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MOLECULAR AND MICROBIOLOGICAL INVESTIGATIONS OF METAL REDUCING BACTERIA *Shewanella putrefaciens*

by

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APPROVAL PAGE

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

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This is to certify that I have read this thesis and that in my opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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MOLECULAR & MICROBIOLOGICAL INVESTIGATIONS OF METAL REDUCING BACTERIA *Shewanella putrefaciens*

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ABSTRACT

Shewanella putrefaceins is a metal reducing bacterium which can use a diversity of organic compounds and metals to obtain the energy needed for its growth. It is a Gram negative, facultative anaerobic bacterium, related to *E. coli.* The tools and techniques that were developed over the past 30 years for *E. coli* are compatible with *S. putrefaciens.* Furthermore, the ability of Shewanella to tolerate oxygen allows easy genetic manipulation in contrast to strict anaerobic bacteria. In this work, we try to engineer a recombinant *Shewanella putrefaceins* as a new and efficient system for producing electricity.

For further molecular cloning studies and strategies a suitable expression system of this enzyme should be investigated to overcome its weak expression in the *E. coli* host organism. Two heterologous expression systems were used up to now. The last one, *E. coli* BL21 (DE3) pLysS, was found more efficient during pilot protein expression studies because of codon usage bias.

In the present work a few constracts were created in order to find the more efficient one for heterologous expression of *Aspergillus niger* glucose oxidase in *E. coli* host cells. Based on the codon preference, the bacterium *E. coli* BL21 (DE3) pLysS was selected as the best potential host for *Aspergillus niger* glucose oxidase expression. A significant increase in expression level of recombinant glucose oxidase was observed for strain BL21 instead of Top 10. This shows that the system of *E. coli* BL21 (DE3) pLysS heterologous production system for fungal gene, glucose oxidase appears more efficient than Top 10 strains.

Keywords: *Shewanella putrefaciens* ATCC 8071, Electricity, Power Generation, Microbial Fuel Cell, Glucose Oxidase, Cloning, *Aspergillus niger*,

METAL İNDİRGEYEN BAKTERİLER GRUBUNA DAHİL OLAN *Shewanella putrefaciens'* in MOLEKÜLER VE MİKROBİYOLOJİK YÖNDEN ARAŞTIRILMASI

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ÖZ

Shewanella putrefaciens birçok metali indirgeme özelliği dolayısıyla hem biyorimediyasyon hem de anaerobik ortamda üretmiş olduğu elektronları sayesinde biyoteknolojik açıdan oldukça fazla öneme sahip bir mikroorganizmadır. Birçok besin ortamında rahatlıkla yaşayabilmesi, oldukça düşük sıcaklıklarda, oksijenli ve oksijensiz ortamlarda olmak üzere pekçok yerde kolayca hayatta kalabilmesi bu mikroorganizma ile çalışılmasını kolaylaştırmıştır. *E. coli* ile olan benzerlikleri kullanılarak yapılan çalışmaların olabilirliğini tahmin etmek daha da kolay olmaktadır.

Bu çalışmada, elektrik ürettiği birçok bilimsel araştırmada ortaya konmuş bu mikroorganizma üzerinde çeşitli genetik manipulasyonlar ile elektrik üretim miktarı arttırılmak istenmiştir. Öncelikle hangi gen üzerinde çalışılacağı tespit edilmiş ve bu amaçla *Aspergillus niger* küfünden izole edilen genomik DNA kullanılarak glikoz oksidaz geni uygun primerler ile çoğaltılmıştır. Amaca uygun olarak seçilen vektör ve transforme edilecek hücre ile glikoz oksidaz geni klonlanmaya ve uygun *E. coli* suşunda klonlanan gen ekprese edilmeye çalışılmıştır. Protein ekpresyon deneylerinde pozitif bulunan klonlardan izole edilen plazmitler elektroporasyon ile *Shewanella putrefaciens*' e aktarılacak ve yine bu canlı içerisinde de proteinin ekpresyonu gözlenmeye çalışılacaktır.

Yapılan birçok denemeden sonra genin vektor içerisine klonlanması oldukça fazla zaman almıştır. Bulunan pozitif klonların ekpresyon suşlarına aktarılıp, protein indüklemesi deneylerinden sonra yapılan SDS-PAGE protein jelinde pozitif

çıkmadıkları görülmüştür. Tekrar başa dönülüp yeni klonlamalar ve yeni pozitif klonlar elde edilmeye çalışılmıştır. Son olarak seçilen *E. coli* BL21(DE3) pLysS suşu ile yapılan IPTG ile protein indükleme çalışmasında istenilen sonuç alınmıştır. Ancak bu aşama çok vakit aldığından üzerinde çalışılması planlanan mikroorganizmaya aktarımı sağlanamamıştır.

Anahtar Kelimeler: *Shewanella putrefaciens* ATCC 8071, Elektrik, Güç Üretimi, Mikrobiyal Yakıt Hücresi, Glikoz Oksidaz, Klonlama, *Aspergillus niger*

This dissertation is dedicated to my mum and dad.

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LIST OF SYMBOLS AND ABBREVIATIONS

SYMBOL/ABBREVIATION

MFC : Microbial Fuel Cell

GOD : β-D-glucose:oxygen 1-oxidoreductase; Glucose Oxidase

H_2O_2 : Hydrogen Peroxide

FAD : Flavine Adenine Dinucleotide

CAT : Catalase

DOE : Department of Energy

rRNA : Ribosomal Ribonucleic Acid

TMAO : Trimethylamine-*N*-oxide

DMSO : Dimethyl Sulfoxide

EPA : Eicosapentaenoic Acid

pfa : Polyunsaturated Fatty Acid

CDC : Centers for Disease Control and Prevention

MFCs : Microbial Fuel Cells

TEA : Terminal Electron Acceptor

PEM : Proton Exchange Membrane

NR : Neutral Red

MB : Methylene Blue

MelB : Meldola's Blue

HNQ : 2-hydroxy-1,4-naphthoquinone

AQDS : Anthraquinone-2,6-disulfonate

RVC : Reticulated Vitreous Carbon

Pt : Platin
COD : Chemical Oxygen Demand
BOD : Biological Oxygen Demand
OD : Optical Density
ATCC : American Type Culture Collection
MEA : Malt Extract Agar
LB : Luria-Bertani
TAE : Tris Acetate EDTA
UV : Ultra Violet
PCR : Polymerase Chain Reaction
CIAP : Calf Intestinal Alkaline Phosphatase
NC : Negative Control
dNTP : Deoxyribonucleotide Triphosphate
SDS-PAGE : Sodium Dodecyl Sulphate- Poly Acrylamide Gel Electrophoresis
TEMED : Tetramethylethylenediamine
IPTG : Isopropyl-1-thio-β-D-galactopyranoside

CHAPTER 1

INTRODUCTION

Genus *Shewanella* are a type of gram-negative, facultative anaerobic bacteria mostly found in aquatic and marine environments and can often be isolated from spoiling fish. Usually, Shewanella are known to be the members of the γ-subclass of the Proteobacteria that are gram-negative rods, 0.4 – 0.7 μm in diameter, 2–3 μm in length, and motile by a single polar flagellum (2). Many types of Shewanella are grown in the laboratory with general growth media following enhancement from environmental samples (12) with a range of salt concentrations, temperatures, and barometric pressures and have distinct roles from food spoilage organisms to symbionts, epibionts, and opportunistic pathogens. (1)

In the absence of oxygen, Shewanella is able to carry out anaerobic respiration by using a broad range of final electron acceptors. This feature not only enables the members of the genus Shewanella to survive in various environments with the capacity of growing naturally almost anywhere, but also to be utilized for bioremediation of contaminated environments by reducing some certain metals and compounds in an altered state (4). For example, Shewanella can convert uranium dissolved in contaminated groundwater to a form unable to dissolve in water, so that the uranium will not spread as the groundwater flows. Hence, areas such as DOE sites contaminated during the manufacture of nuclear weapons can be cleaned and confined.

Shewanella has benefits that make it easier for researchers to study. Escherichia and Shewanella are well known to be related. Tools and techniques developed since 1970s for Escherichia works with Shewanella, which can tolerate oxygen—a useful

ability that makes it easier to work with in the laboratory and is missing in other groups of metal metabolizing bacteria.

1.1 SHEWANELLA AS A VERSATILE ORGANISM

1.1.1 A BRIEF HISTORY OF THE ISOLATION OF SHEWANELLA

The isolation of the first Shewanella was performed in 1931 as one of various contaminating microorganisms in charge of butter putrefaction (5). Derby and Hammer were hesitant to identify the microorganism as *Achromobacter putrefaciens* (5), although the taxon was renamed to Pseudomonas (*Pseudomonas putrefaciens*) following further growth and biochemical characterizations in 1941 (6). In 1960, Shewan *et al.* suggested a classification scheme (7) based on the oxidase positive reaction and motility by means of polar flagella reinforced reclassification into the genus Pseudomonas. On the other hand, Baumann and coworkers introduced a new scheme in 1972 primarily based on moles percent guanine plus cytosine (mol % GC) content of DNA. A numerous strains of nonfermentative marine bacteria previously classified as Pseudomonas was placed into a newly created genus, Alteromonas (8).

The type species isolated in 1931 (5) was changed from *Pseudomonas putrefaciens* to *Alteromonas putrefaciens* (9) in 1977. Finally, based on 5S rRNA sequence data, MacDonell and Colwell (10) proposed reclassification into a new genus, Shewanella, in honor of the late Dr. James M. Shewan and in recognition of his contributions to fisheries microbiology. In 1985, *Shewanella putrefaciens* was born, and no more reclassifications at the genus level have been made up to date. Approximately 40 species are assigned to the genus Shewanella based primarily on DNA at present: DNA hybridization and 16S rRNA sequences. Table 1.1 outlines known Shewanella species and sources of isolation.

1.1.2 SPECIES CHARACTERIZATION

Although the first Shewanella were characterized based on phenotypic characteristics like morphology, standard biochemical reactions, gram stain, and growth at different physiological conditions, molecular analyses have enabled evaluation, comparison, and classification of phenotypically distinct bacteria into the genus Shewanella based primarily on DNA hybridization and 16S rRNA gene sequences. Thus, several phenotypically distinct bacteria have been reclassified under the genus Shewanella, and existing Shewanella species have been assigned new species names or better characterized. Phylogenetic associations based on 16S rRNA gene sequences are presented in Figure 1.1 for well-characterized type strains of Shewanella. Most new Shewanella strains were then classified as *S. putrefaciens* despite indications that the group was more diverse (11). The study of Owen and coworkers (11) showed that the species *S. putrefaciens* included at least four clearly different DNA homology groups (I-IV) based on DNA-DNA hybridization experiments that included several *P. putrefaciens* strains. While the number of species increases, the phenotypic characteristics of the Shewanella are as diverse as the environments in which they live (2,12). The distribution of Shewanella on the earth appears to be rooted in two basic physiological observations: (*a*) their incomparable ability to respire compounds found in the environment and (*b*) their ability to survive at low temperatures.

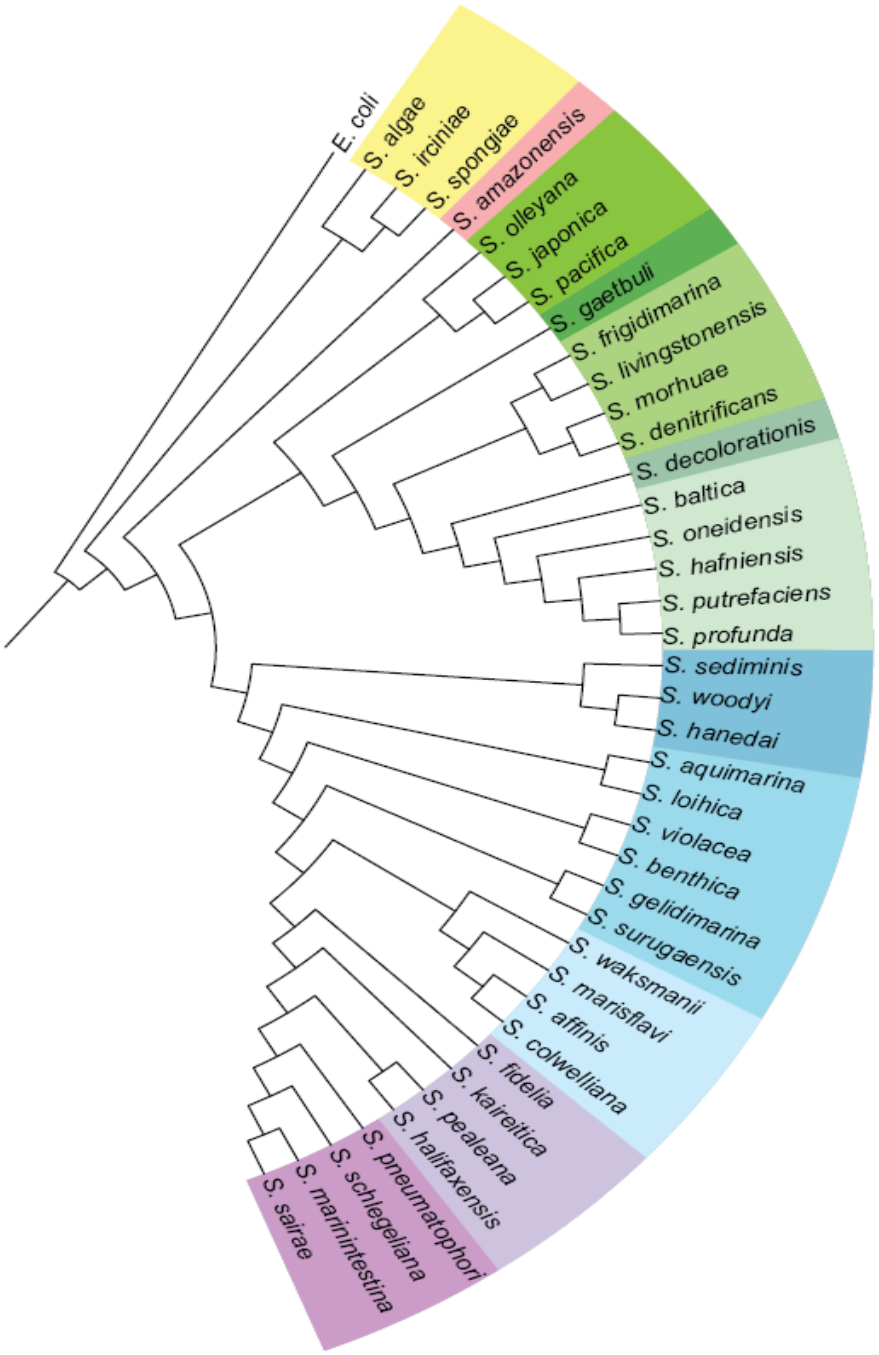


Figure 1.1 Phylogenetic analysis of *Shewanella* type strains.

Some groups of Shewanella are grouped together (*different colors*) that were isolated from similar environments. For instance, several fish intestinal isolates cluster together (*S. pneumatophori, S. schlegeliana, S. marinintestina,* and *S. sairae*). Nevertheless, other examples such as the clustering of the butter surface taint isolate *S.*

putrefaciens with the deep-ocean sediment isolate *S. profunda* indicate that 16S rRNA gene comparisons have restricted predictive power in terms of ecology. *S. abyssi* was not taken into account in this analysis because of some difficulties determining the appropriate 16S rRNA sequence for this type strain (13). Sequences were trimmed to ~1200 bp to smooth the progress of making comparisons between species with incomplete 16S rRNA gene sequences. Accession numbers for sequences can be found in Table 1.1 (14).

1.1.2.1 RESPIRATORY DIVERSITY

As a genus, Shewanella are the most diverse respiratory organisms described so far. There are roughly twenty inorganic and organic compounds that can be respired by Shewanella and these have several insoluble metals and toxic elements. A partial list are as follows:

Trimethylamine-*N*-oxide (TMAO) → Trimethylamine (Me_3N),
Fe (III) Chelate and Fe (III) Oxide → Soluble Fe (II),
Mn (III and IV) Chelates and Mn (III and IV) oxides → Soluble Mn (II),
Sulfur/polysulfide → H_2S,
Sulfite → H_2S,
Thiosulfate→ H_2S,
Dimethyl sulfoxide (DMSO) → Dimethylsulfide,
Arsenate → Arsenite, Fumarate, and Succinate.

A complete list and vivid illustrations can be found in the literature (12). For some compounds reduction has been exhibited but growth has not (e.g., selenite). The respiratory diversity of Shewanella is one of their greatest benefits in terms of survival in the environment. The fact that all isolates seem to be facultative anaerobes and the anaerobic electron acceptors are various, suggests these organisms are normally localized in both oxic and anoxic environments. Oxygen can be limiting in sediments, in intestinal tracts of higher organisms, and in organic-rich flocculates such as marine snow and fecal pellets. Some aquatic systems are permanently or temporarily stratified,

allowing the formation of large anoxic zones. The respiratory diversity of Shewanella allows them to breath almost anywhere. In anoxic environments, Shewanella are likely to respire one if not several compounds. In organic-rich flocculates the electron acceptor may be TMAO or DMSO. In sedimentary environments, the electron acceptors may be insoluble iron or manganese oxide minerals. The mechanism of anaerobic respiration in these organisms is implicit at the genetic level for some compounds (Fumarate, DMSO, TMAO, As, V, Fe, and Mn). Many of these compounds are reduced by terminal reductases located outside of the cell, and reviews have recently concentrated on the molecular details of this process (16, 32). Additionally, it is likely that the list of known substrates respired by Shewanella is not yet complete.

1.1.2.2 LOW- TEMPERATURE GROWTH

Growth at low temperatures (4^{o}C) appears to be a hallmark of the Shewanella genus. Most Shewanella strains are psychrotolerant, meaning that they have the capacity to grow at low temperatures (<5^{o}C), but their optimum temperature is above 16^{o}C. Though some isolates do not demonstrate robust growth at low temperatures, many behave as true psychrophiles with temperature optima below 16^{o}C (2, 17, 18). The capacity to thrive at low temperatures gives these organisms an advantage in permanently cold environments such as the ocean and the Polar Regions. This ability is also useful in environments with large temperature fluctuations. For example, the temperature of Oneida Lake drops dramatically in the winter months, with the lake freezing over completely for several months (19). The physiological changes that take place to allow low-temperature growth in the Shewanella are unknown. However, Abboud *et al.* (20) have described some biochemical parameters and unusual morphological changes during low-temperature growth in *S. oneidensis* strain MR-1, which was isolated from Oneida Lake (Table 1.1).

1.1.2.3 PRODUCTION OF OMEGA-3 FATTY ACIDS

Omega-3 fatty acids are essential nutrients for higher organisms, from bacteria-eating zooplankton to humans. Many organisms, including humans, cannot synthesize these compounds de novo but acquire them through diet. Omega-3 fatty acids are polyunsaturated, typically 18, 20, or 22 carbon units long, and have a double bond occurring at the third carbon-carbon bond from the terminal (omega) end of the molecule. Common omega-3 fatty acids are docosahexaenoic acid and eicosapentaenoic acid (EPA). Some aquatic bacteria, such as those from the genus Shewanella, produce these compounds, although the physiological benefit is unclear. Gene clusters predicted to encode the production of polyunsaturated fatty acids (called *pfa*) have been identified in all 14 sequenced Shewanella genomes (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi). These clusters also appear to be present in the Sargasso Shewanella assemblies generated by Venter and coworkers (21). A wide range of Shewanella isolates have been shown to produce EPA (2, 17, 22, 23, 24), and a *pfa* gene cluster has been cloned from an unsequenced Shewanella strain into a cyanobacterium, conferring the ability to produce EPA (25).

1.1.2.4 PLASMIDS

There are only a few low-temperature expression systems built with cold adapted strains *Shewanella sp.* Ac10 (26), *Shewanella oneidensis* AS52 (27), *S. oneidensis* MR-1 (28), and *S. oneidensis* TSP-C (29) have been reported, based on the broad host range vectors or their derivates. Furthermore, one study has been showed that the gene expression of multiheme cytochrome c_3 from *Desulfovibrio vulgaris* could be overexpressed in *S. oneidensis* TSP-C using pUC-type vectors of *E. coli* (30). Yet, there is no information about specific cloning expression vectors based on the plasmids naturally occurring in Shewanella species. Besides, the information on the naturally occurring plasmids from the environmental isolates of Shewanella species is rather limited (31).

