IDENTIFICATION AND ISOLATION

OF AN AZOREDUCTASE FROM

Enterococcus faecium

By

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ABBREVIATIONS AND SYMBOLS

1. acpD……..acyl carrier protein phosphodiesterase
2. BHI…….Brain heart infusion
3. BLAST.....Basic Local Alignment Search Tool
4. bp…….base pair
5. DNA……...Deoxyribonucleic acid
6. FMN……..Flavin mononucleotide
7. GI……..Gastrointestinal
8. kb……..kilobase
9. kDa……...kilo Dalton
10. µ……micro
11. M……...molar
12. mM……millimolar
13. NADH……Nicotinamide adenine dinucleotide
14. NADPH…….Nicotinamide adenine dinucleotide phosphate

CHAPTER I

Literature Review

Introduction

The earliest evidence of life on earth is estimated to date back nearly 3 billion years. Evidence suggests that microorganisms have dominated the biosphere for approximately 100 million years. Therefore, microorganisms are considered the oldest living systems on earth. Before the 1800's, organisms were classified as being either plants or animals, formally known as the two-kingdom system. In 1866, Haeckel established a third kingdom which he called Protista. The kingdom Protista included algae, fungi, protozoa, bacteria and cyanobacteria (Schlegel, 1992). Most recently, recognition of the ribosomal RNA technique has enabled the construction of phylogenetic relationships between organisms, as demonstrated first by Carl Woese. His work contributed to the formation of the three phylogenetic domains systems, which includes Bacteria, Archaea and Eukarya (Madigan et al., 2003).

Microbes are ubiquitous, diverse and inhabit many environments on earth. These environments range from the extreme cold regions of the Artic to the thermal vent sites found in the depths of the ocean. In addition, they can also be found within and on many different living systems (i.e. humans, animals, etc.). This abundant distribution of

microbes in these environments can be attributed to their amazing phylogenetic and physiological diversity.

As a result, their abundant physiological activities (metabolic capacity, biogeo-chemical cycling, etc.) contribute toward maintaining and sustaining different ecological environments as well as being important for industry (Ketchum, 1988). Even though microorganisms are single cell, they are more flexible metabolically when compared to multicellular organisms. Thus, they are able to survive in many different ecological environments.

During the course of evolution, higher organisms have always interacted with lower organisms. This relationship has been described as commensalisms, predation, parasitism, ammensalism and competition. Therefore, microorganisms play a crucial role in benefiting the welfare of all living systems on earth by maintaining a balance in the environment. Some of the most important roles microbes play involves photosynthesis, metabolism, and production of energy. From this their importance transcends into a wide variety of commercial applications related to the food, drug and pharmaceutical industry. Microbes can also be harmful and cause cellular damage resulting in diseases, which unfortunately overshadows their beneficial role. The overall study of microbial interaction can help devise ways in which the benefits of microorganisms can be increased and their harmful effects reduced (Tortora et al., 2004). For example, microorganisms are excellent experimental models, as microbes have high rates of growth and synthesis, simple cellular architecture, genetic material, and physiological versatility and flexibility. Thus, there will always be many areas of study involving

microorganism.

Normal Microbiota

All life forms, from the time of birth to death, live in a world filled with micro-organisms. These microbes can interact among themselves or with their hosts. The human body is a host and microbes are in contact with the human skin and mucosal surfaces as well as with the gastrointestinal tract (Mackie *et al*., 1999; Schiffrin and Blum, 2002). These microbes are called the normal microbiota or normal flora, and are mostly beneficial thereby they do not cause harm unless they have an opportunity to leave their normal habitat (Drasar and Barrow, 1985).

The Gastrointestinal Tract and Normal Flora

The gastrointestinal tract consists of the oral cavity, pharynx, esophagus, stomach, duodenum, jejunum, ileum, colon and rectum. It is associated with the digestion of food, absorption of nutrients for utilization, and the excretion of waste products (Drasar and Barrow, 1985).

At birth, the fetus is exposed to microbes from the mother and the surrounding environment. These microbes enter through openings to the exterior of the body and eventually colonize in various organs (Tannock *et al*., 1990a, b). Based on the type of neonatal feeding, the type of microorganisms that can populate the GI tract can vary. For example, breast-fed babies are reported to have the following nonpathogenic organisms: *Staphylococci*, *Streptococci*, *Corynebacteria*, *Enterobacteria*, *Lactobacilli*, *Micrococci*, *Bifidobacteria* and *Propionibacteria* (Wijnkoop and Hopkins, 2003). Throughout life the

host is exposed to new microbes, which contributes toward the formation of the normal microflora of the gastrointestinal tract. As humans reach adult hood, the population of microflora remains relatively constant but changes can occur due to age, hormonal changes and pathogenic invasions (Conway, 1997).

The mammalian gastrointestinal tract of a human adult contains up to 1 x 10^{14} colony forming units and more than 500 species of bacteria, which is 10 times more than the number of host cells (Whitman 1998, Moore and Holdeman 1974 and Finegold *et al*. 1975). A majority of these species are considered intestinal flora. They form a dynamic balance that exists between the bacteria, host and their diet. Even though there is constant interaction and movement between the microbes within the GI tract, the intestine tends to maintain a remarkable stability in microbial composition (Savage 1977, Lee 1985 and Conway 1995).

In humans and animals, the GI tract contains a diverse and complex population of microbes which play an important role in the health of the host (Conway, 1995). These microbes enter the GI tract orally via food, water and air. Some of these microbes are inactivated by the acids produced in the stomach and the duodenum while others survive and colonize in the remaining parts of the digestive system. Most of these are harmless and contribute to the fecal bulk while others can invade the intestine and cause disease (Ketchum, 1988).

Different regions of the gastrointestinal tract are populated by different types of bacteria. The oral cavity is inhabited with aerobic, facultative and anaerobic bacteria (Savage, 1999). Within the stomach the low pH and rapid emptying limits microbial

colonization (Cave, 1997). But, bacteria are still present and comprised of the following types of microorganisms: lactic acid bacteria and allochthonous organisms. As bacteria enter the intestine there is a gradual increase in the number of anaerobic bacteria from the proximal to the distal region of the intestine, while aerobic and facultative bacteria are found in the proximal parts. This is due to the decrease in the oxidation-reduction potential (Moore and Holdeman, 1974; Savage, 1977). As with the stomach, pH changes can also occur in the intestine, which dictate the type of microflora that can survive. The large intestine contains the largest population of bacteria, as it has over 100 billion bacteria per gram of feces. The anaerobic bacteria that are predominant in this region belong to the following genera: *Bacteroides*, *Eubacterium*, *Peptostreptococcus*, *Enterococcus*, *Lactobacillus*, *Fusobacterium*, *Clostridium* and *Escherichia* (Moore and Holdeman, 1974; Savage, 1977). These bacteria help in the synthesis of useful vitamins and in the enzymatic breakdown of food (Ketchum, 1988; Schlegel, 1992).

Metabolic Activities of the Intestinal Microflora

One of the most active sites for metabolism in the body is the gastrointestinal tract or gut. The vast communities of bacterial species that inhabit the gut are a source of great metabolic power. For example sulfate reducers and methanogenic bacteria utilize fermentation end products as a source of energy. In addition, these species help to maintain a balance which promotes health and reduces diseases. Specifically, the host is affected by the following bacterial metabolic processes: carbohydrate fermentation, proteolysis and amino acid fermentation, hydrogen disposal, bile acid metabolism, mutagen production, metabolism of neural steroids, transformation of xenobiotic substances, metabolism of lignans and phytoestrogens, immune system development and

modulation, and colonization resistance. The study of the diverse and complex metabolic activities can help us better understand the intestinal ecosystem (Wijnkoop, 2003).

Enterococci: Phenotypic Characteristics of Enterococci

The members of the *Enterococci* family are gram-positive, ovoid, non-sporing bacteria with a GC content of approximately 38-45% and can occur singly, in pairs or short chains. They are catalase negative except for a few that produce pseudocatalase, and some are motile. "They are facultative anaerobic chemo-organotrophs with a complex metabolism resulting in L (+) lactic acid as the major products of glucose fermentation". The cell wall is made up of lysine-D-asparagine peptidoglycan and the membrane consist mostly of straight-chain or mono-unsaturated long chain fatty acids (Hardie and Whiley, 1997). They posses no cytochrome enzymes and are homofermentative. Because the biochemical and phenotypic characteristics of *Enterococcus* was very similar to *Streptococcus*, they were both considered the same (*Streptococcus)* for many years.

Thiercelin was the first to use the name "enterocoque" in 1899 as he described a pathogenic gram-positive bacterium that was isolated from the intestine (Murray, 1990). This organism was later given the name *Streptococcus faecalis* by Andrew and Horder in 1906 after the organism was isolated from a patient suffering with endocarditis (Andrewes and Horder, 1906). For many years isolates were referred to as *S. faecalis*, but in 1919 Orla-Jensen introduced the terms pathogenic and opportunistic pathogens to describe different strains such as *S. faecium* and *S. glycerinaceus* (Orla-Jensen, 1919). In 1937, Sherman identified a different classification scheme which correlated with the

serological scheme developed by Lancefield in 1933. Sherman's classification scheme was based on growth in media, different temperatures, and pH as well as biochemical characterization. In the 1940s and 1950s, more precise biochemical characterization enabled a distinction between *S. faecium* and *S. faecalis*. Some of these characteristics included the inhibition of growth by potassium tellurite, fermentation reactions, and failure to reduce tertrazolium to formazan (Sherman, 1937; Hardie and Whiley, 1997 and Murray, 1990).

It was not until 1984 that *Streptococcus* and *Enterococcus* were recognized as being distinct. The introduction of new molecular and chemotaxonomic techniques like cell wall and lipid chemical analyses, whole genomic DNA-DNA base pairing, DNA-rRNA hybridization and 16S rRNA oligonucleotide cataloguing helped this happen, which improved the classification of bacteria in general (Hardie and Whiley, 1997).

Habitat of Enterococci

Enterococci are distributed widely in nature. They commonly inhabit the gastrointestinal tracts of humans and animals, plants, soil and water (Deibel, 1964). *Enterococci* typically inhabit the feces of most healthy adults and can be found in the lower GI tract but can also be found in the upper GI tract, and lower and upper genital tract (Morrison *et al*, 1997).

Identification and Differentiation of Enterococcus species

Some important reasons for the identification and differentiation of *Enterococci*

species are to determine their susceptibility to selected antibiotics such as vancomycin, beta-lactamase and non-beta-lactamase associated penicillin, ampicillin and gentamicin. Determining susceptibility enables accurate epidemiologic surveillance in hospitals (Facklam and Collins, 1989; Zervos, Kauffman *et al.*, 1987). More recently, the study of *Enterococci* has become important because the bacteria has an enormous and complex metabolic capacity, as evidence suggests that it can impact the health of an individual due to its metabolic activity within the intestine.

The two most common species of *Enterococci* that have gained importance over the last few years are *E. faecalis* and *E. faecium*. Both species have a low degree of DNA relatedness as well as a number of biochemical differences. In addition, the cell walls of both microbes are different, as *E. faecalis* has a demethylmenaquinoes Lys-Ala 2-3 type of peptidoglycan and *E. faecium* has a Lys-D-Asp type with neither menaquinones nor ubiquinones present. Other differences include *E. faecalis*'s ability to produce acid via fermentation of D-tagatose, sorbitol and glycerol, and *E. faecium*'s ability to produce acid via fermentation of melibiose and L-arabinose (Collins, Jones, Farrow Schleifer and Kilppet-Balz. 1984; Facklam, and Carey. 1985; Facklam, and Collins. 1989).

One area of interest related to the metabolic capacity of *Enterococcus* species is their ability to metabolize a diverse group of xenobiotics (man made organics) such as industrial solvents and dyes. This interest has led to the link between xenobiotic metabolism and disease formation such as cancer.

Dyes and Azo Dyes

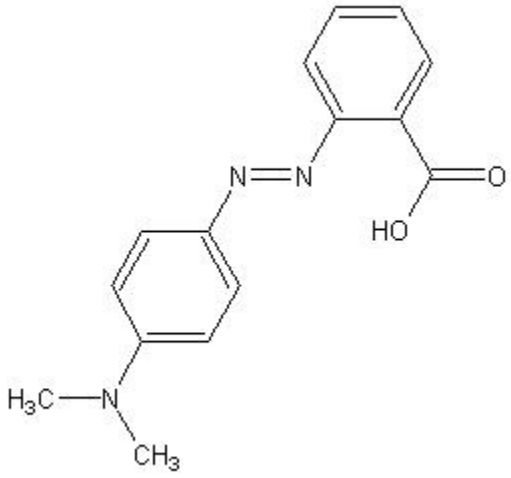
The extensive use of dyes can be traced back to primitive man. They used natural

matter to dye their clothes, decorate their bodies and to paint stories on the walls of caves. The use of yellow, black and red dyes can be dated back to 15000 BC. These natural dyes were extracted from plants, animals, lichens and fungi, and are considered organic aromatic compounds. For example, Indigo is obtained from two species belonging to the genus *Indigofera* and is used for dying cotton work clothes and blue jeans. Inorganic pigments like soot, hematite, ochre and manganese oxide were also used as dyes. Until the discovery of the synthetic dye, dyes of natural origin such as Tyrian purple or mauveine were used extensively as recorded by the English chemist William Henry Perkin in 1856. Since then, the discovery and synthesis of thousands of dyes have replaced the traditional natural dyes (Chippindale and Tacon 1998, Welham 2000).

