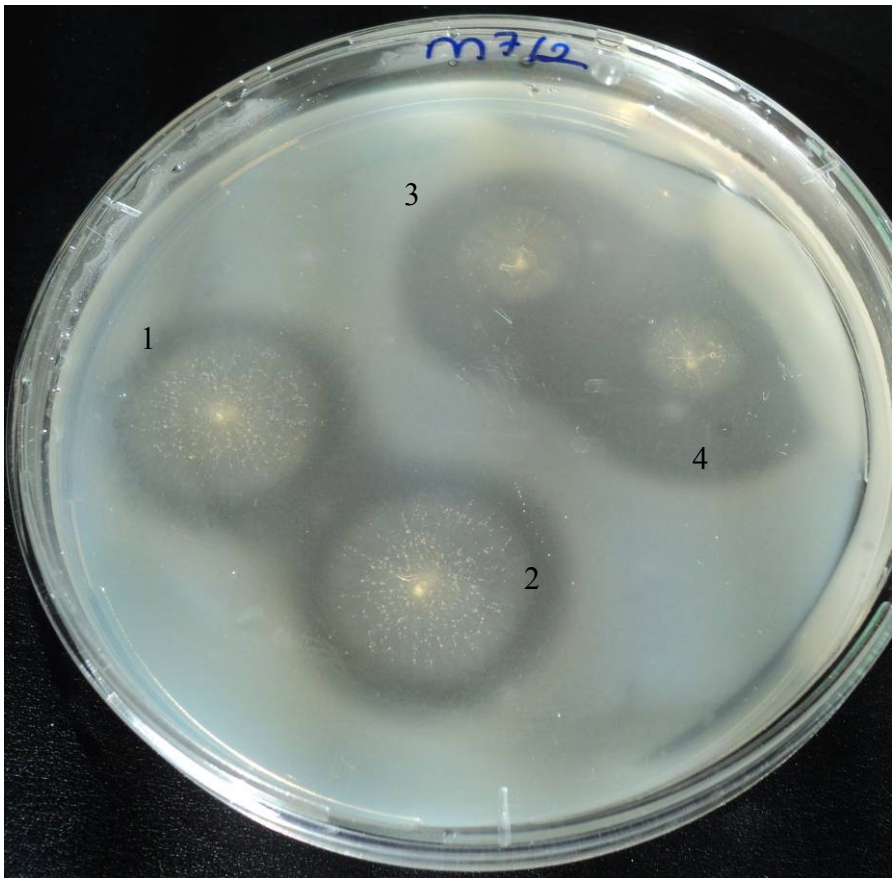


Figure 7.21. Solid media containing polygalacturonic acid (PGA) for detection of enzyme production of the mutated strain M7/2

Table 7.9. Ratio of the diameters of clearance zones of the mutated strain M7/2

Colony	Clearance Zone/Colony (cm)	Ratio (cm)	Average Ratio (cm)
1	3,11/2,15	1,44	1,88
2	3,20/2,67	1,19	
3	2,84/1,39	2,03	
4	3,11/1,13	2,73	