1.1.3 ECOLOGY

1.1.3.1 SYNTROPHY

The diverse distribution of Shewanella is explained by their potential to build syntrophic affiliations with fermentative microbes or those microbes that can use Shewanella's by-products. Most Shewanella strains are nonfermenters, but colonization with fermenters would allow them to employ products of fermentation (lactate, formate, hydrogen, and some amino acids) for anaerobic respiration. By-products produced during anaerobic respiration such as acetate, ammonia, and alanine could be further consumed by acetogens, methanogens, organotrophs, or lithotrophs, depending on the environment (12).

1.1.3.2 MARINE ORIGINS

According to Table 1.1, most of the Shewanella isolates are from marine environments although they have been cultured from nonmarine environments, (see Table 1.1). Under laboratory conditions most Shewanella isolates require some added salt for maximal growth (2). The following are examples of Shewanella isolated from nonmarine environments that potentially have a marine source. The first example is *Shewanella oneidensis* strain MR-1 that was isolated from Oneida Lake which is the largest body of water within New York State. Since this lake, although this is a freshwater lake, was connected the Erie Canal System, which connects Lake Erie with the Hudson River through a series of locks in 1835. Because of the fact that Hudson River flows into the Atlantic Ocean, there might be a risk for contamination of Oneida Lake by sea-faring ships and barges that frequent the Erie Canal System. Although there is no exact evidence for contamination of Oneida Lake by *S. oneidensis.* Venter and his colleagues rely on this hypothesis according to metagenomic studies in the Sargasso Sea (33). Almost two complete Shewanella genome sequences were derived from the Sargasso Sea sequencing effort. And both of them narrowly resemble *S. oneidensis* (33) but there are some differences between them. Amusingly there is a possibility that *S. oneidensis* diverged from its marine origin for at least ~150 years prior to its isolation.

S. oneidensis strain MR-1 grow faster under the conditions of 0.1–0.3M NaCl than in seawater concentrations (0.6 M or 35 g liter^{-1}) or no addition (34). Perhaps caught in the midst of adapting to a freshwater environment, the preference by *S. oneidensis* strain MR-1 for temperate salinity is compatible with recent colonization of Oneida Lake from a marine environment.

Other nonmarine Shewanella isolates can form older colonizations as an example of cultivated from Lake Kinneret (Sea of Galilee) in the Dead Sea Rift Valley, Israel (35). The concentration of salt is very high in this lake (~ 0.2 g liter^{-1}) but there has been a decrease in the concentration of salt in modern times through the diversion of saline springs that feed the lake (36). Besides saline spring inputs into the lake, Mazor and Mero (37) qualified the salinity of Lake Kinneret by the help of interference of seawater that occurred in the Neogene period, some 20 mya. Shewanella may simply be a contaminant in this lake, entering through saline springs, or more interestingly, several million years ago through a geological event. In keeping with these two examples, they show the possibility that some freshwater Shewanella isolates may recently be of marine origin (<150 years in the case of *S. oneidensis* MR-1). But the physiology of most of these strains is not known yet while some freshwater isolates have been described previously. Actually, these bacteria booming in marine, brackish, and freshwater environments attributes to their metabolic and respiratory diversity.

1.1.3.3 PATHOGENICITY

Shewanella most commonly known as secondary or opportunistic pathogens, and the possibility of infections caused by them are very rare; nevertheless, infections are being reported with higher frequency because of better diagnostics in the clinical microbiology laboratory (12, 38). *S. putrefaciens* and *S. algae* are most commonly isolated though human clinical specimens examined thus far are very narrow. The reason for this is a short of criteria for good speciation as an example molecular characterizations, to differentiate between *S. putrefaciens, S. algae,* and other Shewanella species. Additionaly, during isolation of the Shewanella, other bacterial pathogens are also isolated beside the Shewanella. Beacuse of this fact that, the function

of the Shewanella in pathogenesis and their clinical importance have not been understood yet.

On the other side , there are reports of monomicrobial Shewanella infections; patients present clinically with cellulitis in the context of other skin and soft tissue manifestations, bacteremia/septicemia, otitis media or otitis externa, respiratory distress, intra-abdominal infection, pneumonia, and empyema (38, 39). Beside there are some exceptions, *S. putrefaciens* and *S. algae* are vulnerable to common antibiotics used to treat bacterial infections (38, 39), though drug-resistant strains have been documented to emerge during the course of patient treatment (40).

Since earlier isolates have been classified under another genus, in 1963 the first *S. putrefaciens* was isolated from human clinical specimen. From 1963 to 1997, about 75 case of human infection caused by *S. putrefaciens* had been reported (41). There is a strong relation between *S. putrefaciens* infection and an immunocompromised state, and liver disease is a strong risk factor (41). In 1985, three different biovars were before construction of the species *S. algae* in 1990 commonly, but incorrectly, there were reports which identified many human isolates as *S. putrefaciens* rather than *S. algae* (38). After understanding of the fact that *S. algae was* different from *S. putrefaciens* , Gilardi biovar 2 was used to illustrate *S. algae* (43, 44).

Because of the fact that the possibility of Shewanella infections are very low, *S. algae* become the most common human pathogen. In addition to this, *S. putrefaciens* are responsible for nonhuman pathogenicity (38, 42). CDC biotype 1 (*S. putrefaciens*) and CDC biotype 2 (*S. algae*) were recognized as two types of clinical isolates in 1995 by Centers for Disease Control and Prevention (42). But there was need for more detailed molecular characterizations to differentiate into subgroups other than *putrefaciens* and *algae.* A review by Holt *et al.* that was recently published shows important points in differentiating to the clinical microbiologist. On the other hand, DNA:DNA hybridization or 16S rRNA sequences that are examples of molecular characterizations can only distinguish *S. putrefaciens* and *S. algae* from other members of the genus Shewanella.

There has not been information that describe the virulence factors for *Shewanella* clinical isolates yet but according to one study, after comparision many human and nonhuman Shewanella clinical isolates, it was found that *S. algae* was more pathogenic than *S. putrefaciens* on the strength of resistance to antimicrobials, production of hemolysin, and pathogenicity in mice (42). According to the study that examined pathogenicity of various *S. putrefaciens* isolates in mice, it was found that doses ≥1 × 109 organisms per mouse were only lethal (45). Extracellular virulence factors for instance siderophores, exoenzymes, and tetrodotoxin, a potent marine neurotoxin produced by *S. algae* have been determined and by some investigators and may be a part of in pathogenesis (38).

S. algae and *S. putrefaciens have been reported as* opportunistic pathogens in nonhuman species, in only some studies however limited studies show nonhuman infections. In hatchery ponds on the southern coast of China and Taiwan, mass mortality of abalone shellfish was observed because of role of *S. algae and it caused* ulcer disease in the marine fish *Sciaenops ocellata* from a Chinese river (47). At high concentrations of *S. putrefaciens,* it became virulent to juvenile freshwater zebra mussels but at low concentrations of bacteria the mussel may act as a pool for these opportunistic pathogens (48).

1.1.4 APPLICATIONS IN BIOTECHNOLOGY

With a diverse group of electron acceptors, genus Shewanella have able to respire and have shown an adaption to the life in extreme and different environments. Shewanella can easily grow in the lab conditions and are open to genetic manipulation. So, Shewanella have potential to remediate environmental pollutants and in microbial fuel cells (MFCs), where their metabolism have capacity to produce electricity (49, 50, 51, 52).

1.1.4.1 BIOREMEDIATION OF RADIONUCLIDES AND TOXIC ELEMENTAL WASTE

Contaminated environments can be effectively and inexpensively cleaned up by the help of microorganisms which prevent difficulties in efficient bioremediation strategies (53). Shewanella are superior candidates for potential use in pollutant bioremediation among dissimilatory metal-reducing bacteria due to their intrinsic ability to respire using a wide range of electron acceptors (12). The solubility and mobility of elements in soils, sediments, and water can be influenced with their oxidation states. Microorganisms display one means by which changes in oxidation states are catalyzed so that transport into rivers and groundwater can be blocked and cleanup facilitated (12, 54). Applications might contain ex situ remediation strategies and in situ bioremediation in storage tanks or areas of environmental contamination (12). For instance, in manufacturing of nuclear weapons and as a main fuel for nuclear reactors, uranium (U) is used. Some Shewanella can able to play a role in the reduction of U (VI) to the insoluble U (IV) form (12), and this would cause to precipitation and prevent further spread in groundwater at the contaminated site. There is another radionuclide, Technetium (^{99}Tc VII), which is obtain as an end product of nuclear reactor operations and fallout from nuclear weapons testing. Many oxidations states of Technetium (^{99}Tc VII) can be observed but the most reduced form is largely immobile (56). ^{99}Tc can be reduced by *S. putrefaciens* (55, 12, 56), *S. oneidensis* MR-1 (57), and *S. algae* (56) and they can be used in remediation of Tc-contaminated environments and waste streams. Similar to Technetium (^{99}Tc VII), Cobalt (^{60}Co) is a radionuclide and formed after weapons operations. Co(III)EDTA^{-} is the mostly found form of cobalt at contaminated sites and in groundwater, and some Shewanella can use this form of cobalt as an electron acceptor (58, 59). ^{60}Co(III)EDTA is reduced to ^{60}Co(II)EDTA^{2-} which causes reduction of ^{60}Co(III)EDTA^{-} to ^{60}Co(II)EDTA^{2-} leads to immobilization and therefore restricted ransport in subsurface environments. ^{60}Co(II)EDTA^{2-} that is the reduced form is not stable sorbs to mineral surfaces facilitating elimination (58, 59).

Chromium (Cr), mercury (Hg), and arsenic (As) can be reduced by some type of Shewanella (12) but there can be advantages and disadvantages of these reductions. Cleanup efforts can be made possible by the formation of solid oxides as a result of

reduction of soluble Cr(VI) to Cr(III) by some Shewanella (60). But *S. oneidensis* MR-1 reduce ionic mercury [Hg(II)] to elemental mercury [Hg(0)] and this cause an increase in mobility and an oxidation state readily available to form methylmercury, a bioaccumulative environmental toxin (3). In addition to these examples, reduction of arsenate [As(V)] to arsenite by Shewanella cause big problems. Drinking waters are contaminated and poisoned by As(III) since this form of arsenate is mobile (15).

As a result, understanding the role of Shewanella, as well as other microorganisms in the oxidation of target compounds and the results in varying these oxidation reactions are precursor factors to optimize cleanup strategies. Bioremediation strategies consisting Shewanella can be only applied in the laboratory and have not been used outside the lab yet. The future applications of Shewanella in bioremediation strategies consist of cleanup of contaminated global environments and groundwater. Since Shewanella mainly lives in aqua, further studies will be focused on how Shewanella behave in soil and contaminated groundwater environments (1).

1.2 MICROBIAL FUEL CELL

1.2.1 BIOELECTRICITY GENERATION USING A MICROBIAL FUEL CELL

In an MFC, microorganisms can oxidize organic substances and produce ATP energy released electrons from product of oxidation in series of respiratory enzymes. The electrons are then released to a terminal electron acceptor (TEA) which becomes reduced after accepting electrons. For example, oxygen is reduced to water through a catalyzed reaction of the electrons with protons. Many TEAs such as oxygen, nitrate, sulfate, and others readily diffuse into the cell in depending conditions and they accept electrons forming products that can diffuse out of the cell. However, we know that some bacteria can transfer electrons exogeneously to a TEA which is outside the cell such as a metal oxide like iron oxide. These bacteria that can exogenously transfer electrons, called exoelectrogens, can be used to produce energy in an MFC.

An MFC system is shown schematically in Figure 1.2. In the anode chamber, oxygen will inhibit electricity production, so the system must keep the bacteria separated from oxygen (the catholyte in this example). This separation of the bacteria from oxygen can be made by placing a membrane which allows charge transfer between the electrodes, forming two separate chambers: the anode chamber, where the bacteria grow; and the cathode chamber, where the electrons react with the catholyte. The cathode is sparged with air to provide dissolved oxygen for the reaction. The two electrodes are connected by a wire containing a load (i e . , the device which will powered), but in the laboratory a resistor is used as the load. The membrane is permeable to protons which produced at the anode, so that they can transfer to the cathode where they can combine with electrons transferred via the wire and oxygen, forming water. The electrical current produced by an MFC is determined in the laboratory by screening the voltage drop across the resistor using either (a) a voltmeter (intermittent sampling) or (b) a multimeter or potentiostat hooked up to a computer for essentially continuous data acquisition (63).

1.2.2 HISTORY OF MICROBIAL FUEL CELL DEVELOPMENT

Theoretically, most microorganisms can potentially be used as a biocatalyst in MFC. The earliest MFC concept was demonstrated by Potter in 1910 (64). Electrical energy was produced from living cultures of *Escherichia coli* and Saccharomyces by using platinum electrodes (65). This didn't generate much interest until 1980s when it was discovered that current density and the power output could be greatly enhanced by the addition of electron mediators. Unless the species in the anodic chamber are anodophiles, the microorganisms are incapable of transferring electrons directly to the anode. The outer layers of the majority of microbial species are composed of non-conductive lipid membrane, peptididoglycans and lipopolysaccharides that hinder the direct electron transfer to the anode. Electron mediators accelerate the transfer (66). Mediators in an oxidized state can easily be reduced by capturing the electrons from within the membrane. The mediators then move across the membrane and release the electrons to the anode and become oxidized again in the bulk solution in the anodic chamber.

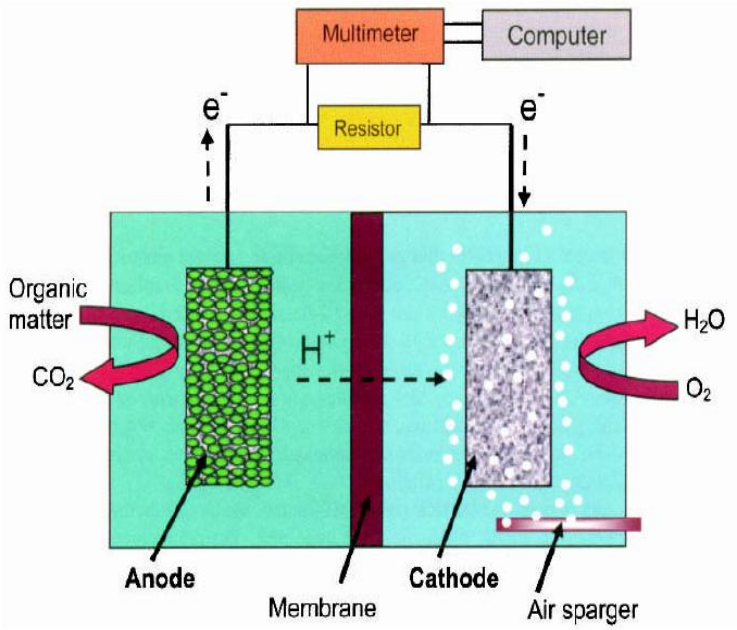


Figure 1.2 Schematic of the basic components of a microbial fuel cell (not to scale).

This cyclic process accelerates the electron transfer rate and thus increases the power output. Good mediators should possess the following features (64): (1) able to cross the cell membrane easily; (2) able to grab electrons from the electron carries of the electron transport chains; (3) possessing a high electrode reaction rate; (4) having a good solubility in the anolyte; (5) nonbiodegradable and non-toxic to microbes; (6) low cost. And how efficient the oxidized mediator gets reduced by the cells reducing power is more important then other features. Although a mediator with the lowest redox would give the lowest anodic redox in theory and maximize the redox difference between anode and cathode (i.e. give biggest voltage difference) it would not necessarily be the most efficient at pulling electrons away from the reduced intracellular systems (NADH, NADPH or reduced cytochromes) within the microorganisms. A mediator with a higher E^o redox would give a higher overall power than a mediator with the lowest redox (64). Typical synthetic exogenous mediators include dyes and metallorganics such as neutral red (NR), methylene blue (MB), thionine, meldola's blue (MelB), 2-hydroxy-1,4-naphthoquinone (HNQ), and Fe(III)EDTA (64, 67, 68, 69, 70). Unfortunately, the instability and toxicity of synthetic mediators limit their applications in MFCs. Some

microorganisms can use naturally occurring compounds including microbial metabolites (endogenous mediators) as mediators. Humic acids, anthraquinone, the oxyanions of sulphur (sulphate and thiosulphate) all have the ability to transfer electrons from inside the cell membrane to the anode (71). A real breakthrough made when some microorganisms were found to transfer electrons directly to the anode (72, 73). These microbes are operationally stable and yield a high Coulombic efficiency (73, 74). *Shewanella putrefaciens* (75), *Geobacteraceae sulferreducens* (76), *Geobacter metallireducens* (77) and *Rhodoferax ferrireducens* (73) are all bioelectrochemically active and can form a biofilm on the anode surface and transfer electrons directly by conductance through the membrane. When they are used, the anode acts as the final electron acceptor in the dissimilatory respiratory chain of the microorganisms in the biofilm. Biofilms forming on a cathode surface may also play an important role in electron transfer between the microorganisms and the electrodes. Cathodes can serve as electron donors for *Thiobacillus ferrooxidans* suspended in a catholyte (78) for an MFC system that contained microorganisms in both anodic and cathodic chambers. *G. metallireducens* and *G. sulfurreducens* (79) or other seawater biofilms (80) may all act as final electron acceptors by grabbing the electrons from cathode as electron donors. Since the cost of a mediator is eliminated, mediator-less MFCs are advantageous in wastewater treatment and power generation (64).

1.2.3 MICROBES USED IN MICROBIAL FUEL CELLS

Depending on the ability of metabolizing organic matters many organisms can transfer electrons to the anode. A list of them and their substrates is shown in Table 1.1. Rich sources for these microorganisms are marine sediment, soil, wastewater, fresh water sediment and activated sludge (103, 104). Recent studies discussed the screening and identification of microbes and the construction of a chromosome library for microorganisms that are able to produce electricity from oxidasing organic matters (105, 106, 107).

Table 1.1 Microbes used in microbial fuel cells

Microbes	**Substrate**	**Applications**
Actinobacillus succinogenes	Glucose	Neutral red or thionin as electron mediator (67; 82)
Aeromonas hydrophila	Acetate	Mediator-less MFC (83)
Alcaligenes faecalis, Enterococcus gallinarum, Pseudomonas aeruginosa	Glucose	Self-mediate consortia isolated from MFC with a maximal level of 4.31 W m^2 (84)
Clostridium beijerinckii	Starch, Glucose, Lactate, Molasses	Fermentative bacterium (85; 86)
Desulfovibrio desulfuricans	Sucrose	Sulphate/sulphide as mediator (64; 87)
Erwinia dissolven	Glucose	Ferric chelate complex as mediators (69)
Escherichia coli	Glucose, Sucrose	Mediators such as methylene blue needed (88; 64; 89)
Geobacter metallireducens	Acetate	Mediator-less MFC (77)
Geobacter sulfurreducens	Acetate	Mediator-less MFC (76; 90)
Gluconobacter oxydans	Glucose	Mediator (HNQ, resazurin or thionine) needed (91)
Klebsiella pneumoniae	Glucose	HNQ as mediator biomineralized manganese as electron acceptor (92; 93)
Lactobacillus plantarum	Glucose	Ferric chelate complex as mediators (69)
Proteus mirabilis	Glucose	Thionin as mediator (94; 95)
Pseudomonas aeruginosa	Glucose	Pyocyanin and phenazine-1 carboxamide as mediator (96; 97)
Rhodoferax ferrireducens	Sucrose, Maltose Glucose, Xylose	Mediator-less MFC (73; 98)
Shewanella oneidensis	Lactate	Anthraquinone-2,6-disulfonate (AQDS) as mediator (99)
Shewanella putrefaciens	Lactate, Pyruvate, Acetate, Glucose	Mediator-less MFC but incorporating an electron mediator like Mn (IV) or NR into the anode enhanced the electricity production (100; 101; 102)
Streptococcus lactis	Glucose	Ferric chelate complex as mediators (69)

Bacteria are able to use several different types of electron acceptors, however MFCs were bacteria able to transfer electrons outside the cell that facinated us. Such bacteria are called *exoelectrogens,* "exo-" for exocellular and "electrogens" due to their ability to directly transfer electrons to a chemical or material which is not the immediate electron acceptor. Usually anaerobes can only transfer electrons to soluble substances such as nitrate and sulfate. These compounds are not synthesized in the cell but can diffuse across the cell membrane and into the cell. However exoelectrogenic bacteria differ from these anaerobes in their ability to directly transport electrons outside of the cell that allows them to function in an MFC.