Dyes contain a chromophore which gives the dye its color upon excitation of electrons and an auxochrome which intensifies the color imparted by the chromophore. There are approximately twenty to thirty different dyes which are classified based on the chemical structure of the chromophore (–C=C-, -N=N-, -C=O, -C=N-). One of the most common and widely used dyes is the Azo dye (Chen, 2004).

The name azo originates from the French word azote, which is the old name for nitrogen. The azo dye is an organic colorant that contains the chromophore –N=N- and when conjugated with an aromatic compound or phenol, renders a brilliant color to the dye. Interestingly the compound 4, 4'-dihydroxyazobenzene is the only naturally occurring azo dye (Gill and Strauch, 1984). Azo dyes are classified based on the number of azo bonds they contain: monoazo (one azo bond), diazo (two azo bonds) etc. (Figure 1).

A



B

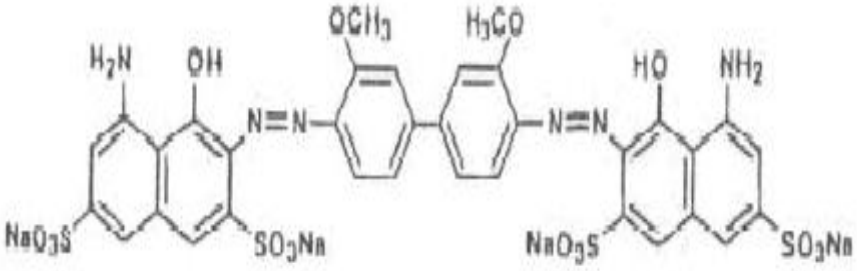


Figure 1: Structure of azo dyes showing the azo bonds (-N=N-) which is used to classify these dyes. A) Methyl red: mono azo dye, B) Direct Blue 15: diazo dye.

Today, azo dyes are important because they are the most widely and versatile class of dyes. Presently there are more than 3000 azo dyes that are being used in different industries such as the paper and textile, food, pharmaceutical, cosmetic, plastic and the rubber industry. The Food and Drug Administration (FDA) has approved the use of some azo dyes in the food, drug and cosmetic industries. The following azo dyes are used as colorants in the food industry: Citrus Red No.2, Allura Red, Tartrazine, Sunset yellow and Orange B. About 80% of azo dyes that are produced annually are used in the dyeing of textiles. Approximately 10% of the dyes used during this process do not bind to the fiber and are released into the sewage systems or the environment. In addition, some soluble reactive dyes are hydrolyzed and eventually released into the environment (Stolz, 2001; Chung, 2000; Rafii, 1995).

Over the past few years there has been an increasing awareness of environmental pollution and its effects on public health. Synthetic dyes or xenobiotic compounds are recalcitrant to biological degradation and are associated with the contamination of ground water and rivers (Pagga U, Brown D (1986); Shaul GM. *et al*. 1991). Waste water containing these dyes is treated using physicochemical techniques such as adsorption, precipitation, chemical oxidation, photodegradation, and membrane filtration. All these methods are expensive, require intensive energy and produce large amounts of hazardous by-products. This has led to the interest of finding biological systems that can be used to treat and eliminate these dyes from waste water and other environments (Stolz, 2001).

Toxic, Mutagenic and Carcinogenic Effects of Azo Dye Reduction

Over the last century, much information has been gathered that related to the

biological activity of azo compounds. For example, azo dyes that are released into the environment can be subjected to anaerobic reduction. Anaerobic reduction can be carried out not only by bacteria found in the intestine or in the environment but also by nematodes such as *Ascaris lumbricoides*, cestodes (*Moniezia expansa*), some fungi (*Phanaerochaete chrysosporium*), and by hepatic enzymes (cytochrome P450). The reduction or the cleavage of the azo bonds results in the formation of aromatic amines, which are considered toxic and potential carcinogens.

In 1945, Miller and Baumann showed the carcinogenicity of the azo dye Butter yellow (DAB: p-Dimethylaminoazobenzene; N, N-Dimethyl-4-aminoazobenzene; Methyl yellow). Later, Ashby *et al.* suggested that the mutagenicity of DAB may be attributed to the product N, N-dimethylphenylenediamine formed due to the reductive cleavage of the dye. It has been established that the toxicity, mutagenicity and carcinogenicity of azo compounds lies in the metabolic products that are formed rather than the parent compound itself. The reductive cleavage of the dye molecule is not only carried out by specific reductases, but can also be carried out by photodegradation (Pielesz, 1999).

Metabolism of Azo Compounds

Sisley and Porscher were the first to show experimentally that azo compounds are metabolized by the reductive cleavage of the azo group. They demonstrated the release of the metabolite sulphanilic acid in the urine of dogs that were fed with Orange I. Later mammalian studies showed the therapeutic properties of azo compounds were due to the reductive cleavage of the azo group from the parent compound (Walker, 1970).

Azoreduction in mammals is said to be catalyzed by two important systems, the hepatic enzymes of the liver and enzymes produced by intestinal bacteria. The bacterial enzyme system is more active than the hepatic system due to the dye being recognized first by intestinal bacteria. Mueller and Miller demonstrated that the azo group present in butter yellow was reductively cleaved when in the presence of rat-liver homogenates under anaerobic conditions (Mueller and Miller 1948). They concluded that the enzyme responsible for this reaction was found in the microsomal fraction of the liver. These results suggested that the enzyme might be a $NADPH_2$-cytochrome c reductase (Mueller and Miller 1949, 1950). In 1967 Hernandez *et al*., found that most of the azoreductase activity from rat liver microsomal azoreductase was attributed to $NADPH_2$-cytochrome c reductase but, hepatic cytochrome P450, which is involved in drug-metabolizing reactions, also plays a role in the breakdown of azo dyes (Hernandez *et al*.). In the 1970's it was shown that many other tissues like kidney, lung, heart, brain, spleen and muscle possessed azoreductase activity due to tissue specific cytochrome P450 enzymes (Walker, 1970).

In 1962, Radomski and Mellinger demonstrated that the metabolism of water soluble azo dyes (Amaranth, Ponceau SX and sunset yellow) was carried out by the gut microflora. This was also confirmed by Childs *et al.* and Fore *et al.* in 1967. They reported that the microflora of the rat intestine reduces azo dyes and organisms isolated from the distal part of the intestine had higher activity than those isolated from the stomach and duodenum. In addition, Roxon *et al*. (1967) showed that the azo dye tartrazine, which is a food colorant, can be reduced by whole cell and crude enzyme preparations of *Proteus vulgaris* isolated from the feces of rat. The azoreductase was said

TABLE 1

Intestinal Bacteria that possess Azoreductase activity	
1. *Bacillus sp.*	2. *Bacteroides fragilis*
3. *Bifidobacterium adolescentis*	4. *Bifidobacterium infantis*
5. *Clostridium paraputrificum*	6. *Clostridium ramosum*
7. *Clostridium sporogenes*	8. *Enterococcus faecalis*
9. *Enterococcus faecium*	10. *Enterobacter aerogenes*
11. *Escherichia coli*	12. *Eubacterium aerofaciens*
13. *Eubacterium biforme*	14.*Fusobacterium prausnitzii*
15. *Klebsiella aerogenes*	16. *Proteus vulgaris*
17. *Pseudomonas aeruginosa*	18. *Salmonella typhimurium*
19. *Staphylococcus aureus*	20. *Shigella dysenteriae*

Chung, K.T *et al*., 1992

to be an NADPH- specific flavoprotein. In 1969, Murrells showed that *S. faecalis* and *E. coli* were able to reduce the azo dye Red 2G (Walker, 1970). Since then, a large number of intestinal bacterial studies have demonstrated azoreductase activity (Table 1). Thus, the impact of azoreduction on the human system was established (Rafii *et al*., 1997).

Aerobic and Anaerobic Azoreductases

Azo compounds can be reductively cleaved by aerobic or anaerobic bacteria. The metabolism of azo dyes aerobically requires specific enzymes called aerobic azoreductases which catalyze these reactions in the presence of oxygen (Stolz, 2001). Zimmermann *et al*. isolated an aerobic azoreductase protein from *Xenophilus azovarans* KF46 and *Pigmentiphaga kullae* K24, in which each protein reduced different azo dyes carboxy-Orange II and carboxy-Orange I. These dyes are not used as a source of carbon or energy as they are unable to further metabolize the reduction products that are formed (Blumel *et al*., 2003). These azoreductases are monomeric flavin-free enzymes that use NADPH as a cofactor. Interestingly, these enzymes exhibit different substrate specificity, and size (21 and 30 kDa), and required the presence of hydroxyl groups in the aromatic ring of the substrate. Finally, these enzymes showed no immunological cross reaction with each other, which supports the fact that the enzymes are evolutionarily different (Zimmermann *et al*. 1982, 1984).

In 2001, an aerobic azoreductase gene was identified, sequenced and characterized in *Bacillus sp. OY1-2*. The enzyme was 20kDa in size, reduced the azo dye methyl red, and produced dimethyl p-phenylenediamine and o-aminobenzoic acid, it also reduced Rocceline and Sumifix Red B in the presence of β-NADPH (Suzuki *et al*., 2001).

Soon after, another azoreductase gene was identified in *E. coli* (AzoR). The azoreductase was an FMN-dependent NADH azoreductase which was 23-kDa in size. It reduced the azo dye Methyl red, and the products formed were similar to the *Bacillus sp*. (Nakanishi *et al*., 2001). Interestingly, the *azoR* gene from *E. coli* was originally thought to encode for the acyl carrier protein phosphodiesterase, which converts holo-acyl carrier protein into apoACP. But, the protein could not convert the holo-acyl carrier protein into the apo form. Rather, it showed characteristics of an azoreductase and the gene was renamed from *acpD* to *azoR*.

In 2004, another aerobic azoreductase (AzoA) was isolated, cloned, purified and characterized in *E. faecalis*. The azoreductase shared 34% identity with the azoreductase (AzoR) from *E. coli*. This enzyme was FMN-dependent and exhibited a broad spectrum of substrate specificity. It was homodimeric protein with a molecular weight of 43kDa and required NADH as an electron donor. AzoA was the first aerobic azoreductase that was characterized from an intestinal microorganism isolated from feces. As previously mentioned, the *azoA* gene was also identified as a putative *acpD* gene (Chen *et al*., 2004). Overall, when all the azoreductases that have been identified are aligned, no significant amino acid similarity exists, which supports the theory that the azoreductases evolved from different origins (Fig. 2).

Less is known about the molecular nature of anaerobic azoreduction but a wide range of organisms have been shown to reduce azo dyes under anaerobic conditions. Previous studies have shown that azo reduction under anaerobic conditions is a "fortuitous, nonenzymatic reduction by enzymatically generated reduced flavins, and inhibition by oxygen is due to regeneration of the oxidized form" (Chen *et al*., 2004).