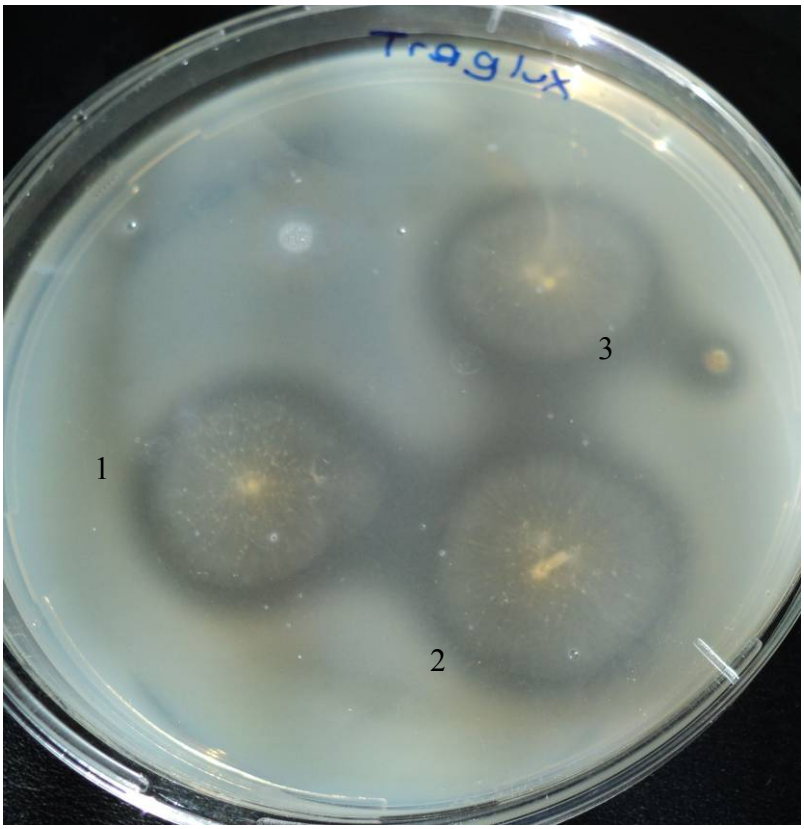


Figure 7.22. Solid media containing polygalacturonic acid (PGA) for detection of enzyme production of transformed strain TraGlux

Table 7.10. Ratio of the diameters of clearance zones to TraGlux colonies and theirs average ratios

Colony	Clearance Zone/Colony (cm)	Ratio (cm)	Average Ratio (cm)
1	2,89/2,58	1,12	1,066
2	3,06/2,78	1,10	
3	2,45/2,35	1,04	

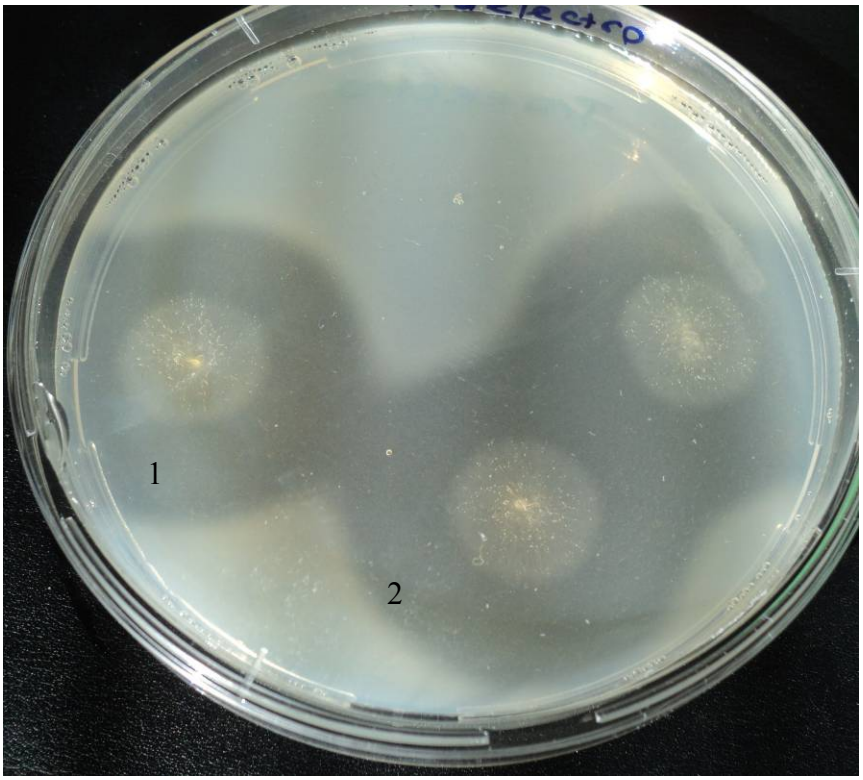


Figure 7.23. Solid media containing polygalacturonic acid (PGA) for detection of enzyme production from spore solution of strain TraElectro

Table 7.11. Ratio of the diameters of clearance zones to TraElectro colonies and their average ratios

Colony	Clearance Zone/Colony (cm)	Ratio (cm)	Average Ratio (cm)
1	4,18/2,14	1,95	2,19
2	4,85/1,98	2,44	

Table 7.12. Average ratio of the diameters of clearance zones to colonies of strains used.