The diversity of bacteria that have exoelectrogenic activity is being discovered nowadays. By studying exoelectrogens from two dissimilatory metal reducing genera (Shewanella and Geobacter), huge amount of information has recently been obtained. With the help of the availability of genome sequences for a number of isolates more about the fundamental nature of electrogenesis can be revealed (62, 61). *E. coli* can also show electrogenic activity. However, this result has not been verified that the reactor remained a pure culture of *E. coli.*

1.2.4 DESIGN OF MICROBIAL FUEL CELLS

1.2.4.1 MICROBIAL FUEL CELL COMPONENTS

In a typical MFC an anodic chamber is separated from a cathodic chamber by a PEM as shown in Figure 1.2. However in a one-compartment MFC the need for the cathodic chamber is eliminated by exposing the cathode directly to the air. Table 1.2 shows a summary of MFC components and the materials used to construct them (56, 45, 66, 87).

Table 1.2 Basic components of Microbial Fuel Cells

Items	**Materials**	**Remarks**
Anode	Graphite, graphite felt, carbon paper, carbon-cloth, Pt, Pt black, reticulated vitreous carbon (RVC)	Necessary
Cathode	Graphite, Graphite, graphite felt, carbon paper, carbon-cloth, Pt, Pt black, RVC	Necessary
Anodic chamber	Glass, polycarbonate, Plexiglas	Necessary
Cathodic chamber	Glass, polycarbonate, Plexiglas	Optional
Proton Exchange system	Protone exchange membrane: Nafion, Ultrex, polyethylene.poly (styrene-co-divinylbenzene); salt bridge, porcelain septum, or solely electrolyte	Necessary
Electrode catalyst	Pt, Pt black, MnO_2, Fe^3, polyaniline, electron mediator immobilized on anode.	Optional

1.2.5 USES OF MICROBIAL FUEL CELLS

1.2.5.1 ELECTRICITY GENERATION

In the MFCs, the chemical energy stored in the chemical compounds in a biomass is converted to electrical energy with the help of microorganisms. In this process chemical energy from the degradetion of organic molecules is converted directly into electricity instead of heat. This results in theoretically a much higher conversion efficiency (>70%) as it occurs in conventional chemical fuel cells than the Carnot cycle with a limited thermal efficiency. In their studies Chaudhury and Lovley (73) showed that *R. ferrireducens* could produce electricity yielding as high as 80% electron. Up to 89% electron recovery as electrocity was also reported (44). During the oxidation of formate with the catalysis of Pt black Coulombic efficiency of 97% was also reported (87). However, due to the low rate of electron abstraction MFC power generation is still very low (45, 98). Storing the electricity in rechargeable devices and then distributing it to end-users is an efficient way to solve this problem (54). Capacitors that works in a pulsed manner were used in their biologically inspired robots named EcoBot I to store the energy generated by the MFCs. For powering small telemetry systems and wireless sensors for which low power is sufficient to transmit signals, MFCs are suitable (45,

76). Especially in underdeveloped regions of the world MFCs can serve as distributed power systems for local uses. According to some researchers, MFCs are perfect energy supply candidate for Gastrobots by self-feeding the biomass collected by themselves (76). MFCs that utilize different fuels like sugar, fruit, dead insects, grass and weed would provide energy to realistic energetically autonomous robots. The robot EcoBot-II is only powered by MFCs for its behaviors such as motion, sensing, computing and communication (56, 90, 87).

The source of power for local consumption is the locally supplied biomass. It also possible to use MFCs in a spaceship since they can supply electricity by degrading wastes generated onboard. Some scientist think that their usage in a human body will expand in the future such as implanting a miniature MFC in a body to provide power for an implantable medical device with the nutrients supplied by the human body (87). The MFC technology is usually chosen for long-lasting power applications. Once the potential health and safety issues brought by the microorganisms in the MFC are thoroughly solved, then they could be applied for this purpose.

1.2.5.2 BIOHYDROGEN

Instead of electricity MFCs can be easily modified to produce hydrogen. Under normal conditions, during the reaction protons are released in the anode and they migrate to cathode to react with oxygen to form water. It is thermodynamically unfavorable to generate hydrogen from the protons and the electrons produced by the metabolism of microbes in an MFC. To overcome the thermodynamic barrier, Liu *et al.* (65) applied an external potential to increase the cathode potential in a MFC circuit. In this case, protons and electrons are produced by the anodic reaction and they are combined at the cathode to form hydrogen. The required external potential to generate hydrogen at an MFC is theoretically 110 mV, much lower than the 1210 mV required for direct electrolysis of water at neutral pH since some energy comes from the biomass oxidation process in the anodic chamber. Approximately 8–9 mol H_2/mol glucose can be produced in MFCs, while it is 4 mol H_2/mol glucose in conventional fermentation (87). Oxygen is not needed in the cathodic chamber in biohydrogen production using

MFCs, because of this MFC efficiencies improve since oxygen leak to the anodic chamber is no longer a problem. To be able to accumulated and stored hydrogen for later usage to overcome the inherent low power feature of the MFCs is another advantage. Therefore, MFCs provide a renewable hydrogen source that can contribute to the overall hydrogen demand (65).

1.2.5.3 WASTEWATER TREATMENT

The MFCs were used to purify waste water early in 1991 (23). Municipal wastewater usually contains high amount of organic compounds that can fuel MFCs. When the power is generated by MFCs in the wastewater treatment process, it can potentially reduce the electricity needed in a conventional treatment process that consumes a lot of electric power aerating activated sludges to half. MFCs result in 50–90% less solids to be disposed of (67).

In addition, some organic molecules such as propionate, butyrate and acetate, can be broken down to CO_2 and H_2O. A hybrid incorporating both electrophiles and anodophiles are especially apposite for wastewater treatment due to the possibility of biodegradation of organics by a variety of organism. MFCs are using certain microbes which are able to remove sulfides as required in wastewater treatment (98). The growth of bioelectrochemically active microbes can be amplified during wastewater treatment which leads to good operational stabilities. Single-compartment MFCs, membrane-less and continuous flow MFCs are accepted for wastewater treatment because of the concerns in scale-up (34, 76, 98). The wastes with rich organic matters such as food processing wastewater, swine wastewater, sanitary wastes and corn stover are great biomass sources for MFCs (45, 74, 76, 87, 98). In some cases it is possible to remove COD up to 80% (54, 56) with a Coulombic efficiency of 80% (66).

1.2.5.4 BIOSENSORS

In addition to aforementioned applications, MFC can be used as a sensor for pollutant analysis and in situ process monitoring and control (40). MFCs are possible biological oxygen demand (BOD) sensors because of the proportional correlation between the Coulombic yield of MFCs and the strength of the wastewater (45). One of the efficeint methods to measure the BOD value of a liquid stream is to calculate its Coulombic yield (76). According to some research there is a good linear relationship between the Coulombic yield and the strength of the wastewater in a quite wide BOD concentration range. Nevertheless, a long response time is required for a high BOD concentration. Because the Coulombic yield can be calculated only after the BOD has been decreased unless a dilution mechanism is in place. Scientists are making research to improve the dynamic responses in MFCs used as sensors (56). A low BOD sensor can also show the BOD value based on the maximum current since the current values increase with the BOD value linearly in an oligotroph-type MFC. In this stage, the substrate concentration limits the anodic reaction. It is also possible to apply this monitoring mode to real-time BOD determinations for either secondary effluents, surface water, or diluted high BOD wastewater samples (23). MFC-type of BOD sensors functions better than other types of BOD sensor because they have supreme operational stability and great accuracy and reproducibility. An MFC-type BOD sensor build up with the microbes enriched with MFC can be kept functional for over 5 years with no additional maintenance (23), far longer in service life span than other types of BOD sensors reported in the literature.

1.3 GLUCOSE OXIDASE

Glucose oxidase which has wide variety of technological applications since 1950’s is a nonhydrolytic enzyme that has fungal origin (108). Glucose oxidase (GOD) is purified mainly from the genus Aspergillus (109, 110) and Penicillium (111, 112) as well as other fungal sources. However the most commonly utilized species for the production of GOD is *Aspergillus niger* (113).

The function of GOD (β-D-glucose:oxygen 1-oxidoreductase) enzyme is to catalyze the oxidation of β-D-glucose to gluconic acid. During the oxidation GOD utilizes molecular oxygen as an electron acceptor and produces hydrogen peroxide (H_2O_2) simultaneously (114). GOD has many commercial applications such as removal of glucose from dried egg; color, flavor and shelf life improvement; removal of oxygen from fruit juices, canned beverages; and from mayonnaise to prevent rancidity. GOD is also used together with catalase in an automatic glucose assay kit (114) and it is mainly involved in biosensors for the detection and estimation of glucose in industrial solutions and in body fluids such as blood and urine (115).

1.3.1 GLUCOSE OXIDASE REACTION MECHANISM

GOD is a flavoprotein that uses molecular oxygen as an electron acceptor to catalyse the oxidation of β-D-glucose to D-glucono-δ-lactone and H_2O_2 (110, 113). This reaction has two steps; a reductive and an oxidative step (Figure 1.3). In the reductive step, the oxidation of β-D-glucose to D-glucono-δ-lactone is catalyzed by GOD. Then D-glucono-δ-lactone is non-enzymatically hydrolyzed to gluconic acid and the flavine adenine dinucleotide (FAD) ring of GOD is reduced to $FADH_2$ (116). In the oxidative step, the reduced GOD is reoxidized by oxygen yielding H_2O_2. The H_2O_2 is then cleaved by catalase (CAT) and producing water and oxygen.

1.3.2 COMPOSITION OF GLUCOSE OXIDASE

GOD obtained from ascomycetes is a dimeric glycoprotein having two identical polypeptide chain subunits which are covalently linked by disulfide bonds (117, 118). In Figure 1.4 the FAD moiety and the conserved active site residues of glucose oxidase from *P. amagasakiense* are shown (119). Each subunit of the *P. amagasakiense* GOD contains one mole of tightly bound, but not covalently linked FAD moiety as co-factor (117, 116). GOD obtained from *P. amagasakiense* is glycosylated with a mannose-rich carbohydrate content of about 11–13% (120, 121). Tyr-73, Phe-418, Trp-430, Arg-516,

Asn-518, His-520 and His-563 are the key conserved active-site residues of GOD ontained from *P. amagasakiense* (Figure 1.4) (116). Arg-516 is the most critical amino acid for the efficient binding of β- D-glucose by GOD, while Asn-518 contributes lesser according to Witt *et al.*'s conclusion (116). For the correct orientation of the substrate and for the maximal velocity of glucose oxidation the aromatic residues Tyr-73, Phe-418 and Trp-430 are important. Also His-520 and His-563 residues form hydrogen bonds with the 1-OH of glucose during the reaction.

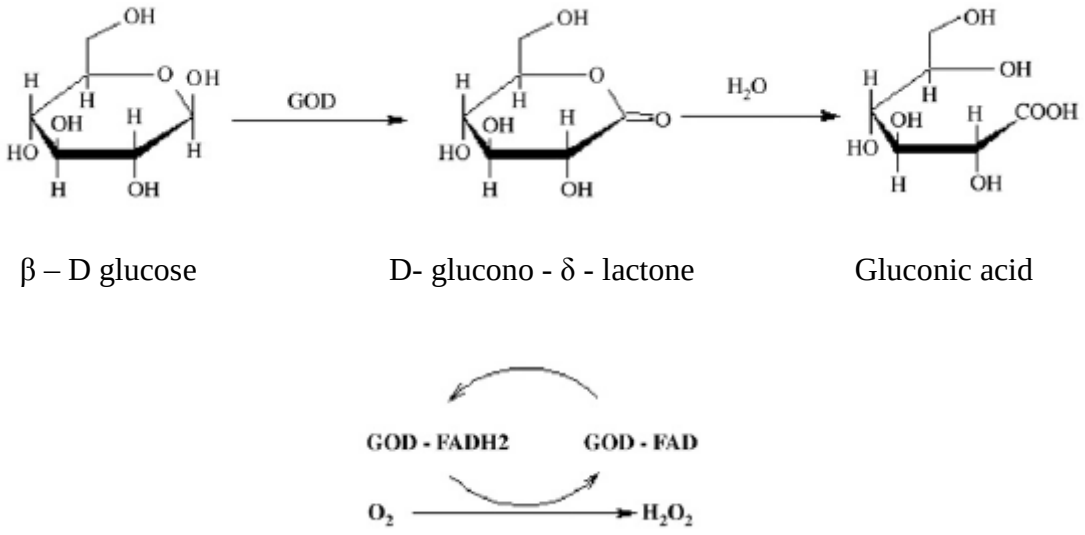


Figure 1.3 Representation of the Glucose oxidase reaction (116)

1.3.3 GENETIC EXPRESSION FOR GLUCOSE OXIDASE PRODUCTION

The fungal GODs are homodimers of about 150–170 kDa. They contain two tightly, but non-covalently bound FAD cofactors, and approximately 11–13% carbohydrate moiety of the high-mannose type. During the production of GODs problems like low productivity and concomitant production of other enzymes such as CAT are encountered. To prevent these, rather than natural sources genetically modified microorganisms for the expression of this enzyme has been strongly suggested (122).

Although GOD is commercially available, there is also much interest in its new forms having useful properties for special applications in biotechnology.

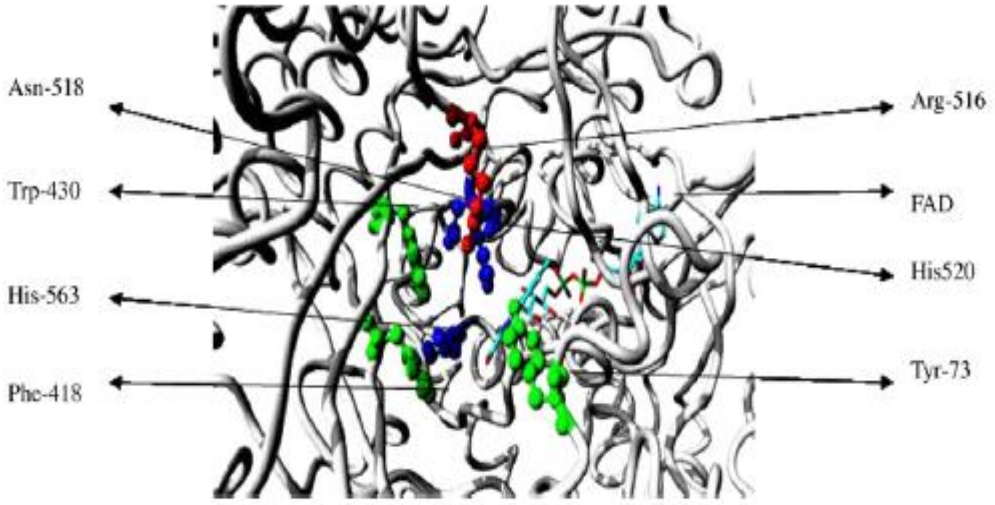


Figure 1.4 Glucose oxidase from *P. amagasakiense* showing the FAD moiety, indicating the key conserved active site residues (119)

The GOD gene of *A. niger* was characterized (110, 123, 124) and its crystal structure was elucidated (125) in order to improve the properties of enzyme by protein engineering. The *A. niger* gene encoding GOD was expressed in *S. cerevisiae* by Malherbe *et al.* (126). They evaluated the transformants for lower alcohol production and inhibition of wine spoilage organisms such as acetic acid bacteria and lactic acid bacteria during fermentation. Also to obtain biopreserved wines with lower alcohol content new strains of *S. cerevisiae* were obtained. An antimicrobial yeast starter culture system, on selective agar plate and in liquid assay was done in order to test this novel product. Due to the production of H_2O_2, a final product of GOD enzymatic reaction and also a known antimicrobial agent, the yeast transformants showed antimicrobial activity in a plate assay. Production of δ- glucono-1, 5-lactone and gluconic acid from glucose by GOD resulted in 1.8–2.0% less alcohol containing wines.

In the last studies, yeasts such as *Hansenula polymorpha* and *S. cerevisiae* have been stated as promising high-yield production systems and recommended for heterologous GOD production. However but further studies showed hyperglycosylation with yeast which may lead to vital limitations of usage (127). There is always limitations at expression of recombinant GOD by using *E. coli* (128) and *S. cerevisiae* (123, 129). When obtained from *E. coli*, 60% of the recombinant protein was inactive, whereas in *S. cerevisiae* the recombinant GOD was hyperglycosylated and showed reduced substrate binding capacity and catalytic activity (130). Methylotrophic yeast *Pichia pastoris* was used as host for expression and secretion of recombinant GOD of the filamentous fungus *P. variabile* P16 by Crognale *et al.* (131). They transformed the gene to *P. pastoris* X33 which is a strain that is largely used for selection on zeocin and large scale growth studies. They showed that *P. pastoris* is an efficient host for expression of both secreted and intracellular heterologous proteins. Fermentation in 3 l fermenter increases the GOD production up to 50 U/ml about four times when compared to *P. variabile* P16 cultivated under optimized conditions.

1.4 HYPOTHESIS

The specific aims of this work presented here: as an ideal model enzyme for bioelectrochemistry assays, glucose oxidase gene from *Aspergillus niger* was selected to clone and overexpress into a prokaryotic expression system, *E. coli* in order to collect much more electrons in a microbial fuel cell system.

After testing the power increase in a model system, *E. coli*, glucose oxidase gene will be transferred for overexpressing of glucose oxidase enzyme into metal reducing bacteria, *Shewanella putrefaciens.* By using the general molecular cloning methods for creating a recombinant *Shewanella putrefaciens*, the possibility of generation electricity will be tested and tried to increase in an MFC.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 CHEMICALS

2.1.1.1 PREPARATION OF MEDIA AND REAGENTS

2.1.1.1.1 SHEWANELLA CULTURE MEDIUM

Luria – Bertani (LB) agar and broth (Merck) were used for the cultivation of *Shewanella putrefaciens.* The composition of the growth media for *S. putrefaicens* was as follows:

Luria-Bertani (LB) broth medium: (per litre)

Tryptone (pancreatic digest of casein)	10 g
Yeast extract	5 g
NaCl	5 g

For the preparation of 500 mL LB broth medium, 10 g powder was suspended in 500 mL of disstilled water. The media were autoclaved at 121^{o}C for 15 minutes, then cooled to 50^{o}C, and stored at 4^{o}C until use.

Luria-Bertani (LB) agar medium: (per litre)

Tryptone (pancreatic digest of casein)	10 g
Yeast Exract	5 g
NaCI	5 g
Agar	15 g

For the preparation of 500 mL LB agar medium, 17.5 g powder was suspended in 500 mL of disstilled water. The suspention was heated to boiling while stirring to dissolve all ingredients completely. The media were autoclaved at 121^{o}C for 15 minutes, then cooled to 50^{o}C. Afterwards, the sterile media were poured into sterile Petri dishes. Petri dishes were cooled in order to solidify the media under aseptic conditions, and then sealed with stretch film and stored at 4^{o}C until use.

2.1.1.1.2 MEDIA FOR THE GROWTH OF ASPERGILLUS

Malt Extract Agar (MEA) was used to grow *Aspergillus niger.* The composition of the growth medium for Aspergillus niger was as follows:

Malt Extract Agar (MEA) medium: (per litre)

Maltose, Technical	12.75 g
Dextrin	2.75 g
Glycerol	2.35 g
Peptone	0.78 g
Agar	15.0 g

Appropriate amount of MEA medium was weighted and dissolved in 500 ml distilled water used for cultivation of this fungus. The suspention was heated to boiling

while stirring to dissolve all ingredients completely. The media were autoclaved at 121^{o}C for 15 minutes, then cooled to 50^{o}C. Then, the sterile media were poured into sterile Petri dishes. Petri dishes were cooled in order to solidify the media under aseptic conditions, and then sealed with stretch film and stored at 4^{o}C until use.

2.1.1.1.3 MEDIA FOR THE GROWTH OF *E. COLI*

LB is the most commonly used medium for the growth and expression of *E. coli*. The preparation and content of this medium as described in Subheading 2.1.4.1. After sterilization of LB broth, the medium was allowed to cool to ~55°C before adding antibiotic (either ampicillin, 100 μg/ml final concentration; or chloramphenicol, 50 μg/ml final concentration).

For preparation of LB plates plus antibiotic; after autoclaving, the medium was allowed to cool to 50°C before adding ampicillin to a final concentration of 100 μg/ml and poured the plates. 30–35ml of medium was poured into 100mm petri dishes. The agar was let to harden. All plates were stored at 4°C for up to 1 month or at room temperature for up to 1 week.

2.1.1.1.4 REAGENTS

Ampicillin Stock Solution

50 mg/ml of ampicillin was dissolved in water, filter sterilized, stored in aliquots at −20°C.

IPTG stock solution (0.1M)

1.2g of IPTG was added deionized water to a final volume of 50ml. Filter-sterilize and store at 4°C.