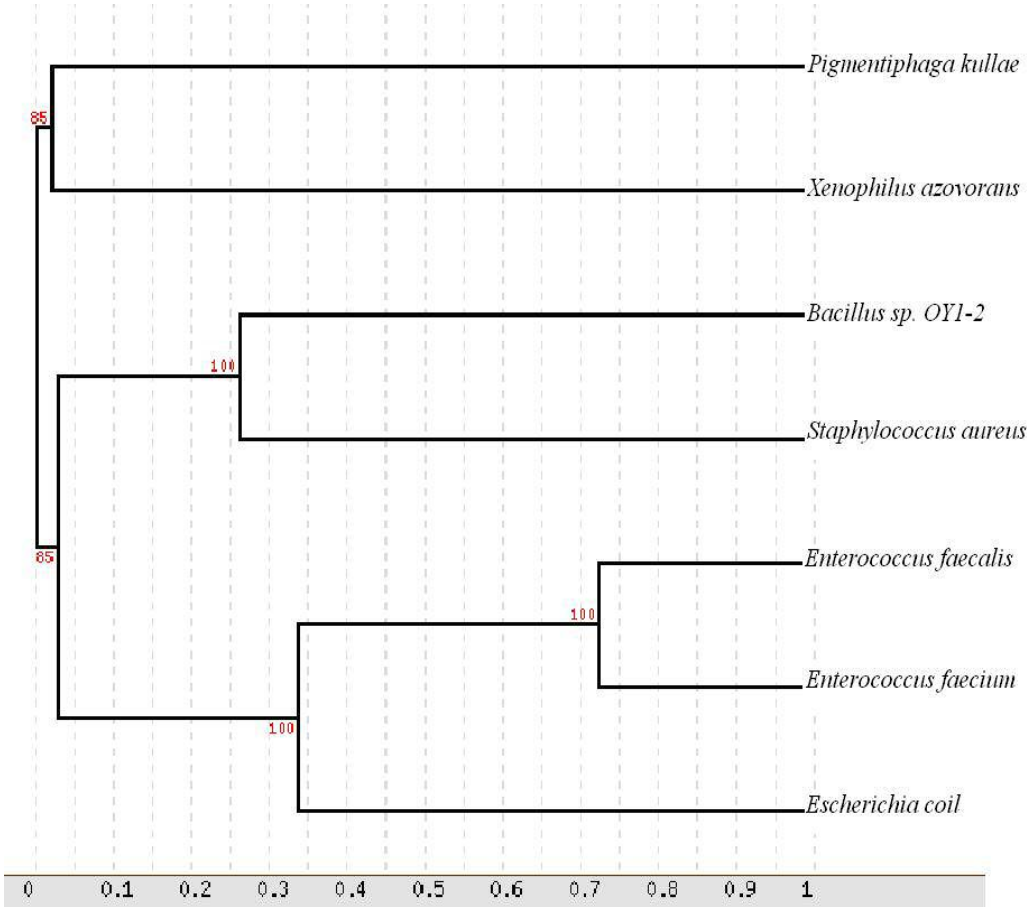


Figure 2: Unrooted phylogenetic tree with bootstrap values that was generated by comparing the acpD gene of *E. faecium* with the different aerobic azoreductases amino acid sequences that have been isolated thus far. The GeneBee software was used to generate this tree.

Further, anaerobic azoreduction is not specific for anaerobic bacteria compared to facultative and aerobic bacteria, yeast and tissues of higher organisms which exhibit specific activity. Bacteria from the human intestine have gained a lot of attention because azo dyes that are ingested are metabolized by anaerobic bacteria and produce carcinogenic aromatic amines. There are many models that are proposed for the reductive cleavage of azo dyes under anaerobic conditions.

The first model proposed by Stolz describes azo dye reduction as being dependent on reduced flavins, as previously mentioned. Under aerobic conditions, oxygen and the azo dye compete for the reduced electron carriers and hence the successful reduction of azo dyes is due to its more optimal redox potential state. The second mechanism is based on studies with facultative anaerobes by Roxon *et al*. (1967) and Walker (1970), which suggested that unspecific reduction of azo dyes was carried out by reduced flavins generated by cytosolic flavin reductases. The limiting factor in this model was the highly polar sulfonated azo dyes as well as the inability of the reduced flavins to cross the cell membrane, thereby suggesting that this process does not generally occur (Roxon *et al*. 1967; Walker 1970).

A third model was proposed for *Sphingomonas xenophaga* BN6. In this model it was suggested that in addition to reduced flavins, quinoid redox mediators were required for the reduction of the azo dye. In this system the quinones acted as redox mediators which were enzymatically reduced by the cells to form hydoquinones, which in turn reduced the azo dye. The quinine reductase activity was observed in the cell membrane and hence the transport of the hydroquinone /quinine redox mediator and the highly polar sulfonated azo dye across the cell membrane was not required (Kudlich *et al*. 1997;

TABLE 2

Redox mediators used for azo dye reduction

Redox mediator	Redox potential (E_0)
methyl viologen	-440
benzyl viologen	-360
Riboflavin	-208
FAD	-219
FMN	-219
Menadione	-203
Neutral Red	-325
Anthraquinone-2-sulphonate	-218
2-hydroxy-1, 4-naphthoquinone	-139

Frank P. van der Zee, 2002

Keck *et al*. 1997). Note other electron carriers can stimulate the reduction of azo compounds as shown in Table 2.

A fourth model proposed by Rafii and coworkers in which they suggested that the dye was not required to be transported across the cell membrane. According to this model experiments associated with *Eubacterium*, *Clostridium*, *Butyrvibrio* and *Bacteroides*, reduce the dye using extracellular enzyme activity. They showed that some of the azoreductase activity was extracellular or supernatant associated (Rafii *et al*. 1990, 1995). One question that still remains unanswered is 'How the extracellular enzymes gain the NADH that is required for the activity of the enzyme in the extracellular environment?'

The last model describes how reduced inorganic compounds that are formed as end products of the metabolic reactions by anaerobic bacteria can also take part in the reduction of azo dyes. Libra *et al*. (1997) and Yoo *et al*. (1999) showed that the formation of hydrogen sulfide by sulfate reducers resulted in the reduction of the azo dye Reactive Orange 96.

The study of azoreductase plays an important role in the environment and also acts as an important human health determinant. Human health is linked to the ability of intestinal microbes to reduce azo dyes. Because azo dyes are recalcitrant, they have the potential of causing harm to humans over a long period of time. Azoreductase studies involving intestinal bacteria is at its infancy and studies in this area can help us better understand the impact that azo dye metabolism has on human health.

The objective of this thesis is to identify the *acpD* gene from the intestinal microbe *E. faecium*, clone and characterize the gene to see if it has azoreductase activity.

CHAPTER II

SCREENING OF INTESTINAL MICROORGANISMS

Introduction

The Human gastrointestinal tract harbors a complex culture of microorganisms. This microbial population differs in the various regions of the gastrointestinal tract and is determined by a variety of factors such as the diet, pH and oxygen requirements. The large intestine is said to contain a rich and complex microflora, consisting of facultative anaerobes and obligate anaerobes.

It has been shown previously that the intestinal microflora of humans and other animals produce several hydrolytic and reductive enzymes that are involved in the metabolism of xenobiotic compounds associated with drugs and food constituents. Most of these reactions take place in the large intestine, as this region contains the largest population of microbes. In the current lab, several major types of human intestinal bacteria were tested to determine their ability to metabolize Phenobarbital, a drug used to treat epilepsy. It was shown that *Bifidobacterium adolescentis* and *Bifidobacterium bifidum* were able to metabolize Phenobarbital into alpha-etyhl-benzeneacetmide (John, G.H. *et al.* 2006). In addition, *Eubacterium biforme* was shown to tolerate the surfactant CTAB, which is normally toxic for a wide variety of bacteria, thereby demonstrating it may have bioremediation potential (John, G.H. *et al.* 2006). Finally, it was shown that *Eubacterium aerofaciens* contained a P450-like protein, which participates in xenobiotic biotransformation.

Currently, there is interest in determining the ability of human intestinal bacteria to metabolize different azo dyes. Azo dyes are the most widely used xenobiotics as they exhibit a wide range of colors due to the presence of the azo bond -N=N-. When these dyes enter the human body, either through ingestion or dermal contact, they are metabolized by intestinal bacteria that posses the enzyme azoreductase. These enzymes cleave the azo bond by reduction to form aromatic amines. Studies have shown that these aromatic amines are potential carcinogens (Cerniglia *et al*. 1982).

Species that belong to the genus *Clostridium*, *Eubacterium*, *Bacteriodes*, *Bifidobacterium* and most recently *Enterococci* have been shown to posses the enzyme azoreductase. Some common azo dyes that these microbes are able to metabolize are Acid Yellow, Amaranth, Congo Red, Direct Blue 15 and Methyl Red. A smaller group of these species have been shown to possess a gene that encodes for azoreductase. Only three bacteria (*Escherichia coli, Clostridium perfringenes,* and *Enterococcus faecalis*) commonly found in the human intestine have been shown to possess a gene that encodes for azoreductase. Thus, there is a need to determine if additional azo genes are present in other types of human intestinal bacteria, as culture studies suggest they may contain azoreductase genes.

Our interest was to determine if our collection of ATCC strains (human intestinal bacteria) possessed ago dye reeducates activity and to select an organism in order to further study the presence of an azoreductase gene(s). According to the literature, the collection of ATCC bacteria in Table 3 was not specifically identified as azoreductase positive cultures. Therefore, we began our study by testing all strains in Table 3. Since the type of media recommended for growth varied for the species in

Table 3; we chose to use one type of media, simple Brain Heart Infusion (BHI), for the following reasons. First it was not known if BHI would be sufficient to support for all the different species in Table 3, as either the recommended media or a complex BHI solution was used in previous studies. BHI would provide an initial selection of cultures that would makeup the first cohort of bacteria that would be studied. Second, we planned on analyzing growth in the presence of dye, and having one type of media would enable a more accurate analysis, as varied growth with different types of media could be due to the different supplements or specific components within each type of media. Third, based on the literature most azoreductase studies used simple Brain Heart Infusion (BHI) to grow and test for azoreductase activity, thereby comparisons could be made. The ATCC cultures included in the study were in lyophilized form or short term liquid cultures (recommended or supplemental BHI). Some short term liquid cultures did not possess labels and were also included in the study.

This chapter describes the scientific methodology used in selecting the current bacterium for the study. Preliminary data suggested that human intestinal bacteria possessed azoreductase activity. BHI was used as a selection media to study the first group of bacteria. Growth in BHI and the ability to reduce azo dyes helped us to narrow down the number of bacteria to focus on. Based on available genomic data and gene similarity, *Enterococcus faecium* was chosen for the study (Chapter 3).

TABLE 3

Simple Brain Heart Infusion Growth (BHI)

ATCC Culture & #	Recommended Growth conditions	Recommended Media	(+ or – growth) *BHI	[1]G seq. (+)	HCB[2]	Ago gene
Bacillus megaterium 14581	Anaerobic, 30 C	Nutrient agar	-			
Bacteroides fragilis 25285	Anaerobic, 37 C	Modified chopped meat	-			
Bacteroides stercoris 43183	Anaerobic, 37 C	Modified chopped meat	+	None	+	
Bifidobacterium adolescentis 15703	Anaerobic, 37 C	Reinforced clostridial media	+	Yes	+	No
Bifidobacterium bifidum 15696	Anaerobic, 37 C	Reinforced clostridial media	-			
Bifidobacterium breve 15700	Anaerobic, 37 C	Reinforced clostridial media	-			
Bifidobacterium infantis 15697	Anaerobic, 37 C	Reinforced clostridial media	+	None	+	
Bififobacterium longum 15707	Anaerobic, 37 C	Reinforced clostridial media	-			
Clostridium perfringens 19574	Anaerobic, 37 C	Beef liver media	+	Yes	-	Yes
Enterococcus faecalis 27274	Anaerobic, 37 C	Todd Hewitt Broth	+	Yes	+	Yes
Enterococcus faecium 6569	Anaerobic, 37 C	Brain Heart Infusion	+	Yes	+	No
Eubacterium aerofaciens 25986	Anaerobic, 37 C	Chopped meat	-			
Eubacterium biforme 27806	Anaerobic, 37 C	Chopped meat carbohydrate	+	None	-	
Eubacterium eligans 27750	Anaerobic, 37 C	Chopped meat carbohydrate	-			
Eubacterium rectale 33656	Anaerobic, 37 C	Chopped meat carbohydrate	+	Yes	-	No
Fusobacterium prousnitzii 27768	Anaerobic, 37 C	Modified E medium	-			
Lactobacillus acidophilus 4356	Anaerobic, 37 C	Tomato juice, yeast extract, milk	-			
Pseudomonas putida 17453	Anaerobic, 37 C	Nutrient agar	-			
Ruminococcus productus 27340	Anaerobic, 37 C	Chopped media	-			
Ruminococcus bromii 27255	Anaerobic, 37 C	Chopped media, carbohydrate	-			
Stretococcus intestinalis 43492	Anaerobic, 37 C	Tryptocase Soy agar, defib blood	-			

*See methods for media preparation and growth conditions

[1]G= genomic sequence information available; HCB: High concentration of Blue 15 (100mM).

Materials and Methods

Preparation of anaerobic simple Brain Heart Infusion (BHI) media

Based on previous studies anaerobic BHI media supplemented Tween 80, fatty acid solutions as well as a solution of the complex vitamins were used to grow all facultative anaerobic microorganisms. To simplify the media, BHI was prepared without Tween 80, fatty acids and complex vitamins. Note: BHI will be referred to as simple BHI. A total volume of 500ml of BHI was made and contained the following: 18.5g of BHI broth, 5.0g yeast extract, 0.2g sodium bicarbonate, and 0.3g cysteine-HCl, dissolved in 425ml of distilled water. To this, 12.5ml of salt solution I (3.0g K_2HPO_4 per liter of distilled water), 12.5ml salt solution II (3.54g KH_2PO_4, 9.0g $(NH_4)_2SO_4$, 0.9g NaCl and 1.8g $MgSO_4$ per 500ml of distilled water), 12.5 ml of $CaCl_2$ solution (0.6g $CaCl_2$ per liter distilled water) and 2ml of Hemin solution (50mg Hemin, 1.0ml of 1N NaOH, 100ml of distilled water) were added and heated to ensure adequate mixing. The pH was adjusted to 7.0 with hydrochloric acid (100%) after which the media was autoclaved at 121^{o}C for 20 minutes. For agar plates, 7g of media was added.