Colony	Average Ratio
M 5/6	4,05
M 7	2,626
M 7/2	1,88
TraGlux	1,066
TraElectro	2,19

CHAPTER 8

CONCLUSION

In this study, the preliminary results of transformation of *Aspergillus sojae* with *vgb* were obtained. ANIp4 shuttle vector was used as a vector in order to transform *A. sojae*. For the selection of transformed *A. sojae* cells, uridine auxotrophic mutants were tried to be selected after UV mutagenesis. However, using a procedure based on the selection of uridine supported growth did not result in *A. sojae* pyrG mutants. Further studies will need to be performed in order to obtain pyrG mutants based on FOA direct selection in addition to uridine and uracil supplements in the growth medium. Also gene replacement with homolog recombination will be used in order to obtain uridine auxotroph strains as an alternative method to UV mutation.

In this study, the transformation of *A. sojae* with *vgb* was tried using both methods of electroporation and protoplasting and tested by sequence analysis and agarose gel electrophoresis. (Alignment with *Vitreoscilla* sp. hemoglobin gene (*vgb*), complete cds; and UvrA (uvrA) gene, partial cds was for strains TraElectro and TraGlux were 100%). Protoplast preparation is a problematic method because in literature fungal protoplasting via enzymes were usually prepared by using the enzyme Novozyme 234 however, this enzyme is no longer available for sale (De Bekker, et al., 2009). According to this knowledge a less common used enzyme glucanex was used in order to prepare *A. sojae* protoplasts.

The *A. sojae* strains used in this study were: Strain M5/6 and M7 which were initially mutated in order to improve the production of exo-polygalacturonase using the wild type *Aspergillus sojae*. Strain M7/2 was UV mutated from strain M7 in order to obtain pyrG negative strains within the scope of this thesis project. Strain TraGlux was obtained by transformation of M7/2 with *vgb* via protoplasting method. Strain TraElectro was obtained by transformation of M7/2 with *vgb* gene via electroporation method; however both strains could not be confirmed due to the contamination occurred in PCR process which carried the contamination to the sequence analysis. In order to obtain the effect of *vgb* gene, M7/2 was compared with TraElectro and TraGlux by PGA zone measurements since this strain was the untransformed strain with the rest of

the characteristics similar to the transformed ones. Other strains (M5/6 and M7) were subjected to PGA zone mearurement analysis as potential control strains in order to compare the effectiveness of the mutation and the transformation process, as they were studied routinely in our laboratuvary. This indicated that *vgb* has a positive affect on PG activity. If TraGlux and TraElectro were compared separately, it was seen that TraElectro had about 2 times higher PG activity compared to TraGlux.

In the future studies the expession of *vgb* will be tested using the methods CO-difference spectra, SDS-PAGE and western blotting analysis. The effect of *vgb* expression will be evaluated in submerged fermentation and the optimum conditions under microaerophilic situations will be determined for the maximum exo-polygalacturonase production.