2.2 METHODS

2.2.1 MICROBIOLOGICAL METHODS

2.2.1.1 CULTIVATION OF MICROORGANISMS

2.2.1.1.1 AEROBIC GROWTH OF *Shewanella putrefaciens* ATCC 8071

S. putrefacience was grown on LB agar at 25°C . For aerobic growth, fresh cultures of 200 ml in 1000 m flasks were shaken continuously on a rotary shaker (Sartorious Certomat IS) at 160 rpm and 25°C. After 36 h of growth, the suspention were pipetted into a 50 ml sterile Falcon tubes and centrifuged at 13,000 X g, for 15 min at 4°C. After centrifugation, the supernatant was discarded and the pellet was washed three times with 10 ml of 50 mM Na-phosphate buffer, pH 7.0, containing 0.1 M NaCl.

After sub-culturing of *Shewanella putrefaciens*, 0.5 ml of fresh culture was aliquoted into 5 sterile 1.5 ml Ependorf tube and stored, following the addition of sterile 50% glycerol in each tube, at -70°C for up to 1 yr.

2.2.1.1.2 ANAEROBIC GROWTH OF *Shewanella putrefaciens* ATCC 8071

Active aerobically grown cultures of *S. putrefaciens* and approximately 2 liter of sterile LB Broth () in a glass bottle were transferred to a glove box anaerobic chamber (Controlled Atmospheric Chamber, Plas By Labs, USA) and degassed with recirculated interior atmosphere (95%N2/5%H2) for 15 min prior to inoculation to remove dissolved oxygen from growth media. 20 ml of an aerobically grown overnight culture was inoculated into 2 liter of sterile LB Broth and incubated without agitation in the anaerobic gas chamber. After 96 h of growth, the cells were transferred and distributed into 50 ml sterile centrifuge tubes under anaerobic conditions. And then all tubes were centrifuged at 13,000 X g, for 15 min at 4°C. The supernatant was discarded and the pellet was washed three times with in sterile anoxic 50 mM Na-phosphate buffer, pH 7.0, containing 0.1 M NaCl. After washing the cells three times, the pellet was resuspended with the same anoxic buffer to the desired cell concentration, estimated by

reading optical density, which will be converted to cell concentration using a pre-established calibration curve (dry cell weight, g *vs.* OD at 660 nm) for the bacteria.

2.2.1.1.3 GROWTH OF *Aspergillus niger*

Stock culture of *Aspergillus niger* was grown on Malt Extract Agar plates (MEA) (Difco, Detroit, Mich.) at room temperature for 5 to 7 days. The fungus was maintained on MEA at room temperature with periodic transfer.

2.2.1.1.4 GROWTH OF *E. coli*

Escherichia coli HB 101, TOP 10 and BL21 (DE3) pLysS served as host for DNA manipulation. *E. coli* transformants were grown at 37°C in the Luria- Bertani (LB) broth and on LB agar plates containing 100 µg of ampicillin and 50 µg of chloramphenicol per ml with shaking.

2.2.2 MOLECULAR METHODS

2.2.2.1 CLONING OF GLUCOSE OXIDASE GENE INTO pBAD / His B VECTOR AND HETEROLOGOUS EXPRESSION OF GOX GENE IN *E. coli* TOP 10

2.2.2.1.1 ISOLATION OF GENOMIC DNA FROM *Aspergillus niger*

DNA was extracted using a High Pure PCR Template Preparation kit (Roche) but with a modification of the manufacturer' s protocol. Approximately 200 mg mycelia was added aseptically to a 1.5 ml microcentrifuge tube and mixed with 200 µl sterile PBS. 10 µl lysozyme (5mg/ml) was added into the solution and the microcentrifuge tube was incubated at 37^{o}C for 30 min in order to lyse the cells. Then 200 µl Tissue Lysis Buffer and 40 µl reconstituted Proteinase K solution were added to lysed cells and

mixed the contents of the tube immediately. The tube was incubated for 10 min at 70^{o}C. After the incubation, sample was mixed with 100 µl isopropanol. One High Pure Fitler Tube was inserted into one Collection Tube. The entire sample was pipetted into upper buffer reservoir of the Fitler Tube. The entire High Pure Tube assembly a standart tabletop microcentrifuge, then the tube assembly was centrifuged for 1 min at 8000 x g. After centrifugation, the Filter Tube was removed from the Collection Tube and the liquid and the Collection tube were discarded. 500 µl Inhibitor Removal Buffer was added to the upper reservoir of the Filter Tube assembly. The centrifugation step was repeated (1min at 8000 x g) and the liquid and Collection Tube were discarded. The Filter Tube was reinserted in a new Collection Tube. 500 µl Wash Buffer was added to the upper reservoir of the Filter Tube. The centrifugation was repeated again. After the centrifugation, the Filter Tube was removed from the Collection Tube and the liquid and the Collection tube were discarded again. The wash step and centrifugation were repeated. Flowthrough was discarded. The Filter Tube-Collection Tube assembly was spinned for 10 s at maximum speed (approx. 13,000 x g) to remove residual Wash Buffer. The Collection Tube was discarded and the Filter Tube was inserted in a clean, sterile 1.5 ml microcentrifuge tube. 200 µl of prewarmed (70^{o}C) Elution Buffer was added to the Filter Tube. The tube assembly was centrifuged for 1 min at 8000 x g.

The eluated DNA was checked by %1 agarose gel electrophoresis under the UV-transilluminator (Gel-Doc, Biorad - USA) and single band was observed near under the loaded well.

Quantification of total DNA was carried out by absorbance at 260 and 280 nm using a Shimadzu UV-VIS spectrophotometer. The absorbance at 260 and 280 nm ratio (260/280) was used to assess the purity of the DNA.

2.2.2.1.2 AMPLIFICATION OF GLUCOSE OXIDASE GENE

2.2.2.1.2.1 DESIGN OF DEGENERATE PRIMERS

One of the most important cloning strategy is the addition of Restriction Endonuclease Sites into PCR product if necessary. For that reason, the oligonucleotide primers were designed to include restriction enzyme recognition sites near their 5' termini.

Suitable forward and reverse primers including the appropriate restriction-enzyme site at the 5' end were designed using a websites that were useful in aiding primer design. A particularly good one used in this study was Primer 3 (www-genome.wi.mit.edu/cgi-bin/primer/ primer3_ www.cgi). In order to amplify the glucose oxidase gene of *Aspergillus niger* using both first and second PCR primers listed on Table 2.1. Primers, F1 and R1 were used to first amplify the glucose oxidase gene as design by Masaki et al. (132). At the end of the first PCR, amplicon expected size was ~2000 bp. A second set of primers, F2 and R2 used for the second PCR was designed in this study. The second protocol (nested PCR) was predicted to amplify a 1818 bp DNA fragment.

Primer F2 (5'-TCCTCGAGACTCTCCTTGTGAG-3'), containing a *XhoI* restriction site (underlined), was used as the forward primer for the second PCR. The reverse primer, R2 (5'- ACCAGCTGCATGGAAGCATAATCT -3'), contained a *PvuII* restriction site (underlined). Both primers were obtained from Iontek (Istanbul, TR).

2.2.2.1.2.2 NESTED PCR CONDITIONS

PCR was carried out in a Thermocycler Techne TC512 Gradient Termal Cycler in thin-walled 0,2 ml PCR tubes. The first PCR protocol was as follows: 2 µl (~200-300 ng) of gDNA was added to a 50 µl reaction containing 4 µl dNTPs (10µM) (Fermentas), 2.5 µl (0.5 µM) forward (F1) and reverse primers (R1) (Iontek, Turkey), 0.25 µl Ex Taq HS DNA polymerase (Takara), and 5µl 10X Ex Taq HS DNA polymerase buffer with

Mg^{++} (Takara), 33.75 µl PCR graded H_2O. The reaction mixture was spin down for 10 s and performed for the First PCR reaction.

First PCR amplification reactions were as described below:

94°C, 2 min for initial denaturation

94°C, 30 sec for denaturation
62°C, 30 sec for annealing } x 30 cycle
72°C, 2.5 min for extension

72°C, 5 min for final extension

Table 2.1 Oligonucleotide primers used for PCR of glucose oxidase gene of *Aspergillus niger*

Primer	**T_M (^0C)**	**Length of primers (bp)**	**Oligonucleotide Sequences**
F1	62.67	22	5'-CCTTTCCTCTCTCATTCCCTCA
R1	57.08	22	5'-AATGCCCTTGTTTGGTAGTAAT
F2	62.67	22	5'- TCCTCGAGACTCTCCTTGTGAG
R2	62.86	24	5'-ACCAGCTGCATGGAAGCATAATCT

The expected PCR product size was 2000 bp. First PCR product was run on a 1% TAE gel stained with ethidium bromide (5 mg/ml) to confirm successful amplification. A 1: 100 dilution of first round reaction was made to be used as template for the second round of PCR. This reaction mixture contains 5 µl 10 X buffer (Takara), 2 µl 0,2 µM of all four dNTPs, 1,5 µl (10 µM) second set of primers, F2 and R2, 0,2 µl Prime Star DNA polymerase, 1 µl of template DNA and 13,3 µl PCR graded H_2O in a 50 µl reaction volume. Totally 5 tubes (250 µl) were prepared by the same way. A second round was performed for 30 cycles. Before 30 cycles of second PCR, initial

denaturation (1,5 min at 94^{o}C) step was performed. After this step, a 30 cycles of melting (30 s at 94^{o}C), annealing (20 s at 63^{o}C), extention (2 min at 72^{o}C) was performed. The reaction was ended with a final extension 10 min at 72^{o}C and cooled to 4^{o}C for storage.

Amplification products were fractionated by electrophoresis in 1% agarosa / TAE gels and appropriate bands (about 1818 bp) excides. The DNA was extracted with a commercial kit.

2.2.2.1.3 GEL ELECTROPHORESIS

1% agarose gel was prepared to visualize the PCR products. 0,4 g powdered agarose (AppliChem, Germany) was dissolved in 40 ml 1 x Tris Acetate EDTA (TAE) buffer by using microwave oven. It was heated on nearly 45 second until boiling by pulse and stirred with 15 second intervals. The gel was cooled to 50°C and 2 µl of ethidium bromide (5 mg/ml) was added. The gel was then poured into horizontal agarose gel platform and and a comb was placed in the gel before polymerization. Afterwards, totally 250 µl of PCR product with approximately 20 µl of 6x DNA Loading Dye (Fermentas) were mixed and loaded on to a large well that is illustrated in Figure 2.1. Then, 1 µl DNA size marker from Fermentas (GeneRuler^{TM} 1kb DNA Ladder) with 2 µl of Loading Dye and 6 µl of ddH_2O mixture were loaded into the other well. The electrophoresis tank was filled with 1X TAE running buffer and the gel was run for 45 minutes at 100 volts. After running, the gel was placed in Gel Doc 2000 (Biorad, Milan, Italy) apparatus and the band was visualised and photographed on a UV transilluminator.

The gel was examined under UV light in order to determine whether DNA band of interest was present on the appropriate place on the gel according to molecular size marker or not. Only a band (about 1818 bp) was detected. This band was isolated from gel as described below. DNA was removed from the gel with the High Pure DNA Extraction Kit (Roche, Germany) as per the manufacturer's instructions.

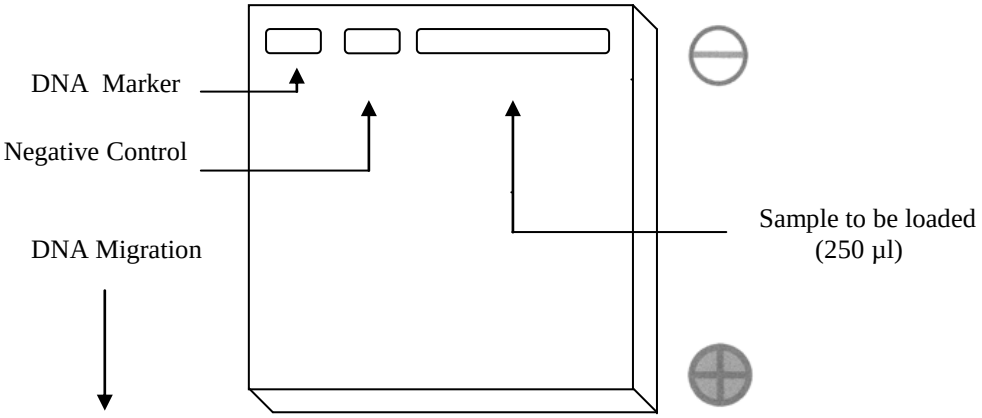


Figure 2.1 Schematic illustration of an agarose gel well preparation before loading the large volume of PCR products.

2.2.2.1.4 DNA PURIFICATION FROM AGAROSE GEL

In this experimental step, the amount of UV exposure time on the DNA was minimised. This was because the UV mutagenises the DNA at a measurable rate. After evaluation of correct DNA band of interest immediatly, proper area was cut using a ethanol-cleaned scalpel and transferred excised agarose gel slice in a sterile 1,5 ml microcentrifuge tube. Gel mass was determined by first pre-weighting the tube, and then the tube with the excised gel slice was re-weighted. 300 µl Binding Buffer for every 100 mg gel slice was added to the microcentrifuge tube. Agarose gel slice was dissolved in order to release the DNA. For that purpose, the microcentrifuge tube was vortexed 15 – 30 s to resuspend the gel slice in the Binding Buffer. The suspention was incubated for 10 min at 56^{o}C. The tube was vortexed briefly every 2 – 3 min during incubation. After the agarose gel was completely dissolved, 150 µl isopropanol was added to the tube for every 100 mg agarose gel slice. The tube was vortexed throughly. One High Pure Fitler Tube was inserted into one Collection Tube. The entire contents of the microcentrifuge tube was pipetted into upper reservoir of the Fitler Tube. The tube assembly was centrifuged for 30 – 60 s at maximum speed in a Standard table top centrifuge at +15 to +25^{o}C. After centrifugation, the flowthrough solution was

discarded and Filter tube with the same Collection Tube were reconnected. 500 µl Wash Buffer was added to the upper reservoir. The centrifugation step was repeated (1min at maximum speed) and the liquid was discarded. The Filter Tube was recombined with the same Collection Tube. 200 µl Wash Buffer was added to the upper reservoir of the Filter Tube. The centrifugation was repeated again. After the centrifugation, the Collection Tube and the flowthrough solution were discarded. Filter Tube was inserted in a clean, sterile 1.5 ml microcentrifuge tube. 100 µl Elution Buffer was added to the upper reservoir of the Filter Tube. The tube assembly was centrifuged for 1 min at maximum speed. After the final step, the microcentrifuge tube was contained the purified DNA (Glucose oxidase gene from *Aspergillus niger*). It was stored at -20^{o}C until use.

Concentration of DNA in the samples was measured by spectrophotometry by taking the OD_{260} reading and using the following formula: micrograms of DNA/ microliter = (A_{260}) * (Dilution Factor) * (50 micrograms/microliter) / 1000.

2.2.2.1.5 CLONING

A basic step in molecular biology is the cloning of a insert DNA into a plasmid vector. This allows the cloned fragment to be replicated upon transformation of the recombinant molecule into a bacterial cell so that the DNA of interest can be investigated further. Cloning is an fundamental part of many experiments, involving library generation and expression studies.

The vector and insert DNA are usually digested with a type II restriction endonuclease that cleaves at specific sites in the DNA. The two molecules must have compatible ends for cloning to proceed. The generation of compatible ends requires the use of restriction and modifying enzymes, which ultimately results in the generation of either blunt or overhanging ends. The cleaved fragments are mixed in the presence of DNA ligase that produces a mixture of products, some of which should consist of the vector containing the inserted DNA fragment.

Lots of vectors are commercially available to make easy cloning for various applications. In Figure 2.2, the basic steps of the cloning a gene are showed. Furthermore, specialized vectors are available for cloning polymerase chain reaction (PCR) products generated by *Taq* DNA polymerase (133). In this part, the DNA insert and vectors were prepared for cloning.

2.2.2.1.5.1 RESTRICTION DIGESTION

The first step in cloning a DNA insert into a plasmid vector is cutting both vector and insert DNA with the appropriate restriction enzyme(s) to generate compatible ends. This may be a simple single digestion or a double digestion with two enzymes in the case of directional cloning. A partial digestion may be needed in situations where there is a lack of suitable sites to use and the restriction enzyme cuts more than once in the molecule.

2.2.2.1.5.1.1 EXPRESSION VECTOR PREPARATION

pBAD/ His B vector (4.1 kb) was digested with *PvuII* (Fermentas) and *XhoI* (Fermentas). A blunt end was generated with *PvuII* restriction enzyme and a sticky end was created with *XhoI* restriction enzyme after digestion. Both restriction enzymes were worked in the same restriction enzyme buffer. As an important point here, the restriction buffer should be completely thawed and thoroughly mixed prior to use.

It is important that the reactions be set up on ice and that the restriction enzyme is added last. When using restriction enzymes, always the enzyme are taken from the freezer and placed immediately on ice. A separate pipet tip is used every time the enzyme is dispensed, to guard against contamination of the enzyme stock.

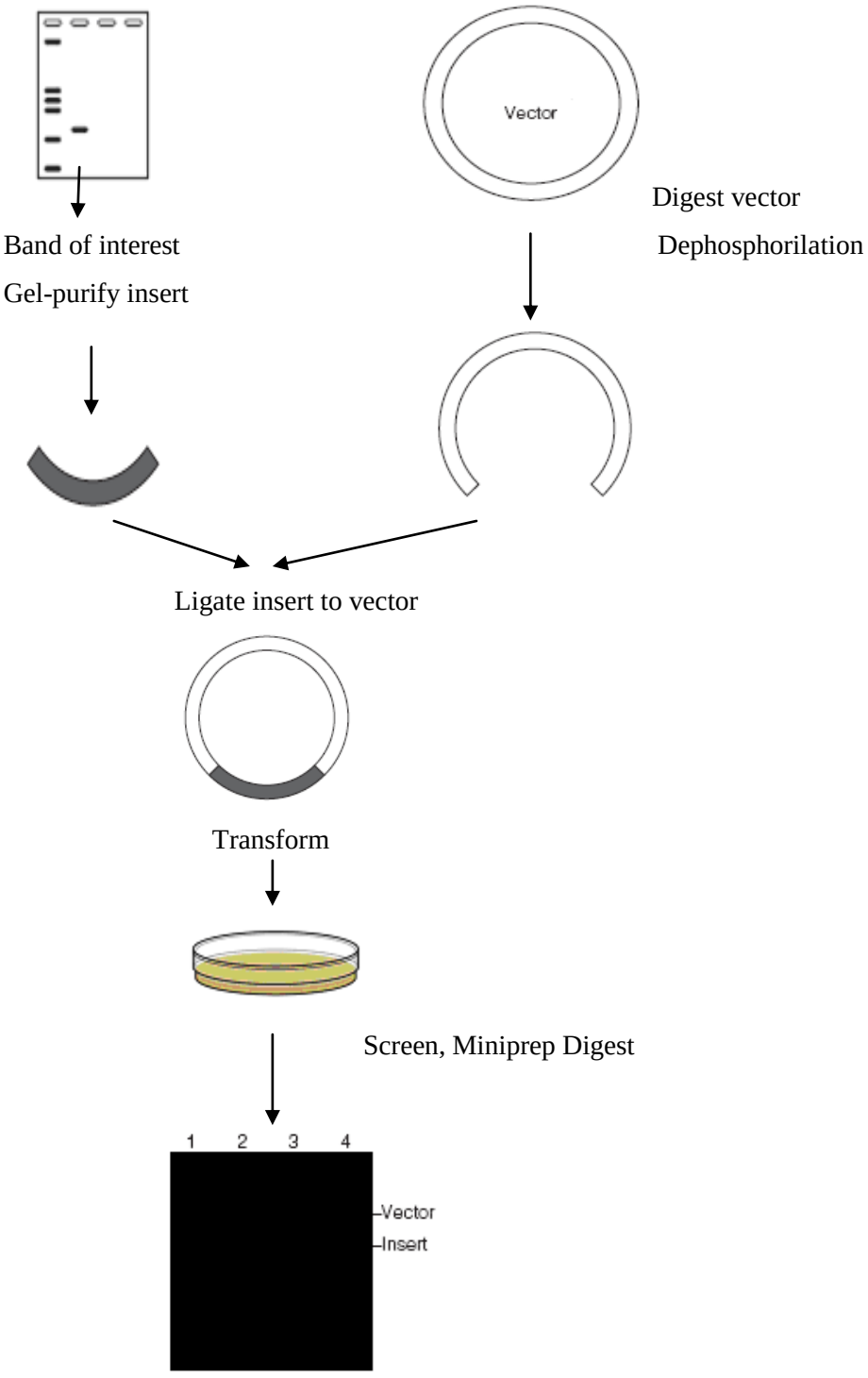


Figure 2.2 The Basic steps of the molecular cloning a gene

The following was added to a 1.5 mL Eppendorf tube on ice :

10X Restriction Buffer	5 µl
Vector DNA (~1 µg)	2,5 µl
XhoI (10u/µl)	0,5 µl
PvuII (10u/µl)	0,5 µl
Sterile ddH_2O	41,5 µl
Final Volume	**50 µl**

The suspention was mixed by pipetting and spinned to ensure that the contents was at the bottom of the microcentrifuge tube. The tube was incubated over night at 37°C. Dephosphorylation of digested vector was performed with CIAP (calf intestinal alkaline phosphatase) enzyme immediately. For that purpose, 2 µl of CIAP (1u/ µl) was added directly to the digested DNA sample to prevent the self- ligation of the cut plasmid (see Figure 2.3). The reaction mixture was incubated at 37°C for 1 hour. Afterwards, CIAP was inactivated by heating at 70 °C for 15 minutes.