After autoclaving, the hot media was placed in the anaerobic chamber which was pumped with nitrogen prior to placing the media in the chamber to create an anaerobic environment. Once the media was cool, 5ml of sterile Vitamin K solution (50mg of menadione was dissolved in 100ml of 95% ethanol) was added using aseptic techniques, followed by aliquoting 5ml of the media into sterile rubber screw cap test tubes. For the agar plates, warm media was poured into sterile Petri plates. The tubes and plates were then incubated at 37^{o}C overnight to check for contamination.

BHI Cultivation of the organisms

Fresh BHI tubes were inoculated with lyophilized sample (dissolved in sterile media) or labeled short term liquid mixed culture(s) and incubated overnight at 37^{o}C. The pure lyophilized cultures were used directly in dye experiments. Some of the short term liquid cultures did not possess a label and were identified as mixed unknowns. The short term liquid cultures were grown in liquid BHI initially. Only those cultures that grew were further examined by streaking onto BHI agar plates for isolated colonies. The plates were incubated for sixteen hours at 37^{o}C in an anaerobic jar. Only two types of colonies were observed based on colony morphology (size and pigment color). Gram stains were carried out on isolated colonies and the colonies were then inoculated into fresh BHI broth tubes. The BHI culture was incubated as before and prepared for the dye reduction experiments and for extraction of genomic DNA. Glycerol stocks (300µl of glycerol was added to 700µl of fresh culture) were prepared for each pure culture and both unknowns and stored at -80^{o}C.

Culture Dye Reduction Assay

To determine if the species in Table 3 were able to reduce azo dyes, Direct Blue 15 was used. In addition, the unknown pure cultures were also tested with Direct Blue 15. After 24 hours incubation, visual examination was used to determine azoreductase activity. See Figure 3 for a representation of different stages of color change in BHI. Direct Blue 15 (10mM, MP Biomedicals), imparts a dark blue color to the media. A loss in the color suggests dye reduction. Once a small group of bacteria or a single bacterium was selected, a more accurate measurement of dye reduction

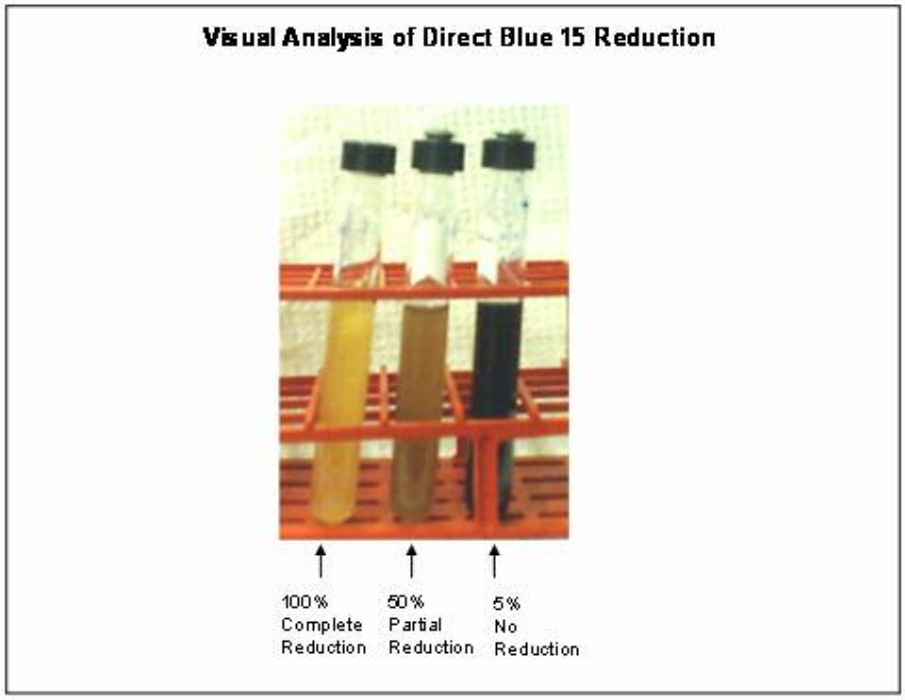


Figure 3: Representation of complete, partial and no reduction of the azo dye Direct Blue 15.

was used involving spectrophotometric analysis. The absorption maximum for the dye was measured using a spectrophotometer. The spectrophotometer was baselined using media only. After 0 to 24 hours of incubation at 37^{o}C, samples of 1ml were collected, the cells harvested by centrifugation and the supernatant tested to check for the reduction of dye absorption using the Shimadzu UV-1601PC UV-visible spectrophotometer. The scan wavelength parameters were set to range between 250nm and 700nm. Three azo dyes were tested and the maximum peak for Direct blue 15 was observed at 609nm, 445nm for tartrazine and 430nm for Methyl red. A final concentration of 10μM was used for all dyes. The control contained media with only dye. For growth analysis, the harvested cells were resuspended in 1ml of water and the absorbance was recorded at 600nm to check the effect of the dye on the growth of the organism.

The maximum peak for each dye after the scan provided a peak profile, which enabled the determination of peak area. The peak area included the following ranges for each dye: 580nm - 630nm for Direct blue 15, 424.5 – 462.5nm for tartrazine and 420 - 440nm for methyl red. The following formula was used to calculate the area under the curve which represents the reduction of the dye:

Result = (Area * Factor) / Divisor Factor = 1.000

Chromosomal / Genomic DNA extraction

To identify the unknown pure cultures (unknown 1 and 2), genomic DNA was extracted for 16s RNA sequencing. Anaerobic bottles containing 100ml of BHI media were inoculated with 0.2 ml of pure unknown culture and incubated at 37ºC until the culture had reached turbidity. The culture was then transferred to 250ml autoclaved Nalgene centrifuge bottles. The cells were spun down for 10 min at 4000g at room

temperature using the Sorvall GSA rotor. The pellet was then resuspended in Solution I [50mM glucose, 25mM TrisCl (pH 8.0), 10mM EDTA (pH 8.0), autoclaved, and stored at 4ºC], 10% SDS, 50µL of 20mg/ml proteinase K, 50µL of mutanolysin and 2.5ml of lysozyme (15mg/ml). The mixture was then incubated for an hour at 37ºC. After incubation 5M sodium chloride and CTAB/NaCl (10% CTAB in 0.7M NaCl: Dissolve 4.1g NaCl in 80ml of distilled water and slowly added 10g CTAB while heating and stirring. Volume was adjusted to 100ml) solutions were added, mixed thoroughly and incubated for 20 min at 65ºC. The nucleic acid in the mixture was then extracted with approximately equal volumes of chloroform/isoamyl alcohol (24:1) and centrifuged at room temperature for 10 minutes at 6000g using the Sorvall SS-34 rotor. The aqueous phase was transferred to a fresh autoclaved 50ml Nalgene tube. Two volumes of phenol/chloroform/isoamyl alcohol (1:1 ratio) was added and centrifuged as before. The aqueous phase was transferred to a fresh 50ml Nalgene tube and 0.6 volumes cold isopropanol was added and incubated over night in a -20ºC freezer to precipitate the DNA. The genomic DNA was then spun down at 6000 × g room temperature for 10 minutes using the Sorvall SS-34 rotor. The supernatant was discarded and the precipitate was washed twice with 70% ethanol to remove excess salt. Excess ethanol was removed by air drying the pellet in the SC110A Savant SpeedVac. The pellet was then resuspended in 1ml TE and RNase buffer (final concentration of RNase is 5µg/ml of TE) and incubated at 65ºC for 20 minutes to remove RNA contamination.

A 0.8% agarose gel (30 ml 1X TAE Buffer, 0.24 g Agarose, and 1.5µL Ethdium Bromide) was made and the samples (5µL of DNA + 3 µL sterile H2O + 2 µL of 6x loading dye) were run out on the gel at 65V for one and a half hours. The gel stained with ethidium bromide and the DNA visualized under UV light. The

concentration of DNA was determined using the Nanodrop ND-1000 spectrophotometer.

PCR of the 16S rRNA gene

To identify the pure cultures, the 16S rRNA primers were used and the amplified product was sequenced. Briefly, the extracted genomic DNA was subjected to PCR amplification of the 16S rRNA gene using the forward primer Bact27f (5' AGA GTT TGA TCM TGG CTC AG 3') and the reverse primer Bact1492r (5' TAC GGY TAC CTT GTT ACG ACT T 3'). The PCR mix contained the following: 37.8µl of sterile distilled water, 5µl PCR buffer, 3.5µl Magnesium chloride, 2 µl dNTPs (PCR nucleotide mix, Fischer Scientific), 1µl DNA of unknown genomic DNA, 1µl Bact27f, 1µl Bact1492r, 0.5µl Taq polymerase (Taq DNA polymerase in Storage Buffer A, Promega) and 50µl of sterile mineral oil as an overlay. The PCR conditions were: initial denaturation: 94º C for 4 minutes, followed by 30 cycles of denaturation, annealing and elongation. Each cycle consisted of: denaturation: 94ºC for 45 seconds, annealing: 56º C for 1 minute, elongation: 72ºC for 1.5 minutes. The final elongation was done at 72º C for 7 minutes. To confirm the presence of the PCR product, the PCR reaction (8µl of product and 2µl of dye) was run on a 0.8% agarose gel at 65V for an hour. The predicted amplified product was approximately 1500 base pairs.

Sequencing the PCR products

Prior to sequencing, the PCR products were purified to remove enzymes, dNTP's and salts, using the Wizard PCR Preps DNA purification system (Promega). Taking care that the oil layer was not collected, 45µl of the PCR reaction was collected and added to a fresh microcentrifuge tube containing 100µl of Direct purification buffer (50mM potassium chloride, 10mM Tris-HCl pH 8.8 at 25C, 1.5

mM $MgCl_2$, 0.1% Triton X-100), followed by briefly vortexing. To this 1ml of resin was added and vortexed every 15-20 seconds for 1 minute. The resin/sample mixture was added Wizard minicolumn and vacuum applied. After breaking the vacuum, 2ml of 80% isopropanol was added and the vacuum was reapplied to drain the solution and dry the resin. The minicolumn was then centrifuged three times at 10,000 x g using a table top centrifuge after which the column was transferred to a clean microcentrifuge tube. The DNA was eluted by adding 30µl of water to the column and centrifuged for 30 seconds at 10,000 x g. The ND-1000 Nanodrop was used to determine the concentration of the DNA. The Bact27F primers were used to sequence (Recombinant DNA/Protein Resource Facility, Oklahoma State University) the unknown samples. The sequence results were analyzed using the nucleotide-nucleotide BLAST tool at the National Center for Biotechnology Information website.

Polymerase Chain Reaction (PCR) of the azoreductase gene

Among the bacteria in Table 3, the bacterium that contained a known azoreductase gene was *E. faecalis*, which also grew in BHI. Therefore, the selected species would be tested to determine if the gene or a similar gene to that of *E. faecalis* existed. At the time of the study only 90% of the genome was complete for *E. faecium*; thereby the PCR technique would help us analyze the genome of *E. faecium* for the presence of a similar *E. faecalis* azo gene. PCR was carried out using 1µl of genomic DNA as template. The control was genomic DNA extracted from *Enterococcus faecalis*. The control *azoA E. faecalis* primers were the same as those used by H. Chen *et al*. The forward primer was named azoFor (5'-gattcatatgtcaaaattattagttg-3') and the reverse primer azoRev (5'-cgggatccgctttatgccgcagctaag-3'). The PCR mixture contained 2µl of each primer in

addition to 79.5µl of sterile distilled water, 10µl of 10X PCR buffer, 3µl of Magnesium chloride, 2µl of dNTP (PCR nucleotide mix, Fischer Scientific) and 1µl of Taq DNA polymerase (Cat. no. 18038-018, Invitrogen). The thermocycling conditions were 30 cycles of denaturing at 94ºC for 1 minute, annealing at 50ºC for 1 minute, extension at 72ºC for 1 minute and final elongation at 72ºC for 5 minutes. On completion the PCR products were run on a 0.8% agarose gel to confirm the presence of the product.