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APPENDIX A

CHEMICAL USED IN EXPERIMENTS

CHEMICAL	CODE
Velvet Pad for Replica Plating	MP Biomedicals 5000-007
Ammonium Sulphate ($(NH_4)_2SO_4$)	Applichem A1032,0500
Agar-Agar	Applichem A0949
Agarose Low EEO	Applichem A2114,0250
Ammonium Heptamolypbdate Tetrahydrate	Merck 1.01182
Ampicillin	Sigma A 9393-5g
Brain heart infusion agar(BHI)	Fluka 70138
Calcium Choloride Dihydrate ($CaCl_2$)	Riedel-De Haën 31307
Coomassie brilliant blue	G-250 Fluka 27815
Copper (II) sulfate-5-hydrate	Riedel-De Haën 12849
$CuSO_4.5H_2O$ Copper (II) sulphate-5-hydrate	Riedel 1kg 12849
D (+)-Glucose	Fluka 49150 1 kg
Disodium Hydrogen Arsenate Heptahydrate	Applichem A3666
EcoRI	Fermentas FD0274
Disodiumsalt(EDTA) (Standart Volumetric Solution 0,1M)	Applichem A 1505,0500
Ethanol	Applichem A2937
$FeSO_4.7H_2O$ (Iron (11) Sulfate Heptahydrate)	Riedel-De Haën 1 kg
FseI	Fermentas
D-Galacturonic acid	Fluka 48280

Gel Stain Gel Star 10000 A Concent	**Lonza Zx 250 ml**
Gene Ruler 100bp DNA Ladder Plus (Fermentas	**50 mg.sm 1F3**
D-(+)-Galacturanicacid	**Fluka 48280 25g**
Glucanex (Lysing Enzymes from *Trichoderma harzianum*)	**Sigma L 1412-10g**
Glycerol	**Sigma G5516**
Boric Acid (H_3BO_3)	**Sigma B 6768-500g**
Hydrochloric acid (HCl)	**Riedel-De Haën**
HindIII Enzyme	**Fermantas FD0504**
Isopropanol	**Riedel-De Haën**
Potassium Chloride (KCI)	**Riedel-De Haën 31248**
Potassium Phosphate Monobasic (KH_2PO_4)	**Riedel-De Haën 1 KG**
Lambda DNA	**Fermentas**
Loading Dye	**Fermentas**
Maltrin	**Pendik Nişasta (Nişkoz H. Maltoz 41521)**
PCR Maxtermix (2X)	**Fermentas 00050331**
Magnesium chloride anhydrous ($MgCI_2$)	**Fluka 63063**
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	**Merck 1.05886**
Manganese (II) Chloride Tetrahydrate ($MnCI_2.4H_2O$)	**Merck 1.05886**
Manganese (II) Sulphate Monohydrate ($MnSO_4.4H_2O$)	**Riedel-De Haën**
Molasses	**Merck 1.02786**
Molybdenium (IV) Oxyde (MoO_3)	**Merck 1.00401.0250**
Sodium Carbonate Anhydrous(Na_2CO_3)	**Applichem A3900.1000**
di-Sodium Hydrogen Phosphate ($Na_2HPO_4.2H_2O$)	**Fluka 71662 1 kg**
Sodium Sulphate (Na_2SO_4)	**Merck 1.06649.1000**

Sodium Chloride (NaCI)	**Riedel-De Haën 13423**
$NaH_2PO_4.2H_2O$	**Riedel-De Haën**
Sodium bicarbonate ($NaHCO_3$)	**Sigma S 8875 500g**
Sodium Nitrate ($NaNO_3$)	**Applichem A 3911.1000**
Sodium Hydroxide (NaOH)	**Merck 1 kg 1.06462.1000**
NheI	**Fermentas FD0973**
PEG 4000	**Applichem**
Peptone	**Merck 1.07214.9999**
Polygalacturonic Acid	**Fluka 81325 50g**
Potassium Sodium Tartate tetrahydrate ($C_4H_4KNaO_6.4H_2O$)	**Sigma S6170**
Qiagen Miniprep Kit	**Quiagen**
Trizma Base	**Sigma T-8404 1kg**
Tryptone	**Applichem A1553.1000**
Tween-80	**Merck 8.22187.0500**
Uridine	**Applichem A0666.0010**
Yeast Extract	**Fluka 70161**
$ZnSO_4.7H_2O$	**Merck 1.03753**

APPENDIX B

MEDIA

B.1. LB Agar Medium

Tryptone	(10 g/l)
Yeast extract	(5 g/l)
NaCI	(10 g/l)
Deionized water	(1000 ml)
Agar	(15 g/ml)

Ingredients were dissolved in 900 ml distilled water by stirring with heating and made up to 1000ml. pH was adjusted to 7,5. Medium was sterilised by autoclaving at 121°C for 15 minutes.