It is important to remove CIAP completely from the mixture, as it may interfere with the efficiency of subsequent ligation reactions. The High Pure PCR Purification kit (Roche, Germany) was used to purify the mixture considering the manufacturers' instructions.

For the another study, pBAD/HisB vector DNA was cut with *PvuII*, linear vector was dephosphorylated as described below. Phosphorylated PCR product and dephosphorylated vector were ligated, then blunt end ligation was performed.

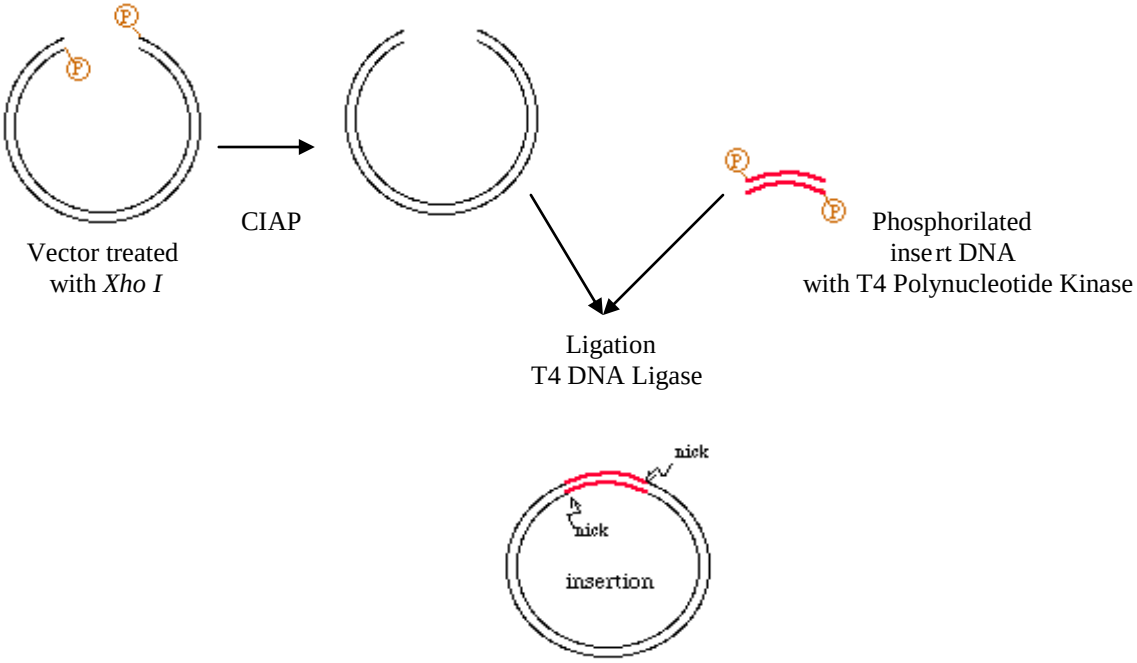


Figure 2.3 Shematic illustration showing the dephosphorilation of digested vector with CIAP and phosphorilation of insert DNA before ligation

2.2.2.1.5.1.2 DNA INSERT PREPARATION

Glucose Oxidase nested PCR product (1818 bp) was digested with *PvuII* and *XhoI* (Fermentas). A blunt end was generated with *PvuII* restriction enzyme and a sticky end was created with *XhoI* restriction enzyme after digestion.

The following were added to a 1,5 mL Eppendorf tube:

Restriction Enzyme Buffer	10 µl
Insert DNA	80 µl
XhoI	1 µl
PvuII	1 µl
Sterile dd H_2O	9,5 µl
Final Volume	**100 µl**

The reaction mixture was pipetted and spinned to ensure that the contents was at the bottom of the microcentrifuge tube. The tube was incubated over night at 37°C. Following the double digestion of insert, mixture was purified to remove the enzyme with a commercially available kit as described in Subheading 2.2.2.1.4.

For the second study, phosphorilation of insert DNA was performed with the given protocol below. The following reaction was set up in a 1,5 mL Eppendorf tube:

Cleaned- up Digested DNA (~250 ng)	80 µl
Kinase 10X reaction buffer	10 µl
T4 polynucleotide kinase	2 µl
100 m*M* ATP	1 µl
Sterile dd H_2O	7 µl
Final Volume	**100 µl**

The solution was incubated at 37^{o}C for 30 minutes. The DNA was purified by using the High Pure PCR Template Purification kit (Roche) (*see* the manufacturers' protocol in Subheading 2.2.2.1.4). Concentration of the phosphorylated insert was determined by spectrophotometer before performing ligation step.

DNA should not be resuspended in, or precipitated from, buffer containing ammonium salts prior to kinase treatment, as ammonium ions are strong inhibitors of bacteriophage polynucleotide kinase (133).

T4 polynucleotide kinase phosphorylates protruding 5' single-stranded termini more efficiently than recessed 5' termini or blunt ends, although the addition of sufficient polynucleotide kinase and ATP allows all termini to be completely phosphorylated (133).

2.2.2.1.5.2 DNA LIGATION

The final step in cloning is the joining of the linear DNA fragments together, referred to as ligation. This involves creating a phosphodiester bond between the 3'-hydroxyl group of one DNA fragment and the 5'-phosphate group of another and is equivalent to repairing nicks in a duplex strand. The enzyme most frequently used to ligate fragments is bacteriophage T4 DNA ligase. T4 DNA ligase (unlike *E. coli* DNA ligase) will join blunt-ended fragments, as well as cohesive-end fragments, efficiently under normal reaction conditions (133).

It is necessary to estimate the concentration of both vector and insert DNA before ligation. This may be estimated by agarose gel electrophoresis when run against molecular-weight markers of known concentration or by using a spectrophotometer. In this step, concentration of both vector and insert DNA was determined using a spectrophotometer by making a 1/100 dilution.

The following reaction was set up using the desired vector : insert molar ratio according to the concentration value (see Table 2.2). It is important to include appropriate control.

Table 2.2 Preparation of Ligation Mixtures

	1	**2**	**3**
	1 : 1	1 : 3	NC
	(molar ratio of vector : insert)		
Vector DNA[a]	2 µl	2 µl	2 µl
Insert DNA[b]	2,5 µl	1 µl	–
10X ligase buffer	1,5 µl	1,5 µl	1,5 µl
T4 DNA ligase (5 U/ µl)	1 µl	1 µl	1 µl
Sterile dH_2O to final volume	15 µl	15 µl	15 µl

[a]The size of vector (pBAD / His B) was 4.1 kb.

[b]The size of insert DNA was about 1.8 kb.

The ligation reaction components were added to three 0,5 ml microcentrifuge tubes, labelled as 1, 2, and 3, according to Table 2.4. The tubes, 1, 2, and 3 were incubated overnight at 16^{o}C. The samples of ligated products were transformed into the host strain immediately.

The ligation reaction should not exceed 0.5% of the transformation reaction volume. Typical ligation reactions use 50–200 ng of vector DNA. Excess DNA may inhibit the transformation. DNA will be less likely to circularize if the concentration of DNA is too high. Many manufacturers now produce rapid DNA ligation kits. These kits make it possible to ligate vector and insert in as little time as 5 min and so are useful when time is limiting and the transformation needs to be performed on the same day (133).

2.2.2.1.5.3 PREPARATION OF COMPETENT CELL

An LB culture was inoculated with *E. coli* TOP 10 cells (directly from the frozen stock without thawing) and the cells were grown overnight at 37°C. 5 mL of this overnight culture was added to 100 mL of LB medium in a 500 mL flask. Cells were grown to an OD_{600} between 0.4 or 0.6. This was the critical value. Do not exceed *0.6.* Culture was chilled on ice for 10 minutes. Then, culture was transferred to a sterile 250 mL centrifuge bottle and the cells were pelleted at 4000 *g* for 5 minutes at 4 °C. The LB medium was decanted off. The pellet was gently resuspended in 30 mL of cold CMG buffer. A large fraction of the cells remained as clumps. The cell suspension was incubated on ice for 15 minutes. The cells were pelleted at 4000 *g* for 5 minutes at 4 °C. The CMB buffer was decanted off. The cells were gently resuspended in 7.2 mL of cold CMB buffer and the suspension was transferred to a 50 mL disposable centrifuge tube. The cells should be thoroughly dispersed. The cell suspension was incubated on ice for 5 minutes. 250 µL of high quality DMSO was added, mixed well, and incubated on ice for 5 minutes. Another 250 µL of DMSO was added, mixed well, and incubated on ice for 5 minutes. The suspension was dispensed into sterile microcentrifuge tubes (250 µL aliquots is generally appropriate) and flash frozen in liquid nitrogen. Competent cells were stored at –80°C. Transformation efficiency was detected with pBAD/ HisB vector.

2.2.2.1.5.4 TRANSFORMATION OF *E. coli* CELL

The transformation was carried out as follows: One tube containing 250 µL of *E. coli* TOP 10 competent cells was removed from – 70^{o}C and thawad on ice. For each transformation, 75 µL of bacterial suspension was aliquoted into a sterile Eppendorf tube. Approximately 1 ng of plasmid DNA (control) was added into the tube containing competent cell for the control of transformation, and 5-10 µL ligation products were added into the other tube and mixed gently. Pipet up and down or vortex the tube was not done. For each set of transformations, a negative control that consists of competent cells without DNA, and a positive control using a standard plasmid were prepared. The transformation mix was placed on ice for 20 min. The transformation mix was transferred to a 42°C water bath and incubated for exactly 45 s. The temperature and time was crucial in this step. The tubes were not shaked. The transformation mix was immediately placed on ice for 2 min. 900 µL of room temperature LB broth was added. The tubes were incubated at 37°C for 30 minutes, shaking vigorously (approx 200 rpm) to ensure good aeration. A range of serial dilutions of the bacteria was prepared from the transformation mix (50 µL and 100 µL from each transformation) and spreaded on to the prewarmed LB agar plates containing 50 µg of ampicillin per ml. The agar plates were incubated overnight at 37°C.

After incubation, at least five colony were selected and each colony was inoculated into 3 ml of LB broth medium containing ampicillin (50 µg /ml). Then each tube was incubated at 37°C until reached with a density of 1.5-5.0 A_{600} units per ml. Arterwards, plasmid DNA was extracted from each transformants using High Pure Plasmid Isolation kit (Roche) and analyzed by restriction digestion.

2.2.2.1.5.5 MINI- PREPARATION OF PLASMID DNA

Mini- preparations of plasmids encoding the glucose oxidase were obtained from bacterial cells using the High Pure Plasmid Isolation Kit (Roche). The procedures were as follows: Before starting the procedure, you must place the Binding Buffer on ice. The bacterial cells from 1.5 ml of *E. coli* culture were pelleted. The supernatant was

discarded. 250 µL Suspension Buffer + RNase was added to the centrifuge tube containing the bacterial pellet. The bacterial pellet was resuspended and mixed well. The resuspended bacterial pellet was treated as follows: 250 µL Lysis Buffer was added and mixed gently by inverting the tube 3 to 6 times. In order to avoid shearing genomic DNA, vortex was not done. The tubes were incubated for 5 min at any temperature between +15°C and +25°C. In this step, the tubes were not incubated for more than 5 min. The lysed solution was treated as follows: 350 µL chilled Binding Buffer was added and mixed gently by inverting the tube 3 to 6 times, and then the suspention was incubated on ice for 5 min. The solution should become cloudy and a flocculant precipitate should form. All tubes were centrifuged for 10 min at approx. 13,000 x *g* (full speed) in a standard tabletop microcentrifuge. After centrifugation: One High Pure Fitler Tube was inserted into one Collection Tube. The entire supernatant of the microcentrifuge tube was transferred into upper reservoir of the Fitler Tube. The tube assembly was centrifuged for 1 min at full speed in a Standard table top centrifuge at +15 to +25^{o}C. After centrifugation, the flowthrough solution was discarded and Filter tube with the same Collection Tube were reconnected. In order to wash the cells 700 µl Wash Buffer II was added to the upper reservoir. The tubes were centrifuged for 1 min at maximum speed and the liquid was discarded. After discarding the flowthrough liquid, the centrifugation was repeated again and the collection tube was discarded. This step was performed to ensure removal residual Wash Buffer. Filter Tube was inserted in a clean, sterile 1.5 ml microcentrifuge tube. 100 µl Elution Buffer was added to the upper reservoir of the Filter Tube. The tube assembly was centrifuged for 1 min at maximum speed. All of tubes contained the eluted plasmid DNA. The eluted DNA was used in such applications as screening of clones and sequencing. The eluted DNA was stored at +2 to +8°C.

2.2.2.1.5.6 SCREENING OF CLONES BY PCR AND RESTRICTION ENZYME DIGESTION

In this method, the PCR reaction achieves multiple goals and can be used to detect the presence of the insert, determine the orientation of the insert based on the selected or designed primers.

The orientation of the insert DNA can be rapidly assessed with colony PCR combining a vector-specific primer and with an insert-specific primer. If your goal is to determine whether the colonies contain the insert of interest or not, vector-specific primers can be used to screen for recombinant plasmids.

In our study, this technique was used to screen for containing of a 1.8 kb (*GOx* gene) insert into the pBAD/HisB vector. Colony PCR was performed with the T7 Promoter Primer and either the insert-specific forward and reverse PCR primer. Ten colonies were chosen from the cloning experiment for analysis.

Colony preparation for PCR was as the following: The transformation plates were examined and ten well-isolated colonies were picked, then transferred to 3 ml sterile LB broth containing appropriate ampicillin for overnight culture and miniprep. Each colony to be screened was labelled. When the colonies incubated at least 3 hours, 10 µl of culture from each tube was taken for performing colony PCR. 10 µl of grown culture from each colony was diluted in 90 µl sterile water, respectively. Each suspention were boiled for 5 min at 95°C in a thermal cycler. 2 µl of the boiled culture was used in each amplification as a template. Positive clones with a suitable size DNA fragment insert were screened by PCR. A master PCR reaction mix was prepare allowing 23 µl per sample. The following was the composition of 240 µl of PCR master mix: 2.5 X 10 µl of 10X PCR buffer, 2.5 X 10 µl of 2 mM dNTPs, 1 X 10 µl of each of the sequencing primers (20 mM) and 16.8 X 10 µl of PCR-grade H_2O, and 0.2 µl X 10 of 1.25 U/ µl of *Taq* polymerase. 23 µl was aliquoted into each PCR tube and 2 µl of bacterial cell suspension was added as template. PCR conditions were as follows: 1 cycle of 94ºC for 2 min for initial denaturation; 30 cycles of 94ºC for 30 sec, 63ºC for 20 sec and 72ºC for 2 min; then a final extention at 72ºC for 10 min before storage at 4ºC. Amplification products (10 µl) were analyzed on a 1% agarose gel containing ethidium bromide. Positive clones with insert glucose oxidase were stored at -70ºC in 50 % glycerol. As an important note here, the amount of bacteria needed for PCR screening is usually very small. Too many bacteria in the mix will inhibit the PCR reaction.

In addition to screen transformants by PCR, a plasmid miniprep followed by restriction digestion was performed. Well-isolated colonies were picked from a plate

and transferred to LB broth culture medium containing the appropriate antibiotic for selection. All cultures were incubated over night with shaking (~250 rpm). The plasmid isolation experiment was performed using a High Pure Plasmid Isolation (miniprep) kit (Roche). Minipreps of several colonies were done as described in Subheading 2.2.2.1.5.5.

Once the DNA was purified, a portion of plasmid was screened by restriction digestion. 0.5-1 µg of plasmid was used in our digest. In order to select the proper enzyme for screening the orientation of the insert, pDRAW32 DNA analysis software was used and *PstI* was selected. After selection of proper enzymes for digestion, reaction mixture was prepared.

The following components were assembled to a 1,5 mL Eppendorf tube:

Restriction Enzyme Buffer	5 µl
Plasmid DNA (~ 1,5 µg)	15 µl
PstI	0.2 µl
Sterile dd H_2O	9.8 µl
Final Volume	**30 µl**

Reaction mixture was incubated overnight at 37ºC. The next day, all tubes were analysed on 1% agarose gel and positive clones were sequenced by cycle sequencing.

2.2.2.1.5.7 DNA SEQUENCING

The insert fragment in the plasmid was determined using the cycle sequencing method and the automated ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Inc). The sequencing reactions were prepared as indicated in the ABI PRISM Big Dye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Inc). 280 ng of double stranded plasmid DNA was added to 8 µl of Terminator Ready Reaction Mix, 3.2 µl (3.2 picomoles) of sequencing primer, deionized water to a final volume of 20 µl. The

forward primer used was the pBAD Forward 5'- TAGCATGACTGGTGGACAGC -3' to sequence one strand and the reverse primer was the pBAD Reverse 5'- TCAGGCTGAAAATCTTCTCTCA -3' for the complementary strand sequence. Before 30 cycles, an initial denaturation was performed (96ºC for 1 minute). The cycle sequencing reaction was performed in 30 cycles of denaturation at 96ºC for 10 seconds, annealing at 60ºC for 5 seconds and an extention at 60ºC for 4 minutes in a Thermal Cycler. The reaction products were then purified by using a commercial kit, High Pure PCR Template Purification Kit (Roche). The eluted DNA was heated 95ºC for 2 minutes. The sample was sequenced on an automated ABI 310 Genetic Analyzer.

After confirmation by sequencing, the clones with correct insert of glucose oxidase fragment were stored at -70ºC in the presence of 50 % (v/v) glycerol. Sequence analysis was performed using the computer program Chromas and the sequences of glucose oxidase gene was compared to GenBank database.

2.2.2.1.5.8 EXPRESSION AND ANALYSIS OF RECOMBINANT PROTEIN

2.2.2.1.5.8.1 PILOT EXPRESSION

Inserts cloned in the correct reading frame were selected for the expression studies. Recombinant colonies and positive control plasmid (pBAD/His/*LacZ*) were inoculated with 5 ml of LB broth containing 50 µg/ml ampicillin and placed in a rotary shaker incubator at 250 rpm and 37ºC for an overnight period. Colonies were left to grow until the bacterial medium reached an A_{600} between 1-2 (Shimadzu). The next day, five tubes were labelled 1 through 5 and added 10 ml of LB containing 50 µg/ml ampicillin. Each tube was inoculated with 0.1 ml of the overnight culture. The cultures were grown at 37ºC with vigarous shaking until an absorbance reading of 0.5 was reached in the visible spectrum at 600 nm. While the cells were growing, four 10-fold serial dilutions of 20% L-arabinose with sterile water were prepared (2%, 0.2%, 0.02%, and 0.002%). At this point, 1 ml cell aliquots were taken and centrifuged at maximum speed for 1 minute. The supernatant was aspirated and the pellet was stored at -20ºC

until use. This was the zero time point sample (uninduced). Stock solutions of L-arabinose were added to each tube in the order showed in Table 2.3.

Table 2.3 L-arabinose stock solutions dilution in tubes 1 to 5

Tube	Volume (ml)	Stock Solution	Final Concentration
1	0.1	0.002%	0.00002%
2	0.1	0.02%	0.0002%
3	0.1	0.2%	0.002%
4	0.1	2%	0.02%
5	0.1	20%	0.2%

All inoculated tubes were grown at 37ºC with shaking for 4 hours. 1 ml samples were taken at 4 hours and treated as centrifugation and feezing steps.

2.2.2.1.5.8.2 SDS-PAGE

Before starting, an SDS-PAGE gel with a 40% polyacrylamide concentration was prepared to analyze the samples collected from induction studies.

When all the samples had been collected from protein induction step, each cell pellet was resuspended in 100 µl of 2X SDS-PAGE sample buffer and 80 µl of sterile distilled water and was vortexed. The cell suspention was boiled for 5 minutes and incubated on ice for 2 minutes. Then, all tubes were centrifuged at 11,000 rpm for 2 minutes. The gel apparatus was assembled and filled with 1X SDS-PAGE running buffer. 15 µl of each sample was loaded on an SDS-PAGE gel and electrophoresed. The samples were saved by storing them at -20°C.