Labeling of probe for Southern Blot

To further analyze the presence of an *E. faecalis* gene in *E. faecium*, southern blot analysis was performed. The AzoA template and the control DNA (DIG kit) were used to prepare the probe. The probe was prepared using the Digoxigenin Random Primed DNA Labeling kit (Roche Applied Science). Briefly, fifteen microliters of 0.075µg/µl of the AzoA template and 5µl of control DNA were added to a 1.5ml microfuge tube and boiled for 10 minutes to denature the DNA, followed by cooling on ice for 2-3 minutes. Labeling was carried out by adding 2µl of 10X hexanucleotide mixture, 2µl of 10X dNTP labeling mixture, and 1µl of Klenow enzyme to the denatured DNA. The reaction was then mixed, centrifuged for 45 seconds, and incubated at 37°C for an hour. The probe was then stored at -20°C.

Southern Blot

Restriction digests of the genomic DNA were set up for the control and the test sample. The 20µl digest contained 12µl sterile distilled water, 2µl 10X buffer, 5µl genomic DNA (approximately 0.574µg/µl- 1.42µg/µl) and 1µl *Eco*RI enzyme (10U/µl). The reaction was incubated at 37ºC for two and a half hours and the digest run on a 0.8% agarose gel at 25V for seven hours. The gel containing the DNA was

hydrolyzed by acid depurination, which involved soaking the gel twice for 15 minutes in 0.25M hydrogen chloride at room temperature. The DNA was then denatured in a solution of 1.5M sodium chloride and 0.5M sodium hydroxide for an hour at room temperature with constant shaking. The DNA was then neutralized by soaking the gel in several volumes of a solution containing 1M Tris chloride (8.0) and 1.5M sodium chloride for an hour at room temperature with constant shaking. In a separate tray, Whatman filter paper was soaked with 20X SSC solution (87.65g of sodium chloride, 44.1g of sodium citrate were dissolved in 500ml distilled water, pH 7.0). Care was taken that all the air bubbles were smoothened out. The prepared gel was then inverted on the filter paper with the underside facing upward. Positively charged nylon membrane (Molecular Biology Boehringer Mannheim) was cut and soaked completely in 2X SSC solution for 2-3 minutes. Care was taken that the membrane was handled with forceps at all times. The membrane was placed over the gel and care was taken that no air bubbles were introduced during the process. The absorption technique was used to transfer the DNA from the gel to the nitrocellulose membrane. Briefly, two pieces of Whatman Filter paper were cut, soaked in 2X SSC and placed over the membrane. A stack of dry paper towels were placed over the filter paper and a weight was applied on top. The setup was left at room temperature without disturbing for sixteen hours. Care was taken that the dish did not run dry. After sixteen hours the gel was separated from the membrane. The membrane was marked and the corner cut to orient the sample positions. The gel was checked for complete DNA transfer to the membrane using a UV light. The membrane was soaked in 6X SSC solution for 5 minutes at room temperature and air dried. Once the membrane was air dried it was crosslinked using the Fisher Scientific FB-UVXL-1000 UV crosslinker and stored between Whatman filter paper at room temperature.

A hybridization tube was filled with 15ml of hybridization solution (DIG Easy Hyb) and warmed to 60ºC for 60 minutes in the Fischer Biotech hybridization incubator. The prepared membrane was put into the hybridization tube, with the DNA side facing away from the glass tube. The membrane was preincubated at 60ºC with constant rolling for 30 minutes and the solution was discarded. The DIG probe was heated to 68ºC in a water bath and then added to the tube. Hybridization was carried out at 60ºC for 18 hours in the hybridization incubator with constant rolling. The membrane was then subjected to post-hybridization washes. The membrane was washed twice with a solution of 2X SSC, 0.1% SDS at room temperature for 5 minutes each. The membrane was then washed twice with 0.1X SSC, 0.1% SDS at 68ºC with constant shaking for 15 minutes. The membrane was rinsed briefly (2-3 minutes) in washing buffer (0.1M Maleic acid, 0.15M sodium chloride; pH 7.5; 0.3% v/v Tween 20) and put into 100ml of 1X blocking solution (10X DIG blocking reagent: Maleic acid buffer, 1:10) for 60 minutes and followed by the addition of 20ml of freshly prepared antibody solution (1:5000 anti-digoxigenin-AP in blocking solution) and incubated for additional 30 minutes. The membrane was washed twice with 100ml of washing buffer for 15 minutes each, followed by incubation for 2-5 minutes in 20ml detection buffer (0.1M Tris-HCl, 0.1M sodium chloride, pH 9.5). The membrane was then transferred to 10ml of freshly prepared color substrate solution (200µl of NBT/BCIP stock solution to 10ml of detection buffer) and incubated in the dark without shaking. After 18 hours the reaction was stopped by washing in 50ml of sterile distilled water. The membrane was air dried and stored at 4ºC.

Results and Discussion

BHI Cultivation and isolation of pure cultures

Prior to this study, no single media was used to for the growth of the organisms shown in Table 3. Different combinations of BHI with supplement, TYG as well as the recommended media were all used. Therefore, all species from Table 3 was grown in BHI media (minus Tween 80, fatty acids and vitamin solutions) to determine which bacterium species were able to grow in simple BHI. Only 8 out of the 21 species were able to grow in BHI (Table 3), thus BHI served as an initial selection media. Note, of the species in Table 3, the species that were not in lyophilized form or were not labeled (short term cultures) were *E. faecium, Bifidobacterium bifidium, Eubacterium rectale, Bacteroides fragilis, Streptococcus intestinalis and Ruminococcus bromii.* Therefore it was likely that these were in the unlabeled short term cultures called unknowns. We set out to determine what species were present in these unlabeled cultures. Only the unknowns that grew in BHI were subjected to colony isolation. Two distinct types of colonies were identified from the unknowns. They were labeled unknown 1 and 2. Unknown 1 had a smooth, convex, medium size and white morphology when compared to Unknown 2 which had a smooth, convex, small size, off-white and moist morphology. Both unknowns were gram positive, diplococci. Once it was established which cultures were able to grow in BHI, they were included in the larger group of positive BHI, which were grown in the presence of high concentrations of Direct Blue 15 (100mM) to determine azoreductase activity. Table 3 shows which organisms were able to grow in BHI. These included *B. stercoris, B. adolescentis, B. infantis, C. perfringens, E. faecium, E. faecalis, E. biforme* and *E. rectale.* Table 3 also shows which microbes from the

positive BHI were able to reduce high concentrations of Direct Blue 15 (100mM), which included *B. stercoris, B. adolescentis, B. infantis, E. faecium, E. faecalis*. The results were based on visual examination of dye color lost at 24 hrs. As determined later in the study, the unknowns were identified using 16S rRNA sequencing, in which they turned out to be *E. faecalis* and *E. faecium* (See 16S rRNA section for details). Note, that from another study, the remaining species that were not identified as lyophilized or label, were purchased from ATCC as well as tested for their ability to grow in BHI, as well as further test for their ability to reduce high concentrations of Blue 15. The data from Table 3 enabled the final selection of the species that would be further studied (Chapter 3). The following criteria were used. First, of the positive BHI and azoreductase species, only *B. adolescentis, C. perfringens, E. faecalis, E. faecium,* and *E. rectale* contained genomic information. Within this group, *E. faecalis* and *C. perfringens* were the only two that possessed an azoreductase gene. Therefore, we decided that the close relationship between *E. faecium* and *E. faecalis* would provide a means of performing molecular analysis in terms of azo gene similarity, thus it was selected as the focus microbe in Chapter 3. Finally, the BHI and azo dye study was valuable in identifying a group of human intestinal bacteria that may possess an azo gene, and prior to the completion of this study it was not known which microbes in Table 3, including the closely related *E. faecium*, was able to reduce high concentrations of Blue 15. Thus, the results from this study identified viable candidates for future azoreductase studies.

PCR and Sequencing of the 16S rRNA gene

The following section describes details of the PCR and sequencing process. The genomic DNA was isolated for both unknowns, and Figure 4 shows a

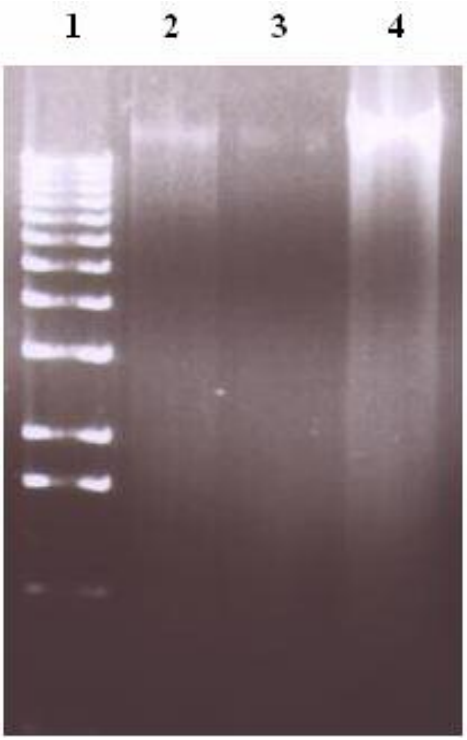


Figure 4: Extraction of genomic DNA. Lane 1: 1kb ladder; Lane 2: *E. faecalis*, Lane 3 and 4: Unknown 1.

representation of the agarose gel containing chromosomal DNA for unknown 1 and the control (*E. faecalis*). The unknowns were identified using the 16S rRNA sequence technique, which is based on PCR amplification. The predicted product of 1500bp was obtained for both unknown 1 and 2 (Figure 5 and 6). The PCR product was purified and the final concentrations were between 0.035 µg/µl - 0.109 µg/µl). The sequence was determined and it was searched using the BLAST tool, which revealed unknown 1 to be *Enterococcus faecalis* with a 99% match and unknown 2 to be *Enterococcus faecium* with a 99% match.

PCR amplification

Since *E. faecium* is related to *E. faecalis* (AzoA gene), it was possible that *E. faecium* may possess a similar azo gene. The complete genomic information for *E. faecium* was not available, thus it was not known if the AzoA sequence was present in *E. faecium*. To test this specific AzoA primers were used to PCR amplify the gene from the chromosomal DNA of *E. faecium*. Figure 3 shows a 0.8% agarose gel and the yield of genomic DNA from the extraction procedure. The concentration of genomic DNA was between 0.3µg/µl and 2.0µg/µl.

The genomic DNA from *E. faecium* (unknown 2) and *E. faecalis* (unknown 1) was used for PCR amplification. *E. faecalis* (unknown1) yielded the predicted PCR product of approximately 800 bp based on agarose gel analysis (Fig. 7). The result confirmed that unknown 1 was *E. faecalis* and it contained an AzoA gene. *E. faecium* did not produce a PCR fragment (Data not shown); confirming the genomic DNA from *E. faecium* did not contain the azo gene similar to *E. faecalis*. To further confirm these results, a southern blot was done using the *E. faecalis* probe.

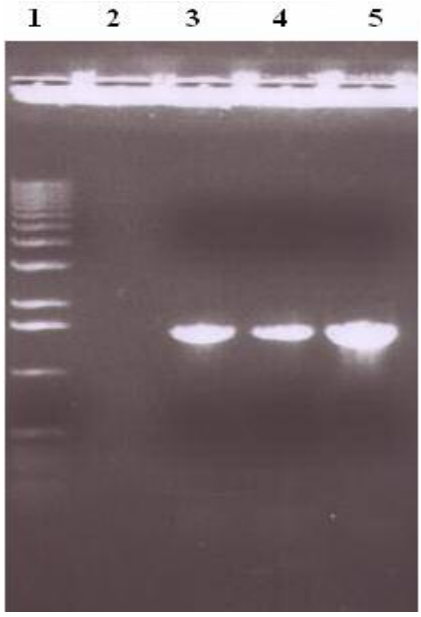


Figure 5: PCR amplification of the 16S rRNA gene. Lane 1: 1kb ladder, Lane 2: empty, Lane 3: *Enterococcus faecalis* (control), Lane 4 and 5: unknown 1.

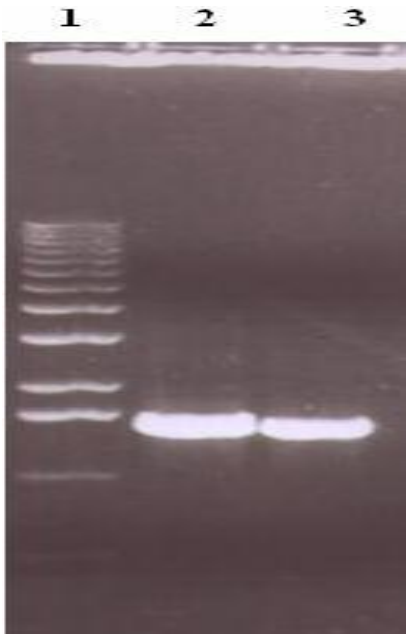


Figure 6: PCR amplification of the 16S rRNA gene. Lane 1: 1kb ladder, Lane 2: *Enterococcus faecalis* (control), Lane 3: unknown 2.