B.2. LB Broth Medium

Tryptone	(10 g/l)
Yeast extract	(5 g/l)
NaCI	(10 g/l)
Deionized water	(1000 ml)

Ingredients were dissolved in 900 ml distilled water by stirring with gentle heating and made up to 1000 ml. pH was adjusted to 7,5. Medium was sterilized by autoclaving at 121°C for 15 minutes.

B.4. LB Agar Medium Supplemented with Ampicillin

Tryptone	(10 g/l)
Yeast extract	(5 g/l)
NaCI	(10 g/l)
Ampicillin	(50 μg/ml)
Deionized water	(1000 ml)
Agar	(15 g/ml)

Ingredients except ampicillin were dissolved in 900 ml distilled water by stirring with heating and made up to 1000 ml. pH was adjusted to 7,5. Medium was sterilized by autoclaving at 121°C for 15 minutes. Ampicillin 100 mg/ml stock solution (see Appendix C) was added to 50μg/ml final concentration after autoclaving.

B.5. LB Broth Medium Supplemented with Ampicillin

Tryptone	(10 g/l)
Yeast extract	(5 g/l)
NaCI	(10 g/l)
Ampicillin	(50 μg/ml)
Deionized water	(1000 ml)

Ingredients except ampicillin were dissolved in 900 ml distilled water by stirring with gentle heating and made up to 1000ml. pH was adjusted to 7,5. Medium was sterilized by autoclaving at 121°C for 15 minutes. Ampicillin 100 mg/ml stock solution (see Appendix C) was added to 50μg/ml final concentration after autoclaving.

B.6. YME (Yeast Malt Extract)

Malt extract	(10 g/l)
Yeast extract	(4 g/l)

Glucose	(4 g/l)
Agar	(20 g/l)

Ingredients were dissolved in 900 ml distilled water by stirring with gentle heating and made up to 1000ml. Medium was sterilized by autoclaving at 121°C for 15 minutes.

B.7.Minimal Agar Medium

$NaNO_3$	(6 g/l)
KCI	(0.52 g/l)
$MgSO_4.7H_2O$	(0.52 g/l)
KH_2SO_4	(1.52 g/l)
D-Glucose	(10 g/l)
Hunter's Trace Elements Solution	(2 ml/l)
Agar	(15 g/l)

Hunter's Trace Elements Solution (In 100 ml of water)

$ZnSO_4.7H_2O$ (2.2 g)
H_3BO_3 (1.1 g)
$MnCI_2.4H_2O$ (0.5 g)
$FeSO_4.7H_2O$ (0.5 g)
$CoCI_2.6H_2O$ (0.16 g)
$CuSO_4.5H_2O$ (0.16 g)
$NH_4Mo_7O_{24}.4H_2O$ (0.11 g)
EDTA. (5.0 g)

In order to prepare Hunter's Trace Elements Solution, all ingredients were mixed and pH was adjusted to 6.5-6.5 using KOH. (Solution was disolved after reaching right pH.) Solution was sterilized by autoclaving at 121°C for 15 minutes.

Ingredients except Hunter's Trace Elements Solution and D-Glucose were dissolved in 900 ml distilled water by stirring with gentle heating and filled to 948ml.

Medium was sterilized by autoclaving at 121°C for 15 minutes. Hunter's Trace Elements Solution and D-Glucose were separately prepared and autoclaved (D-Glucose was dissolved in 50 ml of distilled water) and added to 948ml solution after cooling 50°C and made up to 1000ml.