The gel was run in an electrophoresis apparatus, according to the manufacturer's instructions for the particular electrophoresis system and electrode. When the dye front of the sample buffer reached the bottom of the plate, the gel was removed. In order to

analyze the samples, the polyacrylamide gel was stained with Coomassie blue for 1 h with slow shaking. The gel was incubated in destain solution with slow shaking, until the protein bands were clearly visible. The gel was looked for a band of increasing intensity in the expected size range for the recombinant protein. The uninduced culture was used as a negative control.

The second dimention gel is a vertical slab gel, 6mm thick, poured between two glass plates, one of which is notched. To make 100 ml of running gel solution, 25 ml of lower gel buffer (1.5 M tris, pH 8.8 and 0.4% SDS) 41.64 ml of 30% acrylamide (29.2% acrylamide/ 0.8% bisacrylamide), 33.32 ml of distilled H_2O and 0.333 ml of 10% ammonium persulfate were added to a flask. 50 µl of TEMED was added and the gel mixture was added to a level 25mm below the notch in the notched plate. The running gel was overlayed gently with H_2O and allowed to polymerize 1-2 hours.

To pour the stacking gel, the water was removed from the running gel surface and 20 ml of stacking gel mixture was prepared. The stacking gel mixture has 3 ml of upper gel buffer (0.5 M Tris pH 6.8 and 0.4% SDS), 5 ml of 30% acrylamide (29.2% acrylamide/ 0.8% bisacrylamide), and 12 ml of distilled H_2O. To this mixture, 60 µl of 10% ammonium persulfate was added.

20 µl of TEMED was added. The stacking gel was then poured on top of the running gel up to the notch, layered gently with distilled H_2O and allowed to polymerize 30 to 60 minutes.

The gel was then attached to a standart protein slab gel electrophoresis apparatus. Running buffer (0.025 M Tris base, 0.192 M glycine, 2% SDS) was added to the electrophoresis tank.

Taking time points aliquots were analyzed by SDS-PAGE. The expression control, pBAD/His/*LacZ* vector (Invitrogen) was analyzed in order to verify that the expression protocol, LB medium and L- arabinose solution were correctly prepared. The expression control had an optimum expression point at the fourth hour of induction at a

0.02% of L-arabinose. The expression control was analyzed in a 8% SDS-PAGE resolving gel and 5% stacking gel.

The eight percent SDS-PAGE resolving gel for the control analysis was prepared by mixing 23.2 ml of sterile ultrapure water, 13.3 ml of 30% stock solution of acrylamide mix, 12.5 ml of 1.5 M tris (pH 8.8), 0.5 ml of 10% SDS, 0.5 ml of 10% ammonium persulfate and 0.003 ml TEMED.

The control sample was prepared by mixing 20 µl of control sample with 10 µl of 2X SDS-PAGE sample buffer. The sample was boiled for 5 minutes at 95°C before loading it into the gel. Molecular Weight Standard (Fermentas), was loaded in the same gel for control identification. Samples were seperated by electrophoresis for 40 minutes at 150 volts in the stacking gel and 90 minutes at 200 volts in the resolving gel.

2.2.2.2 MOLECULAR CLONING OF GLUCOSE OXIDASE GENE INTO pGEX-4T1 VECTOR AND HETEROLOGOUS EXPRESSION OF GOX GENE IN *E. coli* BL21 (DE3) pLysS

As a last study, in order to solve the expression proplems, we decided to change our expression vector and host cell. For that purpose, pGEX-4T1 was used as a vector and *E. coli* strain BL21 (DE3) pLysS was served as a host cell. Positive clones, 1, 6 and 11 (these were denominated pBAD/HisB/*GOx1, GOx6 and GOx11, respectively*, from the previous study were grown in 10 ml of LB broth containing 100 µg/ml ampicillin. Mini-preparation of these plasmids were done using a commercial kit protocol as described previously. *HindIII* and *BamHI* restriction enzymes were used for the extraction of glucose oxidase gene from these positive plasmids. Cutting with *HindIII*, Klenow treatment to generate blunt end and *BamHI* reaction to generate a sticky end were performed, respectively.

The following were added to a 1,5 mL Eppendorf tube:

DNA (pBAD/HisB/*GOx1*) (4.5ng)	80 µl
Enzyme Buffer	10 µl
HindIII (10u/ µl)	1,5 µl
Sterile dd H_2O	8,5 µl
Final Volume	**100 µl**

The 60 µl of master mix for these three samples was prepared and aliquoted into three tubes. Lastly, DNA samples were added into these tubes, separately. All tubes were incubated for 3 hours at 37°C. After incubation, purification of the samples were done by Roche High Pure PCR Template Purification kit (Roche). Following the purification, klenow treatment was performed. The digested plasmid after *HindIII* enzyme treatment was mixed with 1 ul Klenow mixture, prepared with 1 ul 10 x Klenow buffer (Promega), 0.5 ul Klenow (2.5 U/ul, Promega), 1 ul of dNTP mix and 8.5 ul dH_2O, and the mixture held at 37ºC for 30 minutes in order to generate blunt end. The reaction was terminated by heating to 70ºC for 10 minutes. After inactivation, all samples were purified and digested with *BamHI* according to the following protocol:

DNA (digested) (2,75µg)	40 µl
Enzyme Buffer	5 µl
HindIII (10u/ µl)	1 µl
Sterile dd H_2O	4 µl
Final Volume	**50 µl**

The suspention was mixed by pipetting and spinned to ensure that the contents was at the bottom of the microcentrifuge tube. The tube was incubated for 3 hours at 37°C. After 3 hours, the samples were loaded onto 1% agarose gel and electrophoresed

for 1 hour and Glucose oxidase was extracted from the gel using the same protocol as described previously.

pGEX-4T1 was digested with *BamHI* to generate a sticky end, and then treated with CIAP, and CIAP inactivation were performed. The DNA was purified again to remove the small oligonucleotides released by the restriction enzymes. Afterwards, linear plasmid was cut with *SmaI*, then CIAP, CIAP inactivation , colon purification, klenow and purification were performed respectively. The concentration of both digested vector and insert were determined and ligation reaction was prepared.

The digested plasmid were ligated and transformed into *E. coli* HB 101. The ligation was carried out in a mixture containing 8 µl digested plasmid solution, 1 µl 10 x T4 DNA ligase buffer, and 1 µl T4 DNA ligase (3U/ µl, Promega) overnight at 25ºC. 5 µl of the ligation product was directly transformed into 75 µl of *E. coli* HB101 competent cell as previously described. The transformation mixture (100 µl) was plated on LB agar plates with antibiotics and incubated overnight at 37ºC. The next day, 4 colonies were picked up and incubated in 3 ml LB broth containing ampicillin for overnight at 37ºC. After incubation, mini-preparation of the plasmids were done. The purified plasmids were controlled by restriction digestion.

In order to delete the GST gene from the original pGEX-4T1 expression vector several steps were done. As a first step, vector DNA was cut with *EcoNI;* second, klenow reaction was performed; third, linear vector was cut with *Not*, fourth, digested vector was electrophoresed on a 1% agarose gel and pGEX-4T1 (~4 kb) without GST gene (~ 900bp) was extracted using extraction protocol as previously described. Positive clones, pGEX-4T1/ *GOx1* and pGEX-4T1/ *GOx4* were used to extract glucose oxidase gene. For that purpose, the following reaction was assembled:

DNA (7µg)	40 µl
Enzyme Buffer	10 µl
BamHI (10u/ µl)	2 µl
Sterile dd H_2O	48 µl
Final Volume	**100 µl**

The tubes were incubated overnight at 37ºC. After incubation, purification was done. Then, klenow treatment for fill in reaction was performed as described above. Another purification step and cutting with *NotI* were performed. Glucose oxidase was extracted from 1% agarose gel. And the concentration of both dephosphorilated vector and digested insert were determined using spectrophotometer to proceed to ligation. Table 2.4 shows the ligation reactions.

Table 2.4 Preparation of Ligation Mixtures for pGEX-4T1(GST Del) and GOx

	1 1 : 1	**2** NC
	(molar ratio of vector : insert)	
Vector DNA[a]	4 µl	4 µl
Insert DNA[b]	12,5 µl	1 µl
10X ligase buffer	1,5 µl	1,5 µl
T4 DNA ligase (5 U/ µl)	1,5 µl	1,5 µl
Sterile dH_2O to final volume	15 µl	15 µl

[a]The size of vector (pGEX-4T1 (GST del)) was 4.0 kb.

[b]The size of insert DNA was about 1.8 kb.

The ligation reactions was carried out overnight at 16°C. The next day, 3 µl of ligation products were transformed into 70 µl of E. coli BL21(DE3) pLysS competent cell. The transformation protocol was in Subheading 2.2.2.1.5.4. After incubation, seven colonies were picked and grown in 3 ml LB medium containing appropriate ampicillin, and then plasmid isolation was performed for each transformant. Each of the plasmid was controlled with *EagI* for the correct orientation of insert. Positive clones were sequenced and used for the pilot protein expression with IPTG. Afterwards, SDS-PAGE analysis was performed in order to observe the band of interest (~67 kDa) as described previously.

CHAPTER 3

RESULTS

3.1 MOLECULAR CLONING of GLUCOSE OXIDASE GENE of *ASPERGILLUS NIGER* INTO pBAD/HisB VECTOR AND HETEROLOGOUS EXPRESSION IN *E. COLI* TOP 10

In this study, in order to express *Aspergillus niger* glucose oxidase gene, pBAD/His B (Figure 3.2) and pGEX-4T1 (Figure 3.3) vectors were used to construct and transcript a recombinant *GOx* enzyme in prokaryotic host cells. Glucose oxidase gene was cloned in two independent experiments to produce the constracts pBAD/HisB/ *glucose oxidase* and pGEX 4T1(GST del)/ *glucose oxidase.* After performing of the plasmid constraction, fungal gene, glucose oxidase was tried to express into different *E. coli* host cells.

The general outline of the cloning strategy in order to constract pBAD/HisB/*GOx* is presented below (Figure 3.1), the details are in the text. The glucose oxidase insert DNA (1.8 kb) lies between the *XhoI* and *PvuII* restriction site and is represented by the GOx. Glucose oxidase was cloned into the pBAD/His B vector using standart cloning techniques, incubating the digested PCR product with the pBAD/HisB expression vector in a ligation reaction for overnight at 16^{o}C and transforming the ligation mixture into competent cells of *E. coli.*

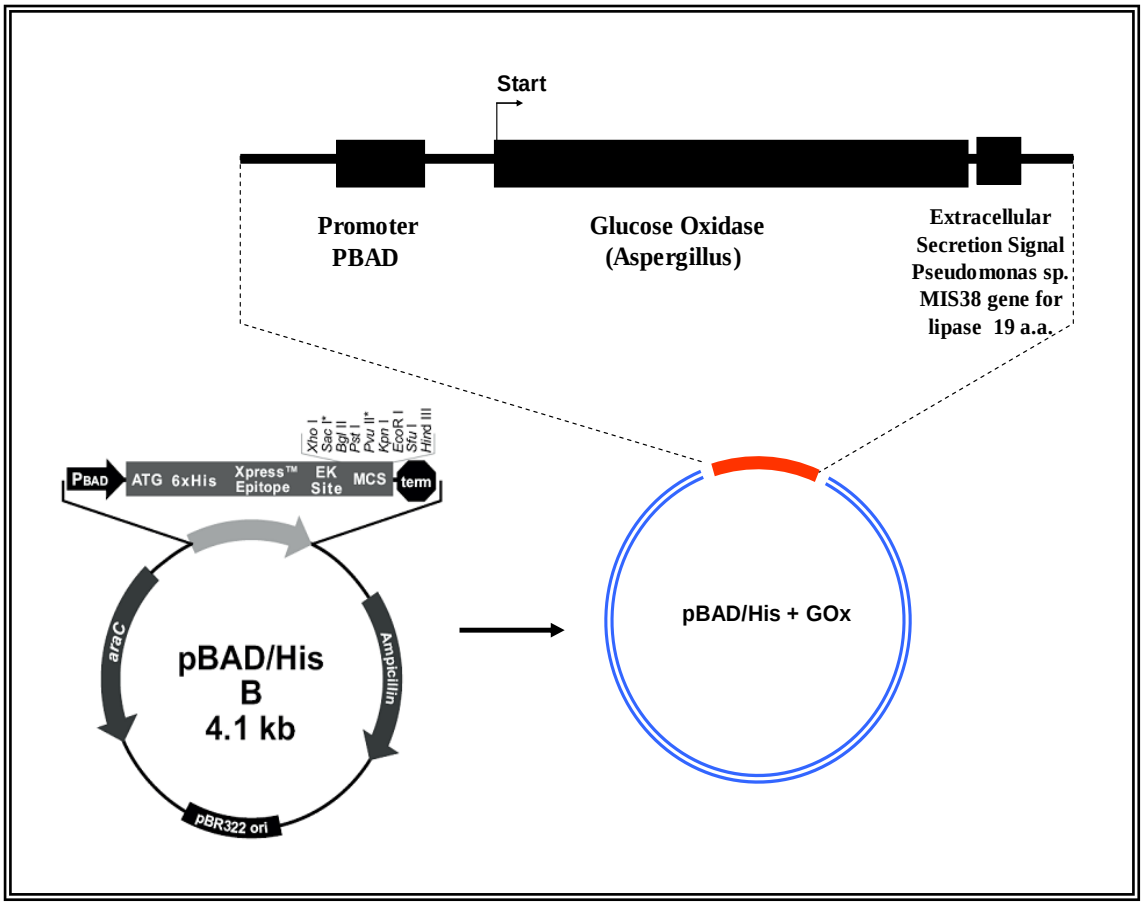


Figure 3.1 pBAD/HisB expression vector (4.1 kb) and cloning strategy.

The genomic DNA isolated from *Aspergillus niger* was quantified spectrophotometrically by absorbance at 260 nm and 280 nm. Table 3.1 shows that the concentration of genomic DNA of *Aspergillus niger* according to the value of 260 nm absorbance. This total DNA solution had a concentration of 1.5 µg/ µL. This demonstrates that the total DNA extracted was high quality to perform the subsequent analysis. This genomic DNA from *Aspergillus niger* was used as template to amplify the glucose oxidase gene by PCR with Prime Star Taq DNA Polymerase (Takara).

Table 3.1 Results of A260/280 ratio of *Aspergillus niger* DNA

DNA Sample	Purity A260/280	Concentration (µg/ µL)
Total genome of *Aspergillus niger*	1.86	1.5

The glucose oxidase gene fragment synthesized by the nested polymerase chain reaction (PCR) was analyzed by agarose gel electrophoresis. Figure 3.4 shows only one band that belongs to glucose oxidase gene and was obtained from total DNA isolated from *Aspergillus niger*. The expected size of the first PCR product was 2000 bp, as demonstrated by its migration on the agarose gel. After performing the nested PCR, 1818 bp was obtained and extracted from agarose gel.

After obtaining PCR product in correct size, pBAD/HisB vector DNA was electrophoresed on a 1% agarose gel for controling the quantity (Figure 3.5). PCR product of glucose oxidase gene and expression vector pBAD/HisB were digested by *XhoI* and *PvuII* restriction enzymes and both digested products were analysed on a 1% agarose-Tris/Acetate/EDTA gel. The concentrations of the both digested DNA insert and plasmid vector were calculated after measurement optical density at 260 nm. Table 3.2 shows the results of the concentration of these products.

Table 3.2 Calculation of the Concentrations of Vector and Insert DNA

	Vector	**Insert**
Dilution Factor	100	100
OD_{260}	0,02	0,1
Calculation of the Concentration	50 x 100 x 0,02 = **100 ng/ µl**	50 x 100 x 0,1 = **500 ng/ µl**

It is necessary to estimate the concentration of both vector and insert DNA before ligation. This may be estimated by agarose gel electrophoresis when run against molecular-weight markers of known concentration or by using a spectrophotometer. In this step, concentration of both vector and insert DNA was determined using a spectrophotometer by making a 1/100 dilution. After performing ligation step with T4 DNA ligase at 16ºC for overnight, all ligation products were transformed into *E. coli* host cell, and then incubated at 37ºC for overnight period. The next day, ten transformants were chosen and mini-preparations of plasmids encoding the glucose oxidase were done for screening positive clones that contain the proper orientation of insert DNA by PCR using the sequencing primers.

As shown in Figure 3.6, only one transformant contains 1818 bp glucose oxidase gene insert. This clone was denominated as *GOx8*. No insert of interest was seen within the other clones. The positive clone containing the insert glucose oxidase was sequenced by cycle sequencing. At the same time, *E. coli* strain TOP 10 containing the positive clone was used for the protein expression studies with L-arabinose. For the control of the our expression system, pBAD/His/*LacZ* (Invitrogen) control plasmid vector contains *LacZ* gene was used. The results of the expression of this gene was shown in Figure 3.9. According to this result, our expression system worked well. The *LacZ* gene (120 kDa) was analysed and easily observed on the protein gel. On the other hand, the result of the pilot protein expression of positive clone (pBAD/HisB/*Gox8*) was negative. We couldn't see any band of interest (67 kDa) on the SDS-PAGE. No induction was observed with L-arabinose.

In order to obtain a sufficient positive clone, we tried to design another recombinant plasmid constraction. Following steps were: Nested PCR (total volume 250 µl) using the same primers and same protocol as previosly described, purification of the 250 µl of PCR product from agarose gel, phosphorilation of the Glucose oxidase insert DNA with T4 Polynucleotide Kinase (Fermentas). At the same time, pBAD/His B vector was cut with *PvuII* in order to generate blunt end. Dephosphorilation of digested plasmid was performed with CIAP, purification and concentration determination were performed after dephosphorilation. Digested plasmid and phosphorilated insert DNA were ligated and ligation products were transformed into host strain *E. coli* TOP 10. The

next day, fifteen colonies were picked up and each colony was inoculated in 3 ml of LB medium containing appropriate ampicillin. The inoculated transformants were incubated overnight at 37ºC and 250 rpm. After incubation, PCR using sequencing primers was done for checking whether the plasmids contain insert DNA or not. Analysis of the cloned PCR product by amplification of the region of the insert by PCR indicates that 7 out of 15 clones contained the insert of interest. Figure 3.7 shows gel electrophoresis of the amplified insert fragments by PCR. Additionally, the orientation of the insert DNA was observed by restriction enzyme digestion with *PstI*. As shown in Figure 3.8, 6., 8. and 11. clones contained the insert of interest with the correct orientation. The clones, 6, 8, 11 were sequenced. After performing cycle sequencing, 11. clone was selected for the expression studies. Once sequenced plasmid selected for the protein expression was denominated pBAD/HisB/*GOx11*.

Sequence analysis was performed using the computer program pDRAW32 and the sequences of glucose oxidase gene was compared to GenBank database. After identifying bacterial harboring plasmids with the insert in correct orientation, we proceeded to use the *GOx11* plasmid to carry out pilot protein expression studies with L-arabinose. The proteins obtained from the pilot protein expression experiment were analyzed by a SDS-PAGE.

After performing the pilot protein expression protocol, again no induction was observed on the protein gel electrophoresis (data was not shown). The expressed protein is expected to have a molecular weight of 67 kDa approximately.