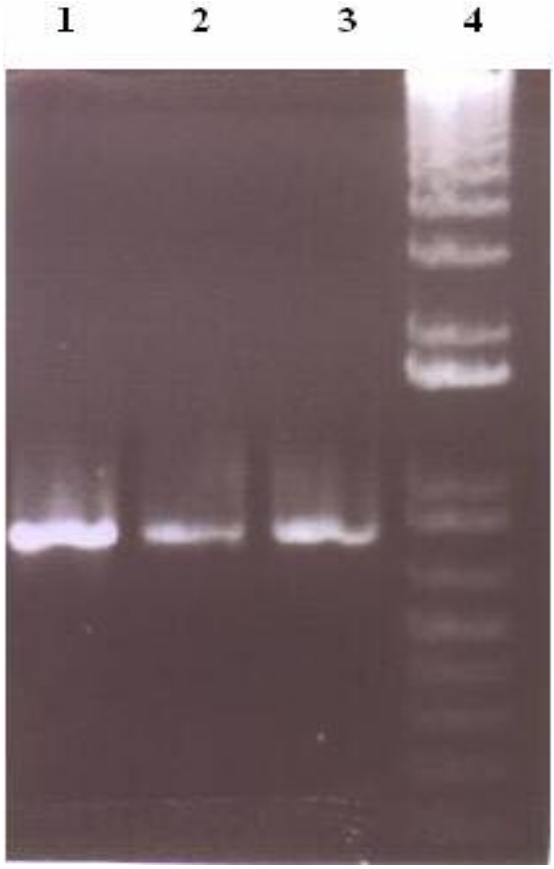


Figure 7: PCR amplification of the azoreductase gene showing the presence of the azoreductase gene in both organisms. Lane 1-2: Unknown 1, Lane 3: *Enterococcus faecalis* (control) and Lane 4: 1kb ladder.

Southern Blot

Figure 8 shows the positive signal for the control and unknown, which are weak due to the low concentration of genomic DNA used (lane 2 and 3), compared to the concentration of genomic DNA used for lane 4. The results confirmed the presence of a gene similar to the azoreductase gene from *E. faecalis* (*azoA*) in the unknown 1.

Spectrophotometric Dye Reduction Assay

E. faecium was selected as the microbe to further test in this study, as it grew in BHI, it grew in high concentrations of Blue 15, it reduce high concentrations of Blue 15, it had available genomic information (90% complete), and was similar to an already similar *E. faecalis* which possessed an azoreductase gene (AzoA). To study more closely the ability of *E. faecium* to reduce azo dyes, the monoazo dyes tartrazine and methyl red and the diazo dye Direct Blue 15 were tested. Table 4 shows the decrease in dye absorbance or peak area for the three dyes. *E. faecium* (Unknown 2) reduced all three dyes after 3 hours. The same data is represented in Figure 9 as a line graph. The graph demonstrates the ability of *E. faecium* (unknown 2) to reduce the dyes completely after 3 hours. In addition, the growth results indicate that *E. faecium* (unknown 2) possess the ability to reduce both mono and diazo sulfonated dyes without any inhibitory effects on the bacterial growth rate (Fig. 10). Finally, the culture supernatants did not show any azoreductase activity which indicates that the enzyme is not an extracellular enzyme but an intracellular enzyme (Data not shown).

In conclusion, we have identified a group of intestinal bacteria that are able to grow in simple BHI and reduce high concentrations of Direct Blue 15. After careful consideration of the data, we selected unknown 2 which was identified as *E. faecium* from a mixed culture using16S rRNA sequencing. *E. faecium* was one of the

Figure 8: Southern Blot hybridization using the *E. faecalis azoA* probe. Lane1: empty, Lane 2: *E. faecalis*, Lane 3- 4: unknown 1.

original ATCC cultures, as predicted earlier, as it was mixed with *E. faecalis*. The specific *E. faecalis* primers and PCR probe showed *E. faecium* was different. The selected microbe *E. faecium* is a gram positive, facultative anaerobe which inhabits the GI tract of humans and it was further tested and shown to grow in the presence of and reduce the mono and diazo sulfonated dyes to colorless end products which were detected visually and spectrophotometrically. Genomic analysis revealed that *E. faecium* was a potential candidate to further identify the azoreductase gene. Based on these results we hypothesize that the gram positive intestinal microbe *Enterococcus faecium* possesses a gene which codes for the production of the enzyme azoreductase which results in the breakdown of mono and diazo dyes. Chapter 3 describes this in more detail.

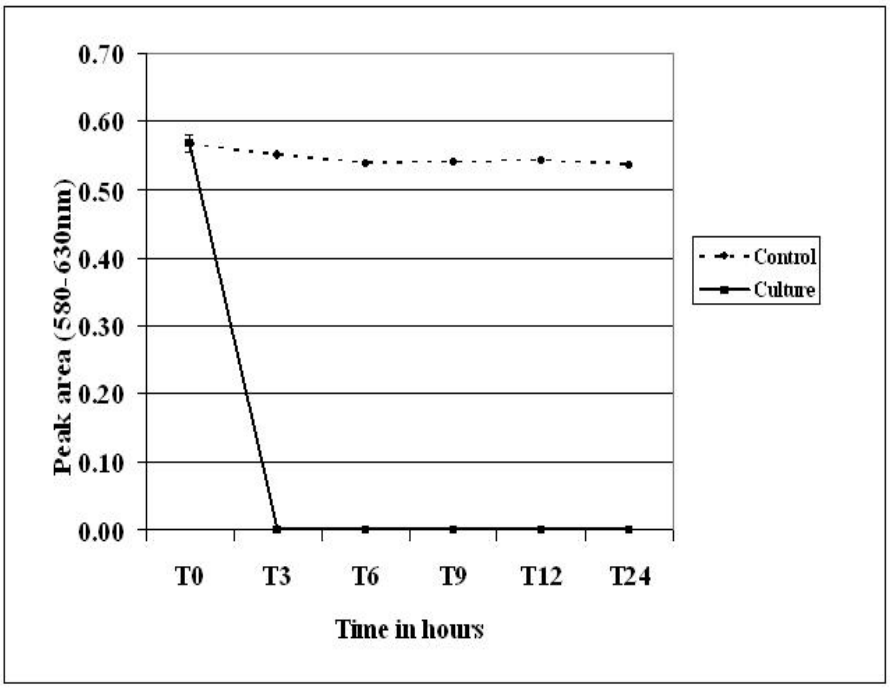
TABLE 4

Culture Reduction of the azo dyes Direct Blue 15, Tartrazine and Methyl red

Time in hours	Direct Blue 15[1]		Tartrazine [2]		Methyl Red[3]	
	Absorption (O.D.)					
	Control	Culture	Control	Culture	Control	Culture
0	0.57	0.57	0.55	0.55	0.06	0.07
3	0.55	0.00	0.59	0.00	0.06	0.00
6	0.54	0.00	0.53	0.00	0.05	0.00
9	0.54	0.00	0.58	0.00	0.05	0.00
12	0.54	0.00	0.57	0.00	0.04	0.00
24	0.54	0.00	0.52	0.00	0.04	0.00

1. Absorbance values of Direct Blue 15.
2. Absorbance values of Tartrazine.
3. Absorbance values of Methyl Red

A



B

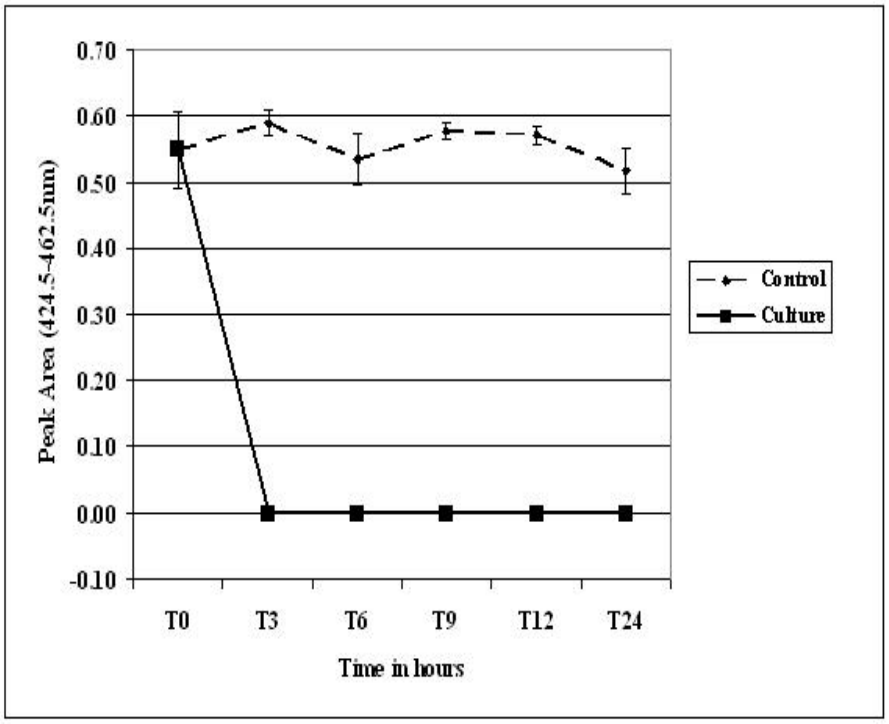


Figure 9: Whole cell dye reduction. (A) Reduction of Direct blue 15 (B) Reduction of Tartrazine.

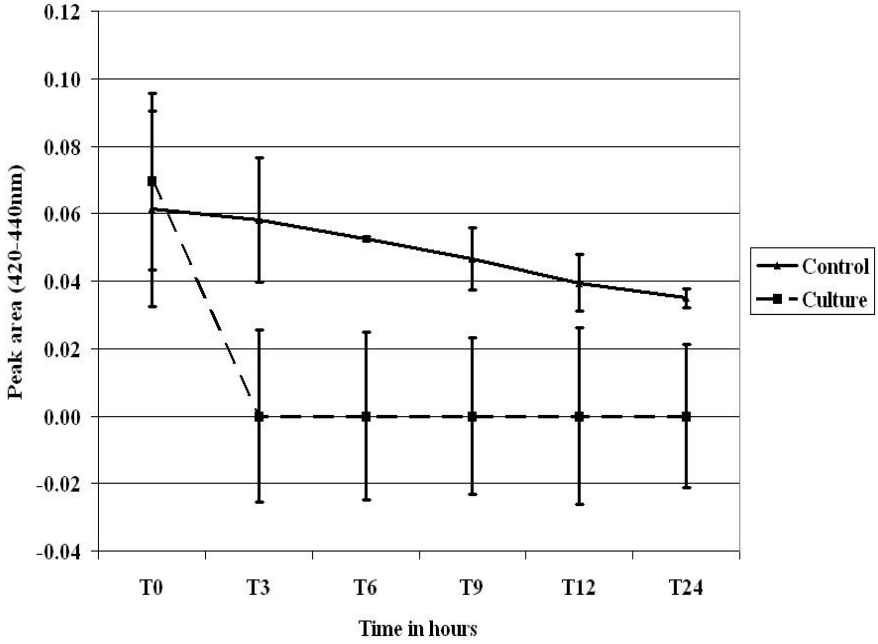


Figure 9: Whole cell dye reduction. (C) Reduction of Methyl red.

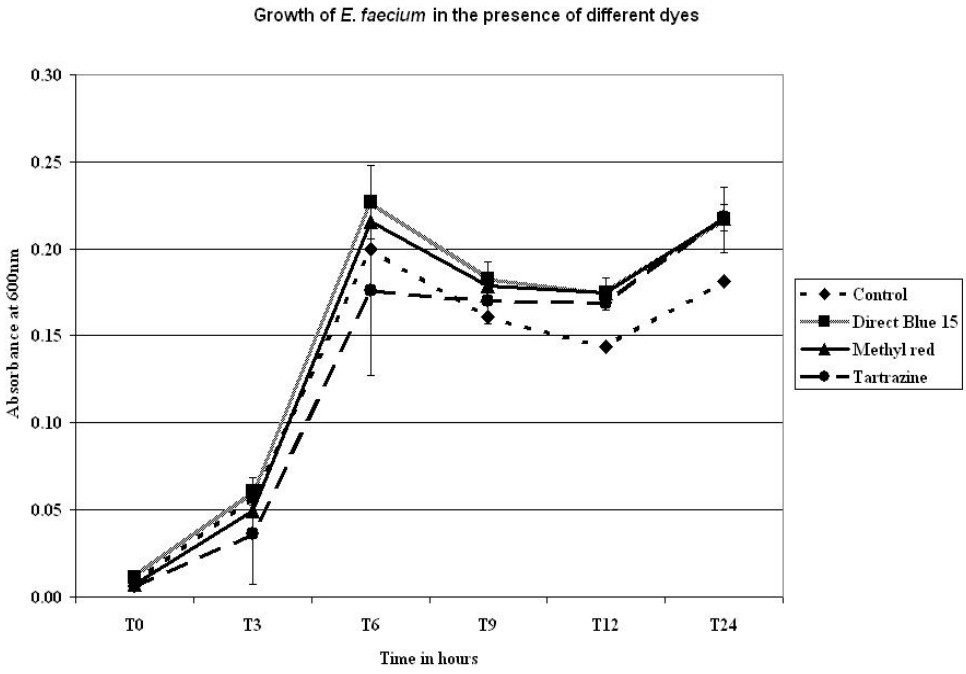


Figure 10: Effect of dye on the growth of *Enterococcus faecium*.