B.8. Minimal Broth Medium

$NaNO_3$ (6 g/l)
KCI (0.52 g/l)
$MgSO_4.7H_2O$ (0.52 g/l)
KH_2SO_4 (1.52 g/l)
D-Glucose (10 g/l)
Hunter's Trace Elements Solution (2 ml/l)

Hunter's Trace Elements Solution (In 100 ml of water)

$ZnSO_4.7H_2O$ (2.2 g)
H_3BO_3 (1.1 g)
$MnCI_2.4H_2O$ (0.5 g)
$FeSO_4.7H_2O$ (0.5 g)
$CoCI_2.6H_2O$ (0.16 g)
$CuSO_4.5H_2O$ (0.16 g)
$NH_4Mo_7O_{24}.4H_2O$ (0.11 g)
EDTA. (5.0 g)

In order to prepare Hunter's Trace Elements Solution, all ingredients were mixed and pH was adjusted to 6.5-6.5 using KOH. (Solution was disolved after reaching right pH.) Solution was sterilized by autoclaving at 121°C for 15 minutes.

Ingredients except Hunter's Trace Elements Solution and D-Glucose were dissolved in 900 ml distilled water by stirring with gentle heating and filled to 948ml. Medium was sterilized by autoclaving at 121°C for 15 minutes. Hunter's Trace Elements Solution and D-Glucose were separately prepared and autoclaved (D-Glucose

were dissolved in 50 ml of distilled water) and added to 948 ml solution after cooling 50°C and made up to 1000ml.

B.9. Minimal Agar Medium Supplemented with Uridine

$NaNO_3$ (6 g/l)
KCI (0.52 g/l)
$MgSO_4.7H_2O$ (0.52 g/l)
KH_2SO_4 (1.52 g/l)
D-Glucose (10 g/l)
Hunter's Trace Elements Solution (2 ml/l)
Uridine (10ml stock 2,4% /l) (0,24 mg/ml)
Agar (15 g/l)

Hunter's Trace Elements Solution (In 100 ml of water)

$ZnSO_4.7H_2O$ (2.2 g)
H_3BO_3 (1.1 g)
$MnCI_2.4H_2O$ (0.5 g)
$FeSO_4.7H_2O$ (0.5 g)
$CoCI_2.6H_2O$ (0.16 g)
$CuSO_4.5H_2O$ (0.16 g)
$NH_4Mo_7O_{24}.4H_2O$ (0.11 g)
EDTA. (5.0 g)

In order to prepare Hunter's Trace Elements Solution, all ingredients were mixed and pH was adjusted to 6.5-6.5 using KOH. (Solution was disolved after reaching right pH.) Solution was sterilized by autoclaving at 121°C for 15 minutes.

Ingredients except Hunter's Trace Elements Solution D-Glucose and Uridine (0.24mg/ml) were dissolved in 900 ml distilled water by stirring with gentle heating and filled to 948ml. Medium was sterilized by autoclaving at 121°C for 15 minutes. Hunter's Trace Elements Solution and D-Glucose were separately prepared and autoclaved (D-Glucose was dissolved in 50 ml of distilled water), 10 ml stock solution

of uridine was filter sterilized and added to 948ml solution after cooling 50°C and made up to 1000ml.

B.10. Minimal Broth Medum Supplemented with Uridine

$NaNO_3$ (6 g/l)
KCI (0.52 g/l)
$MgSO_4.7H_2O$ (0.52 g/l)
KH_2SO_4 (1.52 g/l)
D-Glucose (10 g/l)
Hunter's Trace Elements Solution (2 ml/l)
Uridine (10ml stock 2.4% /l) (0,24 mg/ml)

Hunter's Trace Elements Solution (In 100 ml of water)

$ZnSO_4.7H_2O$ (2.2 g)
H_3BO_3 (1.1 g)
$MnCI_2.4H_2O$ (0.5 g)
$FeSO_4.7H_2O$ (0.5 g)
$CoCI_2.6H_2O$ (0.16 g)
$CuSO_4.5H_2O$ (0.16 g)
$NH_4Mo_7O_{24}.4H_2O$ (0.11 g)
EDTA. (5.0 g)

In order to prepare Hunter's Trace Elements Solution, all ingredients were mixed and pH was adjusted to 6.5-6.5 using KOH. (Solution was disolved after reaching right the pH.) Solution was sterilized by autoclaving at 121°C for 15 minutes.