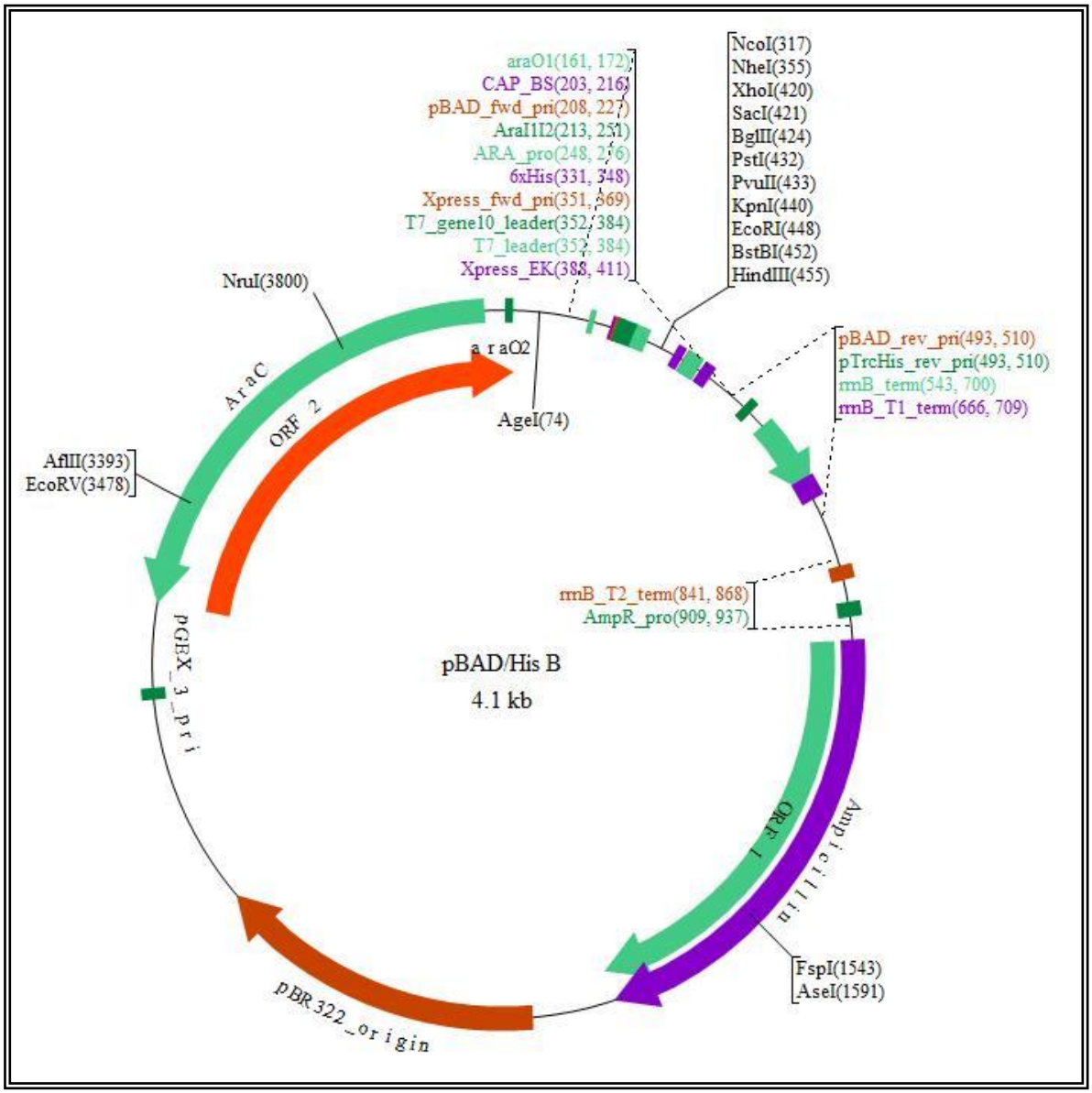


Figure 3.2 pBAD/His B expression vector (4.1 kb) map.

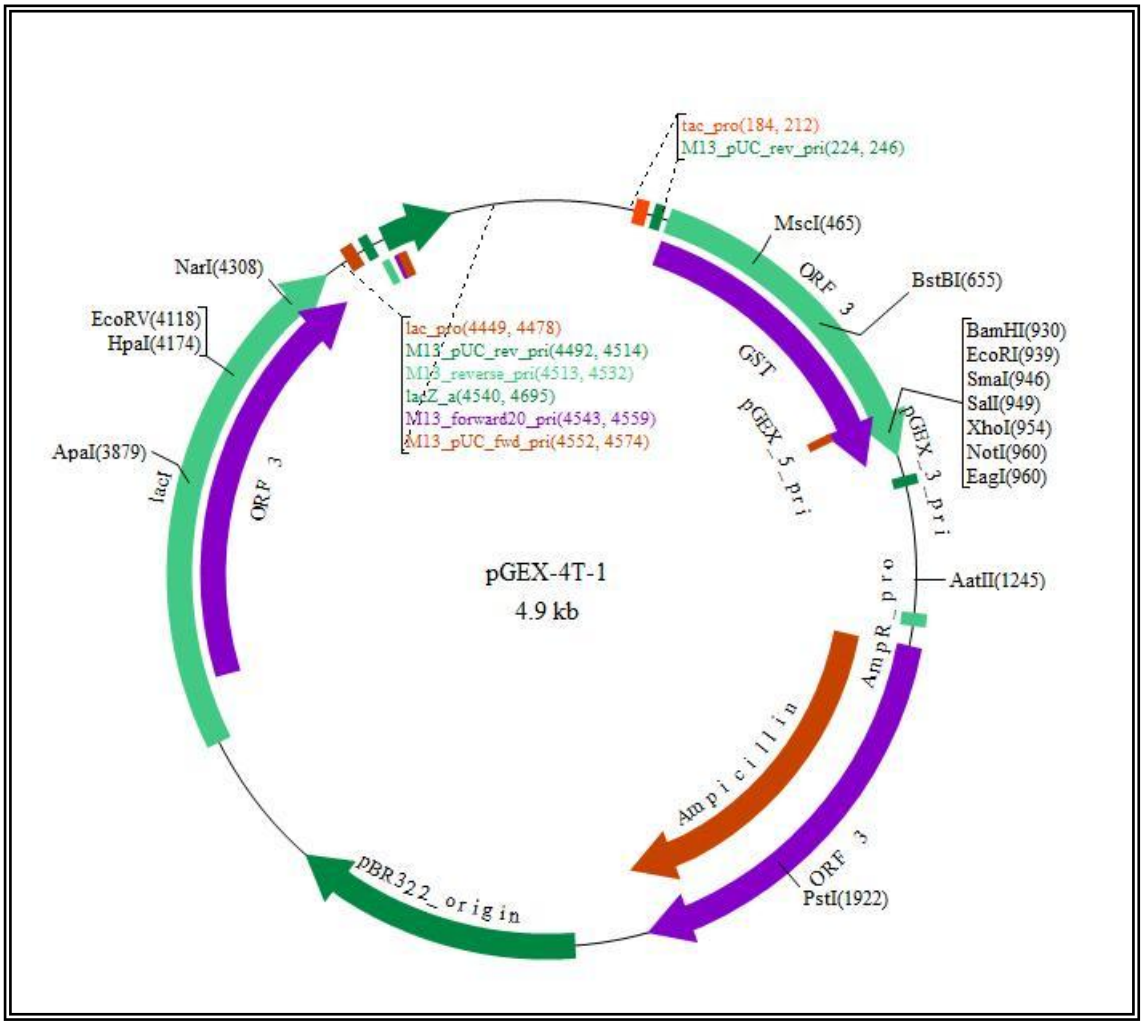


Figure 3.3 pGEX-4T-1 expression vector map.

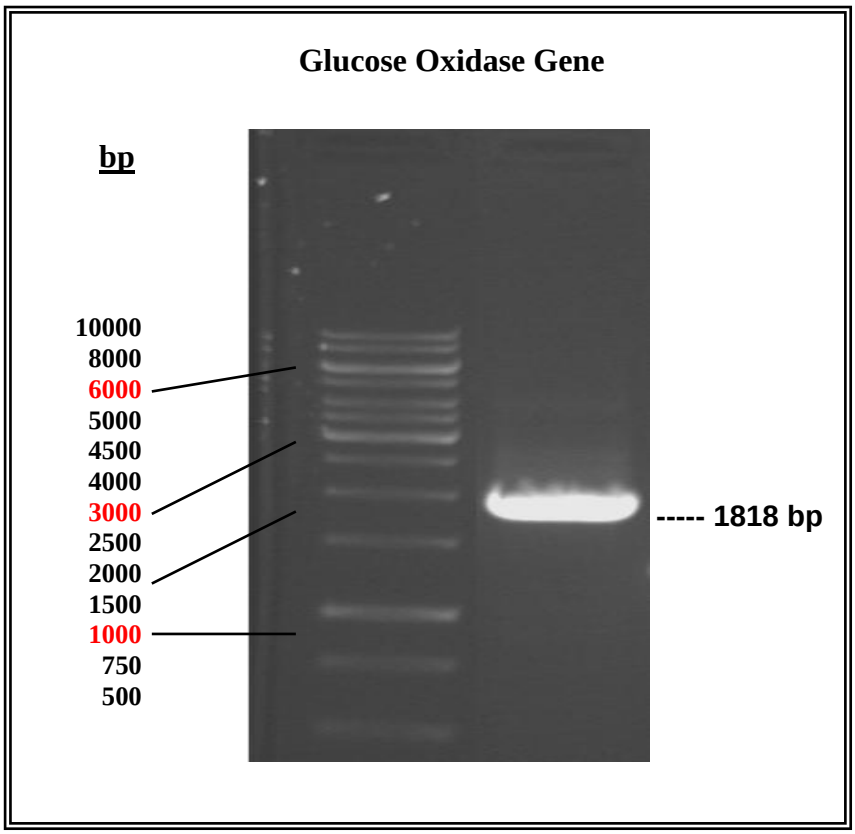


Figure 3.4 : Glucose oxidase gene seen on the 1% agarose gel.

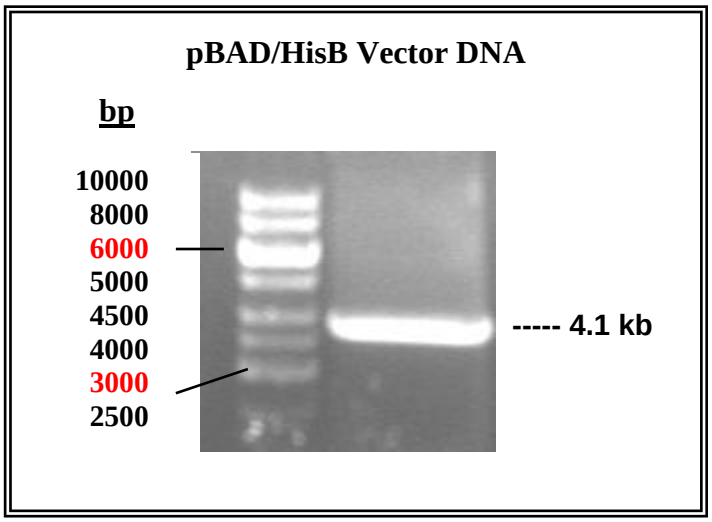


Figure 3.5 : Gel image of pBAD/His B vector DNA on 1% agarose gel.

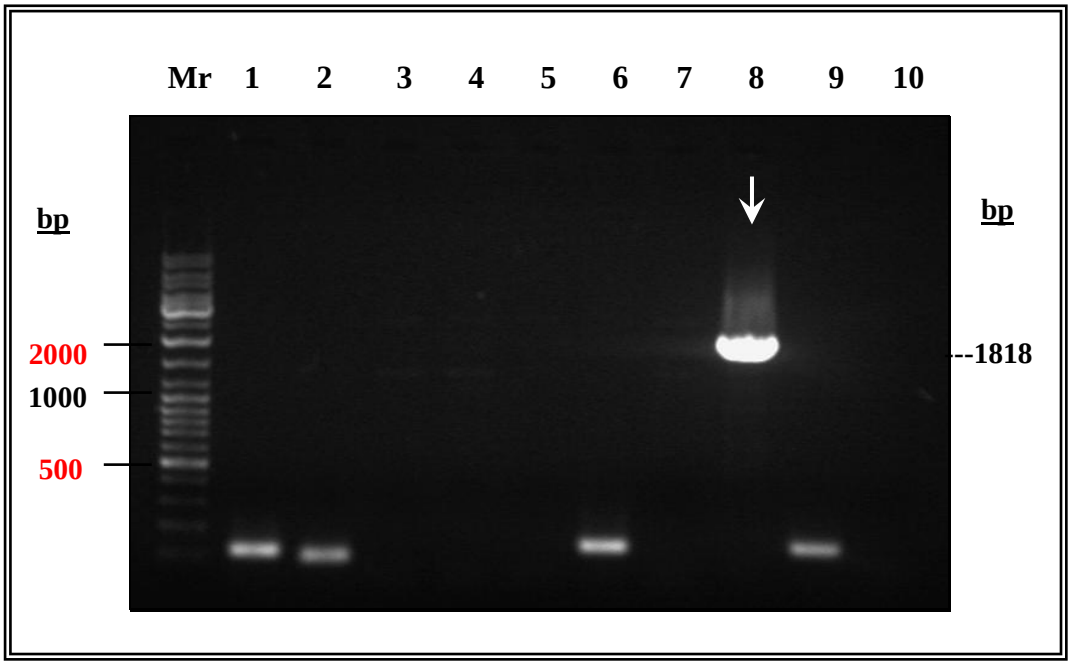
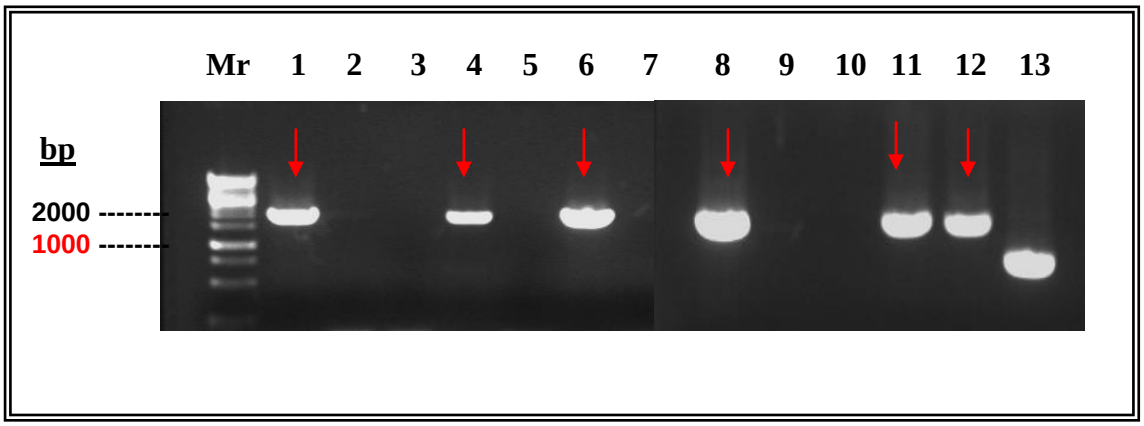


Figure 3.6 Agarose gel electrophoresis of ten well-isolated colonies containing plasmid insert amplification by PCR

Plasmid DNAs from 10 clones were used as template in PCR reactions using primers specific for the pBAD/His B vector. The PCR products of each clone were run on seperate lanes (1-10). Lanes 1, 2, 3, 4, 5, 6, 7, 9 and 10 contain the plasmid without insert. Only lane 8 contains insert of expected size (1818 bp). Lane Mr contain DNA Ladder Mix (Fermentas).



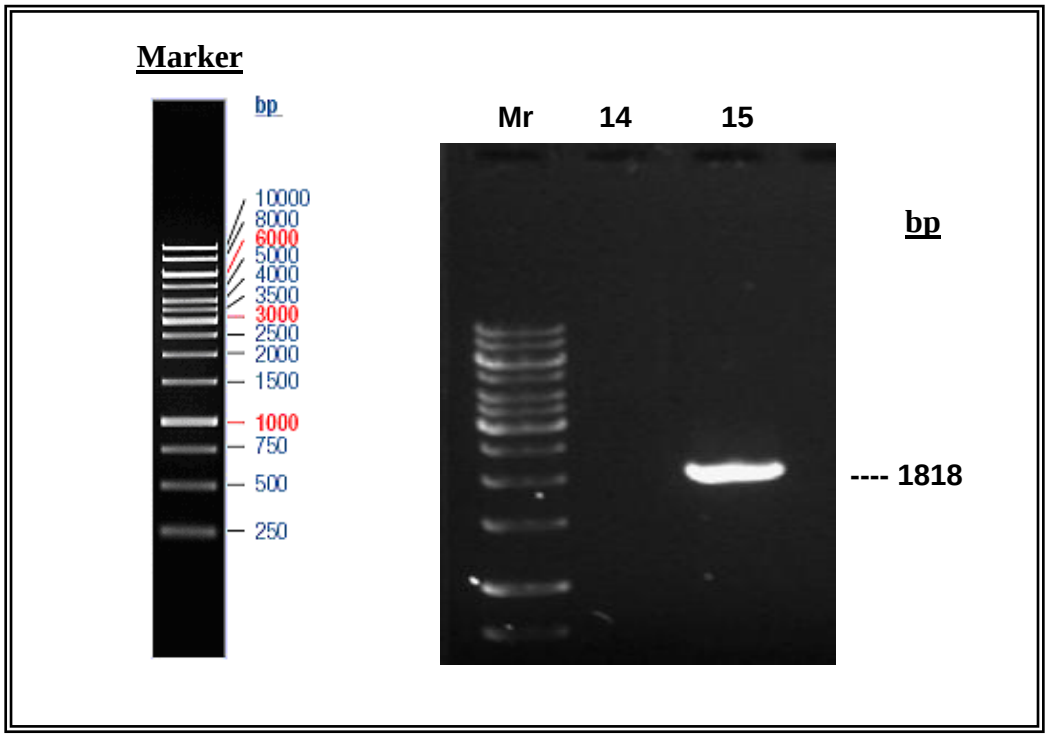


Figure 3.7 Analysis of transformants by PCR.

Colonies were suspended in 50 µl sterile water, boiled for 5 minutes, and 5 µl of the suspention was used in each amplification. The DNA was amplified by PCR in 25 µl volumes. Amplification products (15 µl) were analysed on a 1% agarose gel containing ethidium bromide. In the figure above, 6 colonies contain the insert of interest (glucose oxidase, 1818 bp) and in the figure below contain 1 positive clone additionally. Lane Mr contains the 1 kb DNA Ladder from Fermentas.

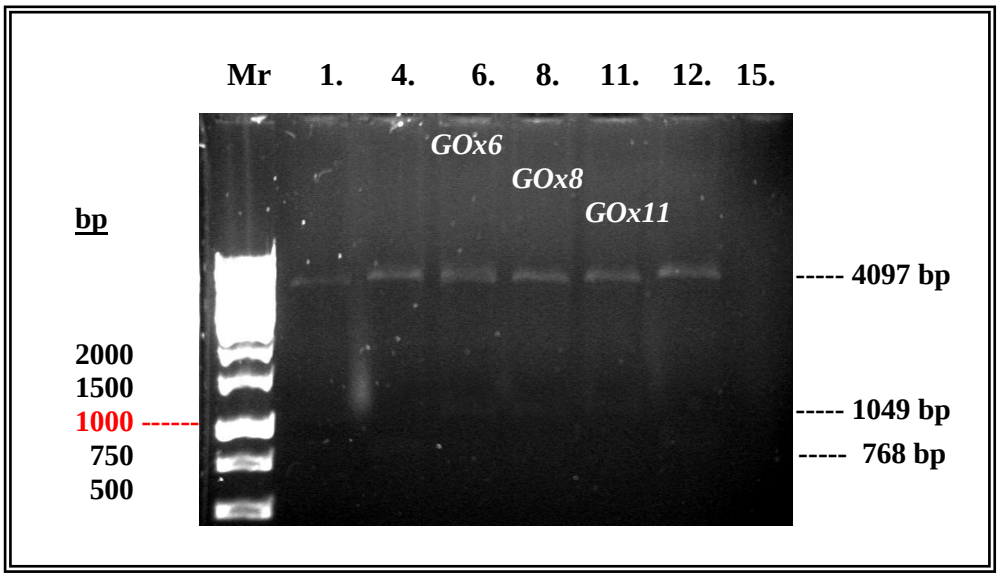


Figure 3.8 Agarose gel electrophoresis of plasmid digestion with *PstI*.

Lane Mr contains the 1 Kb DNA Ladder (Fermentas). Plasmid DNAs from seven different colonies, which were digested with *PstI* and aliquots of the digestion products of each clone were run on seperate lanes (2-8). The clones, number 6, 8 and 11 contain insert of the expected size. They were denominated *GOx6*, *GOx8* and *GOX11*, respectively. The positive clones with the correct orientation of the insert DNA shows 3 bands. These are 4097 bp, 1049 bp, and 768 bp, respectively.