CHAPTER III

Cloning, expression, purification and characterization of azoreductase in *E. faecium*

Introduction

An important function associated with intestinal bacteria is their ability to metabolize xenobiotics, such as azo dyes (Chung, KT. *et al*., 1978; Brown, JP. 1981; Cerniglia, CE., 1982; Manning, BW., Cerniglia, CE. and Federle, TW., 1985). Azo dyes are characterized by the presence of one or more R1-N=N-R2 bonds, and are widely used in the paper, textile, plastic, pharmaceutical, food, cosmetic, enamels, and drug industries (Collier *et al*. 1993; Dillon *et al*. 1994; Levine, 1991). The entrance of these dyes into the human body by either environmental contamination or ingestion of food and drugs can result in exposure to potential carcinogenic metabolites (Cerniglia *et al*. 1986; Bragger *et al*. 1997; Brown *et al*. 1983). Azo dye metabolites are produced after being reductively cleaved at the –N=N- position, and are considered toxic aromatic amines. For example, the metabolism of the azo dye Direct Blue 15 yields 3, 3' dimethoxybenzidine, a potential carcinogen (Cerniglia, CE., 1982). In addition *in vivo* and *in vitro* experiments support the toxicity of these metabolites (Morgan *et al*. 1984; Chung KT. 1983). The mechanism associated with toxicity involves the interaction between the microbial intestinal and/or by hepatic enzymes (Levine WG. 1991; Chung and Cerniglia, 1992). Intestinal bacteria first produce aromatic amines which can serve as the toxicant or the metabolite can be

further activated by hepatic enzymes (Drasar and Hill, 1972; Hill, 1974), a process known as bioactivation.

The enzyme produced by bacteria that is involved in reducing azo dyes is called azoreductase. The azoreductase gene has been cloned and characterized from various types of microorganisms such as *E. faecalis* (Chen H. *et al*. 2004), *Xenophilus azovorans* KF46F (Blumel *et al*. 2002), *Bacillus sp*. OY1-2 (Suzuki Y. *et al*. 2001) and *E. coli* (Nakanishi M. *et al*. 2001). These organisms metabolize azo dyes aerobically and the properties of these azoreductases vary. For example, the azoreductase from *E. coli* is a 46kDa monodimer, requires FMN as a cofactor, and uses NADH as an electron donor (Nakanishi M. *et al*. 2001); compared to *Bacillus sp*. OY1-2 which is 19kDa in size, is not FMN associated, and uses NADPH (Suzuki Y. *et al*. 2001). Interestingly, the deduced protein sequences from known azoreductase genes do not show significant sequence homologies.

Enterococci are associated with a variety of infections like endocarditis, respiratory and skin lesions (Morrison *et al*. 1997). One example would be *Enterococcus faecium* a predominant gram-positive cocci found to inhabit the human intestine. Due to its presence in the gastrointestinal tract, *E. faecium* has the potential to make an impact on the health of an individual, particularly as it concerns azo dye metabolism. *E. faecium* is related to the more fully characterized *E. faecalis*, but the degree of DNA relatedness is low as well as its biochemistry (Murray 1990).

The methods used to isolate and purify azoreductase have been laborious, expensive and time consuming. To simplify the process, we have used a more molecular approach. Recently *E. faecium* has been shown to reduce the azo dye Methyl red. The *acpD* gene commonality was a mean of identifying new azoreductase

genes. For example, the AzoR from *E. coli* was originally identified as an *acpD* gene which codes for the protein acyl carrier protein phosphodiesterase, but was later determined to be a gene that codes for an azoreductase (Nakanishi M. *et al*. 2001). We hypothesize that the *acpD* gene from *E. faecium* may also be responsible for the azoreductase activity. Based on a 67% amino acid sequence homology with the azoreductase from *E. faecalis*, a *acpD* gene from *E. faecium* was identified, cloned, expressed, purified and characterized for its ability to metabolize the azo dyes Direct blue 15, Tartrazine and Methyl red.

Materials And Methods

Bacterial strains, plasmids and culture conditions

Enterococcus faecium ATCC 6569 was grown in Brain Heart Infusion (BHI) broth supplemented with Vitamin K solution under anaerobic conditions, and used for genomic DNA extraction (Current protocols in Molecular Biology).

The pCR2.1-TOPO (Invitrogen) plasmid was used for cloning of the PCR fragment and pET15b (Novagen) was used for overexpression of the protein. For recombinant DNA studies, *E. coli* TOP10 (Invitrogen) competent cells and NovaBlue (DE3) (Novagen) cells were used. The *E. coli* strains were grown in Luria-Bertani (LB) medium containing ampicillin (50μg/ml) at 37^{o}C.

Culture conditions for azo dyes activity

E. faecium from a BHI agar plate was inoculated into anaerobic BHI broth tubes containing a final concentration of 10μM of azo dye and incubated at 37ºC for 24 hours. The three dyes tested were Direct Blue 15 (MP Biomedicals), Tartrazine (Sigma) and Methyl red (Sigma). Samples were analyzed by harvesting the cells for 5 minutes at 14,000g. The supernatant was analyzed at time zero and every three hours for a decrease in the optical density of the azo dye using the Shimadzu UV-1601PC UV-visible spectrophotometer. The peak area for each time point was calculated by measuring the absorbance of the dye and using the formula:

Result = (Area * Factor) / Divisor Factor = 1.000

Genome analysis and PCR amplification of the *acpD* gene

A BLAST search using the protein sequence of *E. faecium* AzoA was used to determine protein sequence homology in *E. faecium*. An *acpD* gene with 67% homology was identified as a putative *acpD*. The gene was rescued using PCR amplification. Genomic DNA was isolated from *E. faecium* and used as the template to amplify the *acpD* gene (*azoM*). The forward primer (EfciumAzof) 5'GGGGAGAAGGTAAGCATATGGCATCTG 3'contains the *Nde*I restriction site upstream of the start codon, and the reverse primer (EfciumAzor) 5'CCCCAAAAAGAGGGATCCGGGAAAATATCC 3'contains the *Bam*HI site after the stop codon.

The PCR was performed in a Perkin Elmer DNA Thermal Cycler 480, and the conditions were 30 cycles: each cycle consisting of denaturing at 94^{o}C for 1 minute, annealing at 55^{o}C for 1 minute, extension at 72^{o}C for 1 minute and a final extension at 72^{o}C for 7 minutes. The PCR reaction mix contained sterile distilled water, 1X PCR buffer (Invitrogen), 1.5mM Magnesium chloride (Invitrogen), 0.2mM dNTP mix (Fischer Scientific), 10ng genomic DNA, 50pmol of forward and reverse primers, 2.5U Taq DNA polymerase (Invitrogen) in a final reaction volume of 100µl. The amplified PCR products were run on a 0.8% agarose gel and the DNA eluted from the gel using the Qiagen QIAquick gel extraction kit.

Cloning and expression of AzoM

The amplified PCR product was directly cloned into the pCR2.1-TOPO vector (Fig. 11). The insert was sequenced using plasmid specific primers (M13 forward and reverse primers). The plasmid was digested with the restriction enzymes *Nde*I and *Bam*HI (Invitrogen) to release the insert. The released insert was eluted from a 0.8%

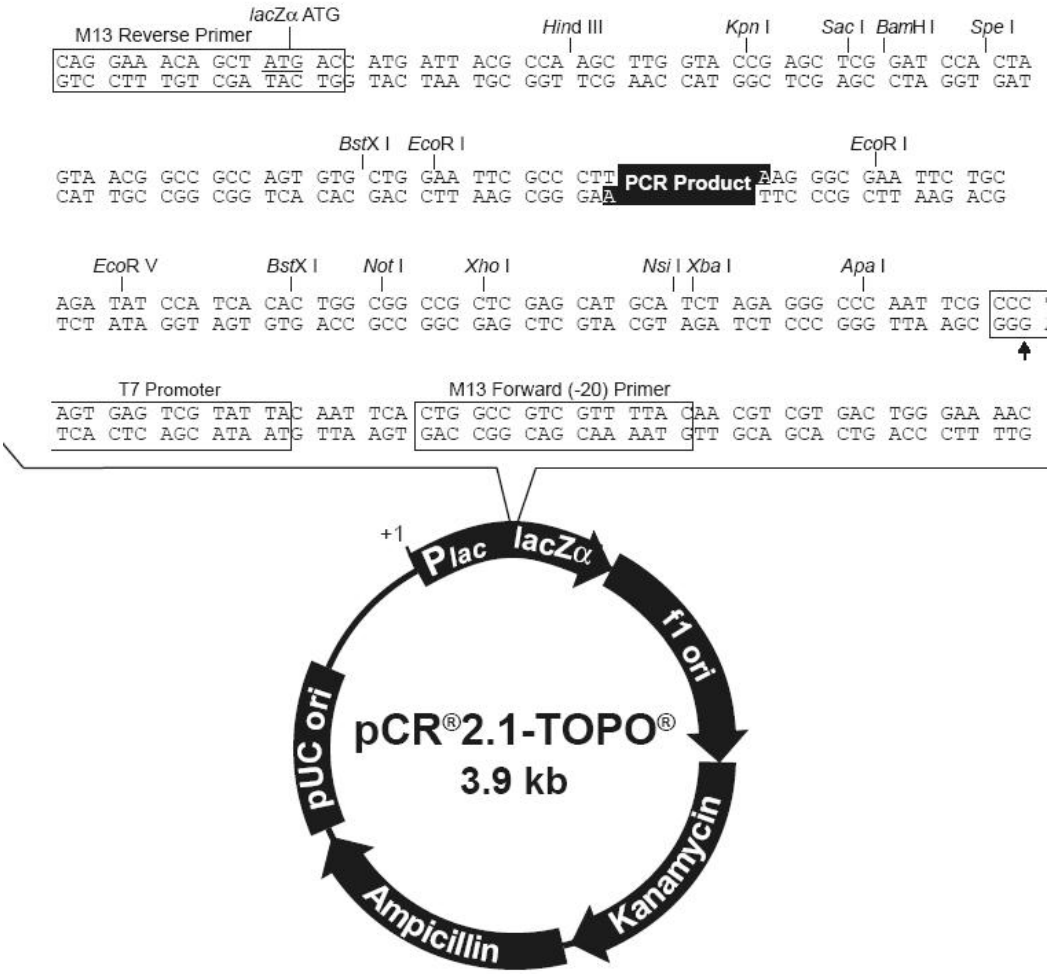
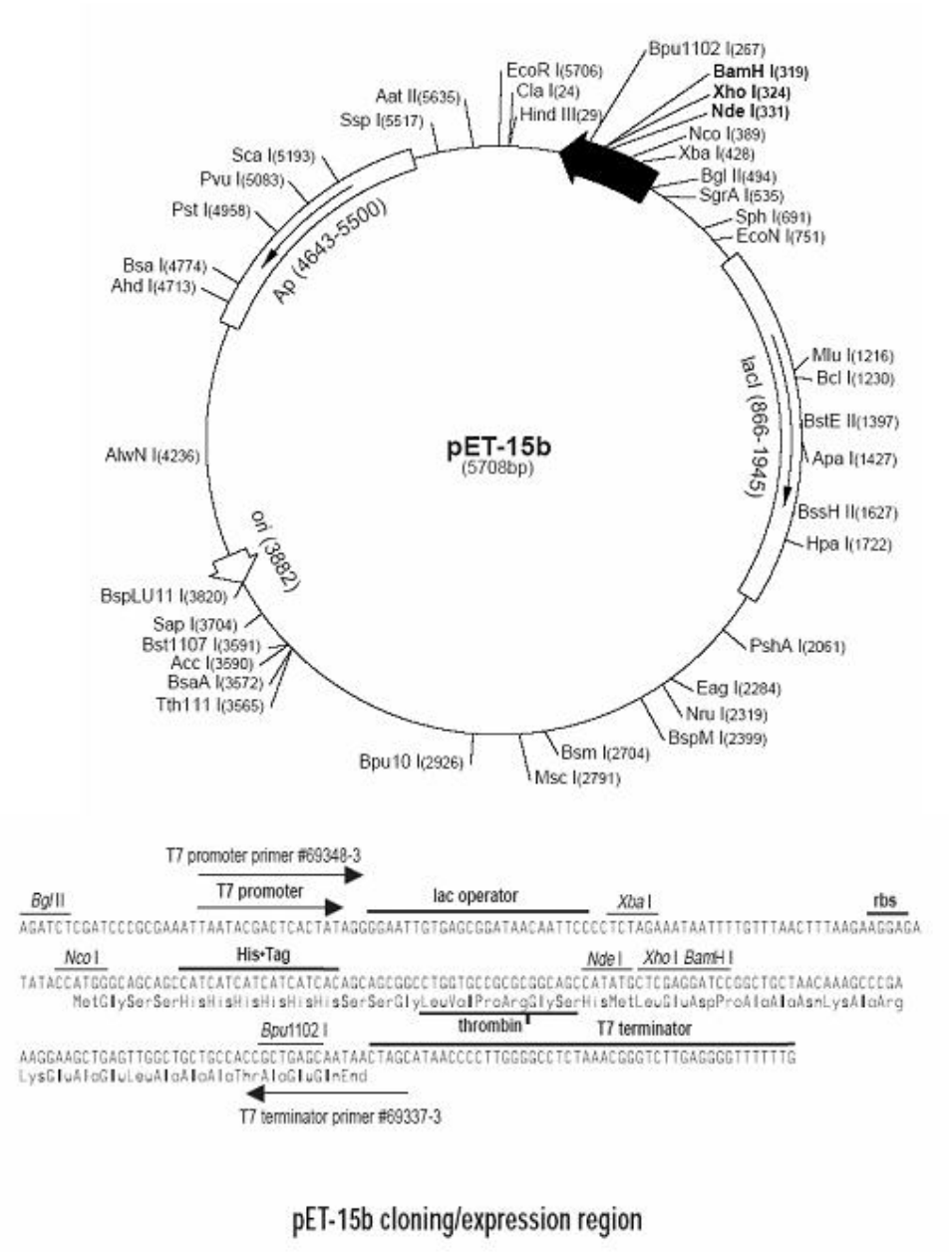