Ingredients except Hunter's Trace Elements Solution D-Glucose and uridine (0.24 mg/ml) were dissolved in 900 ml distilled water by stirring with gentle heating and filled to 948ml. Medium was sterilized by autoclaving at 121°C for 15 minutes. Hunter's Trace Elements Solution and D-Glucose were separately prepared and autoclaved (D-Glucose are dissolved in 50 ml of distilled water), 10 ml stock solution of

2.4% uridine was filter sterilized and added to 948 ml solution after cooling 50°C and made up to 1000ml.

B.11. Molasses Agar Slants

Glycerol (45 g/l)
Peptone (18 g/l)
Molasses (45 g/l)
NaCI (5 g/l)
$FeSO_4.7H_2O$ (15 mg/l)
Agar (20 g/l)
Stock Solutions (5ml/l of each)

Stock Solutions

KH_2P0_4 (1.2%) 5 ml/l
$MgSO_4.7H_2O$ (1%) 5 ml/l
$CuS0_4.5H_2O$ (0.24%) 5 ml/l
$MnSO_4.5H_20$ (0.3%) 5 ml/l
KCI (10%) 5ml/l
5ml/l of each below listed stock solutions are used, and stored at 4°C.

Ingredients were dissolved in 900 ml distilled water by stirring with gentle heating and filled to 1000 ml. While medium was warm enough, 10 ml of this media was added to 18x180mm glass tubes. Medium was sterilized by autoclaving at 121°C for 15 minutes and tubes were left to solidify in slant position.

B.12. PGA

Mineral salts solution (500 ml/l)
Yeast extract (1 g /l)
Agar (15 g /l)

Polygalacturonic acid (PGA) (5g 500 ml/l)
Distilled water (500 ml/l)

The mineral salts solution

$(NH_4)SO_4$ (2 g/l)
KH_2PO_4 (4 g/l)
Na_2HPO_4 (6 g/l)
$FeSO_4.7H_2O$ (0,2 g/l)
1000 times concentrated salt solution

The 1000 times concentrated salt solution

$CaCI_2$ (1,32 g/l)
H_3BO_3 (0,01 g/l)
$MnSO_4$ (0,011 g/l)
$ZnSO_4.7H_2O$ (0.125 g/l)
$CuSO_4.5H_2O$ (0,078 g/l)
MoO_3 (0,01 g/l)

$FeSO_4.7H_2O$ had to be dissolved in d-H_2O before adding to the mineral salt solution. Mineral salt solution and PGA were autoclaved separately from the yeast extract and agar. After autoclaving, all the components of the media were mixed together. pH of the media was adjusted to pH 5 by using 5N HCI.

APPENDIX C

STOCK SOLUTIONS

C.1. Ampicillin 100 mg/ml Stock Solution

1 g of ampicillin was weight and added to a 15 ml tube to which 10 ml of sterilized deionized was added. This solution was inverted and vortexed to mix it completely. Final volume was adjusted to 10 ml. Stock solution was filtered through a 0.2 μ syringe filter into a new tube. 1 ml portions or less were filled into in sterile 1.5 ml tubes and stored at -20°C.

C.2. 50X TAE

242 g of Tris base was dissolved in deionized water. 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA (pH 8.0) were added. The final volume was adjusted to 1000 ml with deionized water.

C.3. 1X TAE

20 ml of 50**X** TAE buffer was taken and 980 ml of deionized water was added to obtain 1**X** TAE buffer.