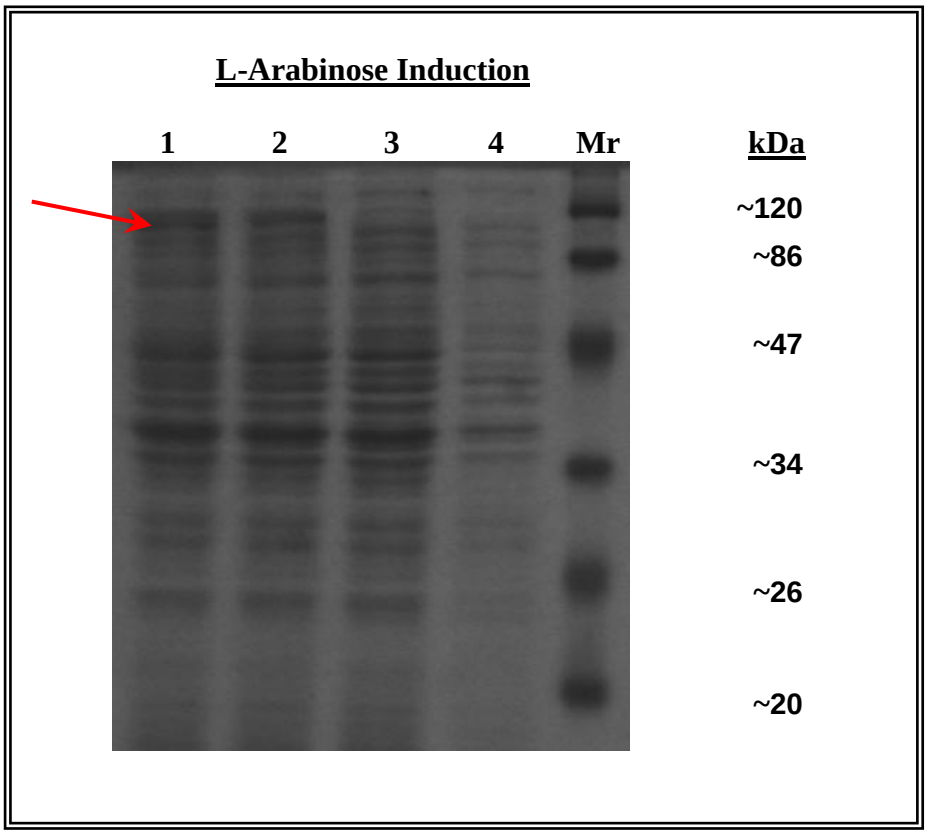


Figure 3.9 SDS-PAGE analysis of the bacterial cell containing pBAD/ His/ *lac*Z for the control of L-arabinose expression system.

Lane 1, 2, and 3: TOP10/pBAD/HisB/*lacZ* induced by 20%, 2%, and 0.2% concentration of L-arabinose in test tube culture after 4 hours, respectively. Lane 4: TOP10/pBAD/HisB/*lacZ* uninduced control sample (zero time point). Lane Mr: Prestained Protein Molecular Weight Marker (Fermentas). Red arrow shows the 120 kDa of *lac*Z gene.

3.2 MOLECULAR CLONING of GLUCOSE OXIDASE GENE of *ASPERGILLUS NIGER* INTO pGEX-4T1 VECTOR AND HETEROLOGOUS EXPRESSION IN *E. COLI* BL21 (DE3) pLysS

The ends of the glucose oxidase gene were used to design degenerate primers for nested PCR. After a first PCR using F1 and R1 as primers, a band of about 2000 bp in size was amplified. To confirm the authenticity of this band, nested PCR was performed using diluted first PCR product as template and F2 and R2 as primers. This fragment (1818 bp) was extracted from the gel, cloned into pGEX-4T1 expression vector (Figure 3.10) and transformed into strain BL21 (DE3) pLysS and the clones with the insert of the fragment were screened restriction digestion with *XhoI* (Fermentas) (see Figure 3.11). Then, positive clones were sequenced. Sequence analysis revealed that the deduced amino acid sequences at the 5'- and 3'- ends of the fragment were identical to native glucose oxidase sequence. The glucose oxidase gene has an open reading frame of 1818 bp encoding 605 amino acids.

The most effective pGEX-4T1(GST del)/*GOx* expression transformant was selected by protein expression studies. Recombinant *GOx* expression was confirmed by SDS-PAGE analysis. Additionally, for the control of the IPTG expression system, pGEX-4T1 original vector was induced in order to express the GST gene (Figure 3.12). Recombinant *GOx* was secreted into the bacterial cell almost as a single band on SDS-PAGE gel, while no corresponding enzyme activity was detected in cultures.

The expression vector pGEX-4T1/*GOx* was transformed into *E. coli* BL21 (DE3) pLysS host cell. Four colonies were isolated and the expression of the recombinant *GOx* was determined. Transformant No.1, in which the level of transcription was very strong was selected for further studies. However, there was no significant enzyme activity in the β-glucosidase assay.

Heterologous expression of glucose oxidase from pGEX-4T1/*GOx* was achieved within 5 h of induction with 50 uM IPTG at 30^{o}C in *E. coli* strain BL21 (DE3) pLysS, which supplies tRNAs for codons that are rarely used in *E. coli*. The choice of such a bacterial host strain for protein expression was dictated by the observation that, due to

its high GC content. Expression of recombinant glucose oxidase was observed by SDS-PAGE analysis.

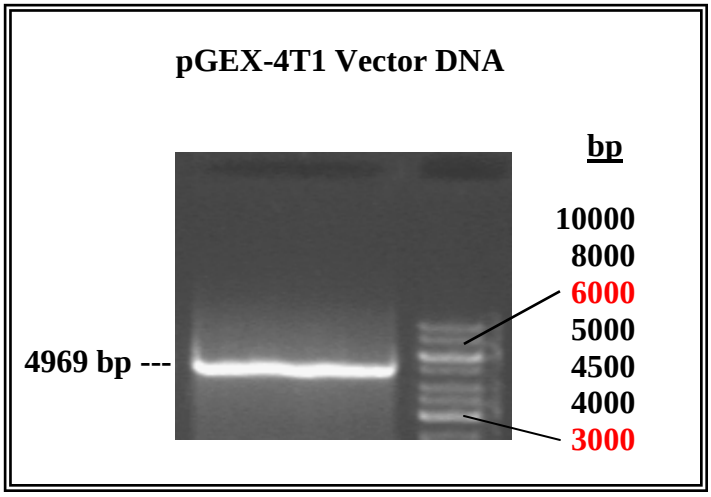


Figure 3.10: Gel image of pGEX-4T1 vector DNA on 1% agarose gel.

A) B)

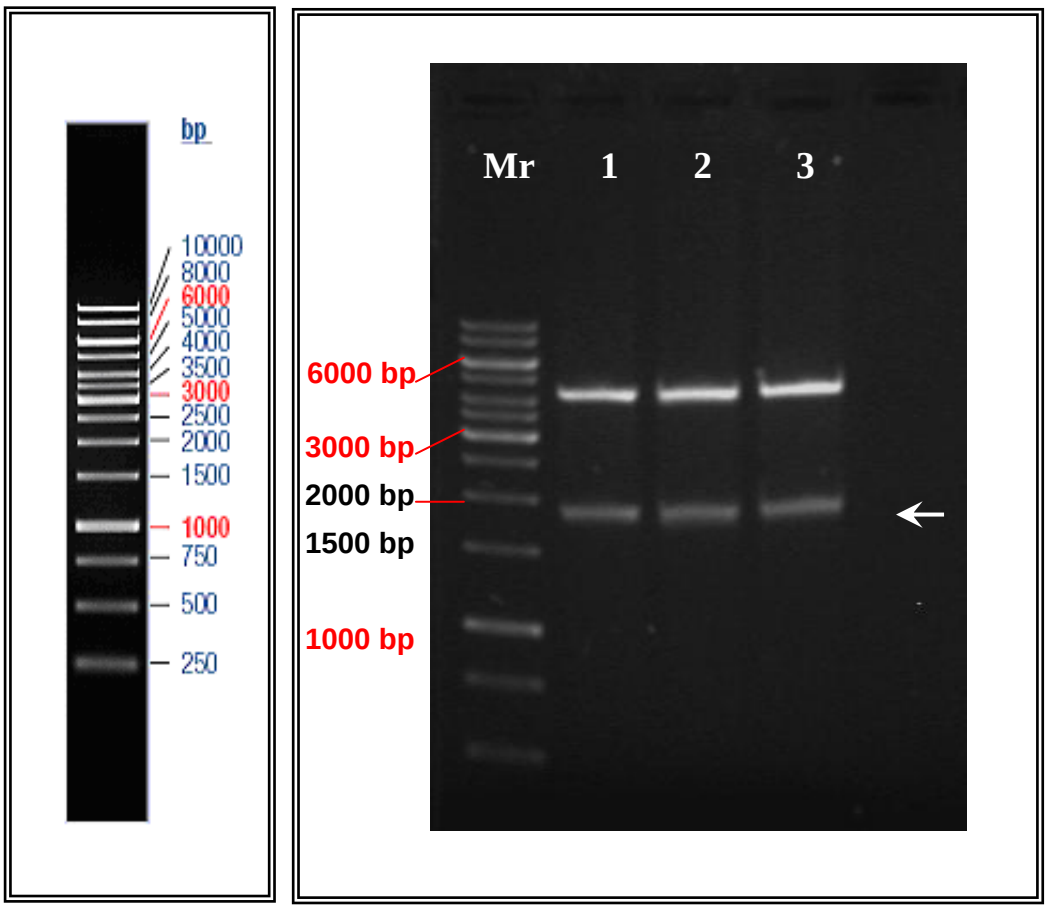


Figure 3.11 Control of the orientation of the insert DNA with *XhoI*.

A) Bands of the 1 kb molecular marker (Fermentas). B) Lane Mr: 1 kb Molecular DNA ladder, Lane 1, 2 and 3 indicates the positive clones. White arrow shows the glucose oxidase gene (1818 bp).

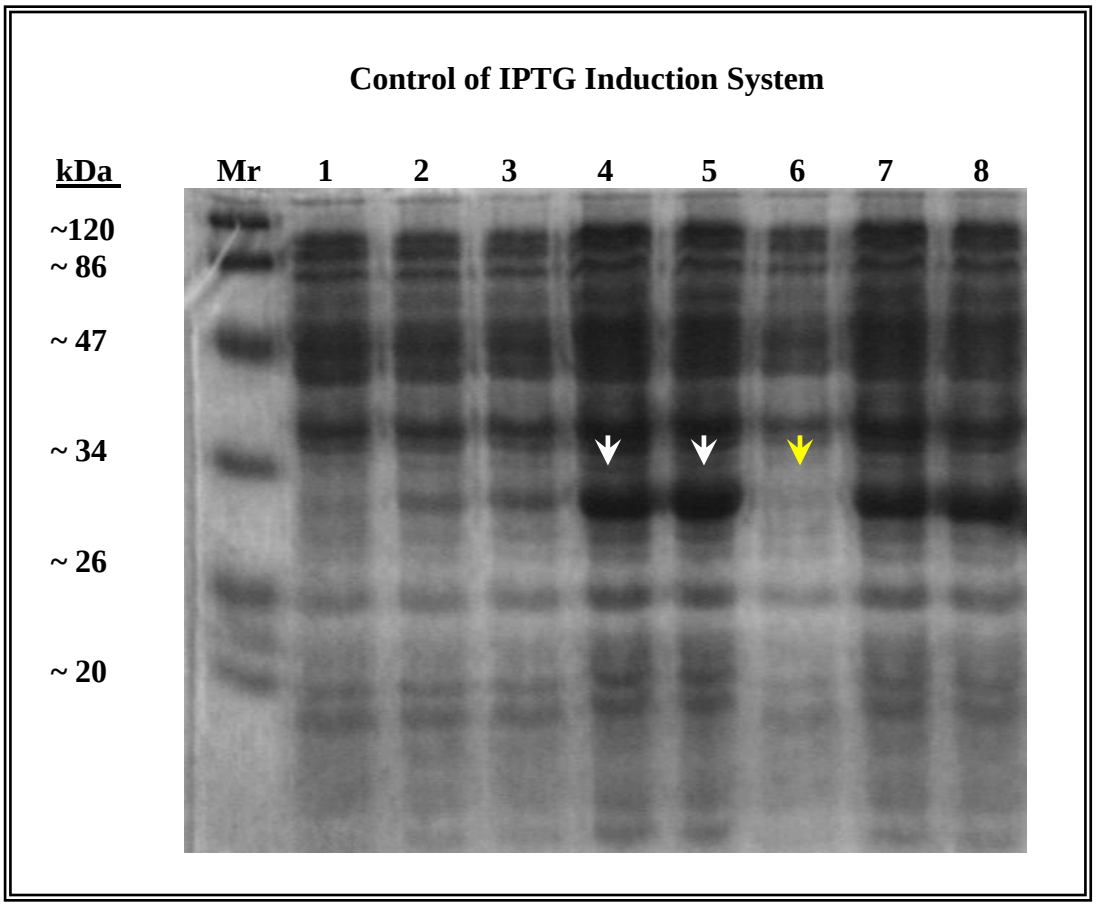


Figure 3.12 Control of the IPTG expression system.

SDS-PAGE of bacterial cell *E. coli* strain HB101 containing original pGEX-4T1 vector. (Lane 1 and 6: bacterial cell containing original plasmid before induction by IPTG (zero time point); Lane 2, 3: bacterial cell containing original plasmid 1 h after induction by IPTG; Lane 4, 5, 7 and 8: bacterial cell containing original plasmid 4 h after induction by IPTG; Lane Mr: Prestained Protein Molecular Weight Marker (Fermentas). The white arrows show the GST gene (27 kDa) induction. The yellow arrow shows uninduced negative control, there is no band of GST.

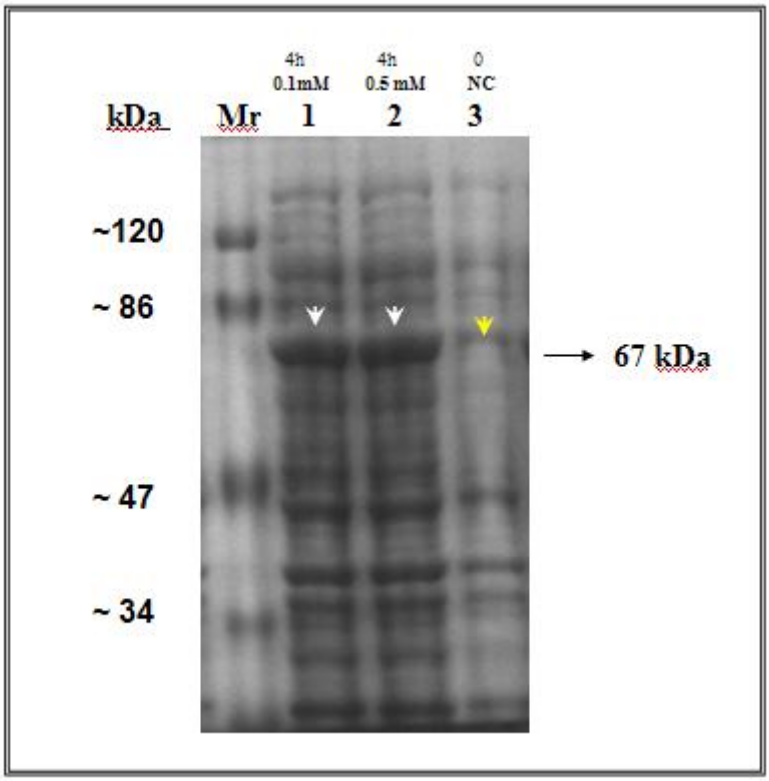


Figure 3.13 SDS-PAGE analysis after 4 hours of IPTG induction of the pGEX-4T1/ *GOx* recombinant vector in the *E.coli* BL21 (DE3) pLysS cells

SDS-PAGE of bacterial cell *E. coli* strain BL21 (DE3) pLysS containing recombinant pGEX-4T1/*GOx* vector. Lane 1 shows bacterial cell containing pGEX-4T1/*GOx* plasmid after 4 h of 0.1 mM IPTG induction. Lane 2 shows bacterial cell containing pGEX-4T1/*GOx* plasmid after 4 h of 0.5 mM IPTG induction. Lane 3 contains bacterial cell harboring pGEX-4T1/*GOx* plasmid before IPTG induction. It was used as negative control sample (zero time point). Lane Mr: Prestained Protein Molecular Weight Marker (Fermentas). The white arrows show the Glucose oxidase gene (67 kDa) expression. The yellow arrow shows uninduced negative control, there is no band of Glucose oxidase.

CHAPTER 4

DISCUSSIONS & CONCLUSIONS

Shewanella putrefaciens is a metal reducing bacterium which can use a diversity of organic compounds and metals to obtain the energy needed for its growht. *Shewanella* is grown robustly and very easily in the laboratory and is suited to genetic manipulations. These organisms show great potential for remediation of various environmental pollutants and in microbial fuel cells (MFCs), where their metabolism is harnessed to generate electricity.

Shewanella can be used in microbial fuel cell applications. Energy can be harvested from biomass when bacteria oxidize organic compounds and utilize an electrode as a final electron acceptor. Mechanisms of electron transfer by *Shewanella* to electrode surfaces have not been fully elucidated, but outer membrane cytochromes appear to be important.

A microbial fuel cell is an electrochemical device which converts the chemical energy of fuel to electrical energy by the catalytic actions of microorganisms. Several types of biofuel cells including microbial fuel cell and enzymatic biofuel cell have been well documented in the literature.

In MFCs, electrons obtained from the oxidation of organic matter can be passed by respiratory enzymes to the electrode, creating current flow. Protons must then diffuse to the cathode to combine with the electron and oxygen to form water. The power generated is in proportion to the organic content of the sediment.

It is very hard to study with enzymatic fuel cell due to enzyme instability in a long time reaction. In this study, *Shewanella putrefaciens* and microbial fuel cell were selected because of the some advantages. One of the most important features of *Shewanella* is to be a facultative anaerobic bacterium. It can grow easily either oxic or anoxic conditions. Additionally, *Shewanella* can grow anywhere and in a lots of media. It is not hard to grow it. So, it is very easy to work with this bacterium.

Shewanella is related to *Escherichia,* a bacterium well known to microbiologists. Tools and techniques developed over the past 30 years for *Escherichia* work with *Shewanella.* In this work, we tried to create an efficient *Shewanella putrefaceins* in order to incrase the production of electron.

Fungal gene encoding for glucose oxidase enzyme have been cloned using a variety of different molecular methods. In this study, a few strategy was used to clone glucose oxidase from *Aspergillus niger.* Degenerate primers were designed to amplify the glucose oxidase sequence. Glucose oxidase gene has an open reading frame of 1818 bp encoding for 605 amino acids.

Additionally, several host cells were used but BL21 (DE3) & pGEX-4T1 systems was observed to express glucose oxidase more efficiently when comparing the other host cells, *E. coli* TOP10 and *E. coli* HB101 strains . pGEX-4T1 vector contains tac promoter, which can be exclusively recognized by T7 RNA polymerase. BL21 (DE3) is a genetically engineered *E coli* strain containing the T7 RNA polymerase gene with LacUV5 promotor. With pGEX-4T1(GST Del)/*GOx* transformed into BL21 (DE3) host cell, IPTG was utilized to induce *E. coli* to express T7 RNA polymerase. The Gox gene was then transcribed through interaction with T7 promotor. Moreover, the codon preference in *E. coli* must be considered. More preferred codons were employed to minimize the G+C content.

In this work, two different systems were used for the cloning and expression studies of glucose oxidase gene. In the first system, pBAD/HisB - *E. coli* TOP10, we couldn’t see any remarkable expression on SDS-PAGE gel. On the other hand, a consedirable expression was observed in the second system, pGEX-4T/ *E. coli* BL21 (DE3) pLysS with IPTG induction. However, due to time constrains, we have focused

on the cloning of glucose oxidase gene encoding for its production in *E. coli.* After detection of enzyme activity, we will continue to transfer the positive recombinant plasmid into *Shewanella putrefaciens.*

In the past few years, the *E. coli* BL21 (DE3) p*LysS* has been reported to be a very successful high-level expression host for many different proteins. In this study, we investigated *E. coli* BL21 (DE3) p*LysS* as a potential host for heterologous glucose oxidase expression. No significant GOx enzyme activity was detected in the lysed culture of pGEX-4T1/*GOx* transformant after induction, although SDS-PAGE analysis revealed that the corresponding protein band was very strong. This luck of activity is unlikely to result from enzyme inactivation.

Although the enzyme activity of recombinant glucose oxidase has not been determined, studies to constract recombinant plasmids and to express of recombinant glucose oxidase in different *E. coli* host cells have been performed. Determination of the enzyme activity of recombinant glucose oxidase will therefore be performed.

In summary, Expression of recombinant GOx using *E. coli* and *S. cerevisiae* has always shown limitations. In the case of *E. coli,* 60% of the recombinant protein was inactive, whereas the recombinant GOD expressed in *S. cerevisiae* was hyperglycosylated and thus characterized by reduced substrate binding capacity and catalytic activity. Crognale et al. (131) used methylotrophic yeast *Pichia pastoris* as host for expression and secretion of recombinant GOD of the filamentous fungus *P. variabile* P16. They transformed the gene to *P. pastoris* X33, a strain largely used for selection on zeocin and large scale growth studies. They demonstrated *P. pastoris* to be an efficient host for expression of both secreted and intracellular heterologous proteins.

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