Figure 11: Map of the TOPO vector that was used to clone the amplified PCR product. Map adapted from the invitrogen TOPO kit manual.



pET-15b cloning/expression region

Figure 12: Map of pET-15b cloning and expression vector showing the T7 promoter region and terminator region used for overexpression of the protein, the HisTag region and the restriction sites NdeI and BamHI. Figure adapted from pET vector manual.

agarose gel using the QIAquick gel extraction kit (Qiagen). The purified DNA was then ligated into the pET15b vector (Fig. 12), using a 1:6 vector to insert ratio. Ligation was carried out by T4 DNA ligase (Invitrogen) at 4^{o}C for 16 hours.

The recombinant plasmid named pAzoM was transformed into *E. coli* DH5α cells. For overexpression of the protein, the NovaBlue (DE3) expression cells were transformed with pAzoM. The azoM gene was sequenced from the pET15b using the T7 promoter and terminator primers (Novagen)**.**

Purification of the enzyme

NovaBlue (DE3) cells containing pAzoM was inoculated in a LB/Amp (100µg/ml) tube and incubated over night at 37^{o}C with shaking at 300 rpm. The cells were harvested and resuspended in 1ml LB/Amp (100µg/ml) and transferred to 1 liter of LB/Amp (100µg /ml). The 1 liter culture was incubated at 37^{o}C with shaking at 300 rpm. Upon reaching an OD_{600} of 0.6, the cells were induced for 3 hours with Isopropyl-1-thio-β-galactopyranoside (IPTG final concentration of 1mM). The induced culture was centrifuged at 6000xg for 10 minutes at 4^{o}C to harvest the cells. The supernatant was discarded, and the pellet air dried and stored at -20^{o}C overnight.

The cells were prepared for analysis (Sample + 1X loading buffer) with a 12.5% SDS-PAGE gel to confirm the expression of the protein. The pellet was thawed for 15 minutes; lysis/wash buffer (20% glycerol, 50 mM sodium monobasic phosphate, 750mM sodium chloride, 20mM Imidazole in double-distilled water pH 8.0) was added and the sample vortexed to disrupt the pellet. Lysozyme (final concentration of 0.1mg/ml) was then added and the sample was incubated on ice for 30 minutes. The cells were disrupted by sonication using a Sonic 300 dismembrator with an intermediate tip and relative output of 60%. Sonication was performed 10

times for 5 seconds each and incubating on ice after each pulse. The sample was centrifuged at 10,000g for 30 minutes at 4^{o}C using a Sorvall SS-34 rotor to remove cell debris. The supernatant was then subjected to purification using 50% Ni-NTA slurry (50% suspension in 30% ethanol, precharged with Ni^{2+}). In a centrifuge tube 4ml of supernatant was mixed with 1ml of the Ni-NTA slurry.

All protein purification steps were carried out at 4^{o}C. The 50% Ni-NTA slurry was washed with lysis/wash buffer to remove ethanol. To allow the protein to bind to the resin the tubes containing the samples were fixed horizontally to a Biodancer (New Brunswick Scientific) and incubated for an hour with constant rotation at a speed of 8. The sample was centrifuged for 2 minutes at 3800rpm using a Sorvall SS-34 rotor. The pellet was then washed three times with lysis/ wash buffer (Appendix), and centrifuged as above. The protein was eluted by resuspending the pellet in 1.5ml of elution buffer (20% glycerol, 50 mM sodium monobasic phosphate, 750mM sodium chloride, 250mM Imidazole in double-distilled water pH 8.0) and centrifuging at 3900rpm. This step was repeated four times and the protein was collected after each elution. All samples were stored at -20^{o}C.

Dialysis was carried out using the Spectra/Por molecular porons membrane tubing (25mm width, 16mm diameter, MW: 12-14000kDa). The protein was dialyzed in dialysis buffer (50mM potassium phosphate buffer pH 7.0, 10mM Magnesium chloride, 10mM sodium chloride, 10% glycerol) for 6 hours with constant stirring at 4^{o}C. Fresh dialysis buffer was added and dialysis was continued for another 16 hours with constant stirring. The protein was concentrated using the Millipore Ultrafiltration system. The concentration of the protein was determined using the BSA standard curve.

Enzyme assay

All enzyme assays were carried out in a quartz cuvette with a total reaction volume of 1ml. The activity of the enzyme was assayed by measuring the decrease in the optical density for the azo dye (430nm for methyl red) with a Shimadzu UV—1601PC UV-visible spectrophotometer. The 1ml reaction mixture contained 25mM potassium phosphate buffer pH 7.0, different concentrations of the azo dye methyl red (17μM and 31μM), different concentrations of NADH (0.1mM, 0.5mM) and a different amounts of the enzyme (100μg, 250μg, 500μg) were used for the assays. Enzyme denatured by boiling and the addition of a few drops of HCl was used as a control for all enzyme assay experiments. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the decolorization of 1μM of azo dye per minute. Enzyme reactions were carried out aerobically at room temperature and the reactions were initiated with the addition of enzyme. All reactions were done in triplicates. A time course experiment was carried out for 2 minutes and readings were acquired every second.

Effect of pH and Temperature on enzyme activity

To determine the effect of pH on the activity of the enzyme, a pH range of 4.0 to 9.0 was studied using different buffer systems. The three different buffer systems used for the experiments are: pH 4 and 5 Sodium acetate (pKa 4.76), pH 6 and 7 potassium phosphate (pKa 6.86) and pH 8 and 9 Tris- HCl (pKa 8.06). A 2 minute time course experiment was carried out to check the effect of pH on the activity of the enzyme and readings were acquired every second.

Four different temperatures (12, 28, 38 and 53) were chosen to check the optimum temperature for the activity of the enzyme. The reaction mixture was

preincubated at the particular temperature for five minutes. The reaction was initiated by the addition of enzyme. An initial reading was recorded at time 0 and the reaction was incubated for 2 minutes. After 2 minutes another reading was obtained. All temperature experiments were carried out similarly.

Results and Discussion

Culture conditions for azo dye activity

Based on visual observation *Enterococcus faecium* grown in the presence of 10 μM azo dye (Direct blue 15, tartrazine and methyl red) resulted in the lose of dye color (Fig. 3).

The decrease in dye absorbance for all three dyes was observed for *E. faecium* (Fig. 9). There was a significant decrease in absorbance at 3 hours; and times points beyond 3 hours showed very little change. The data suggests that after a period of three hours there was complete reduction of all three dyes (Table 4). In addition the azo dyes show very little inhibitory effects on the bacterial growth rate (Fig. 10). The culture supernatants did not show any azoreductase activity which indicates that the enzyme is not an extracellular enzyme.

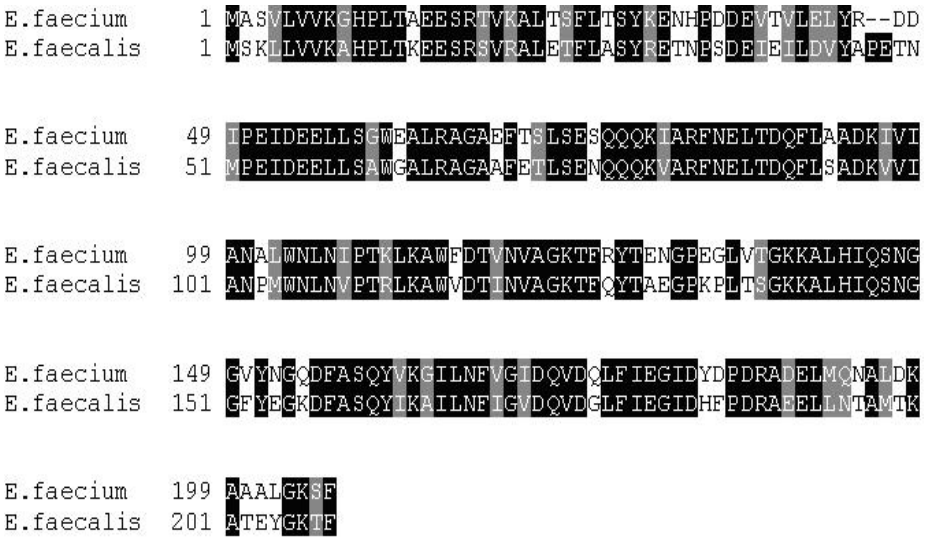
Identification of the *E. faecium* acpD gene

The AzoA protein sequence from *E. faecalis* was used to carry out a BLAST search against the *E. faecium* database using the protein-protein BLAST tool at the National Center for Biotechnology Information database. The search resulted in a single acyl carrier protein phosphodiesterase (acpD) protein that was 206 amino acid in length and showed 67% primary structure identity with AzoA (Fig. 13A) and 42% identity with the AzoR from *E. coli* (Fig. 13B).

Cloning and expression of the acpD gene from *E. faecium*

Specific primers were designed to amplify the *acpD* gene from *E. faecium* using the genomic DNA that was extracted. The PCR resulted in a 730bp amplified

A)



B)

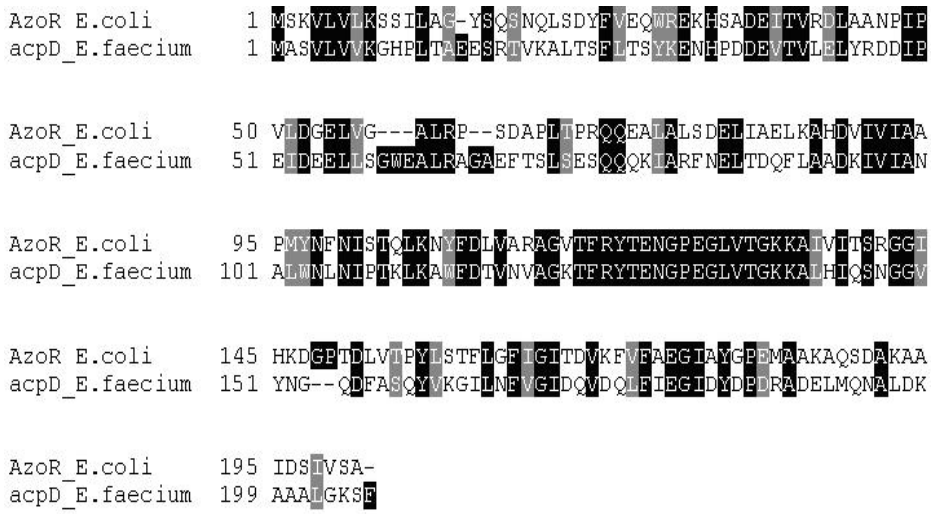


Figure 13: Amino acid sequence homology. A) 67% sequence identity between *E. faecium* acpD and *E. faecalis* azoA, B) 42% sequence identity between *E. coli* AzoR and *E. faecium* acpD.

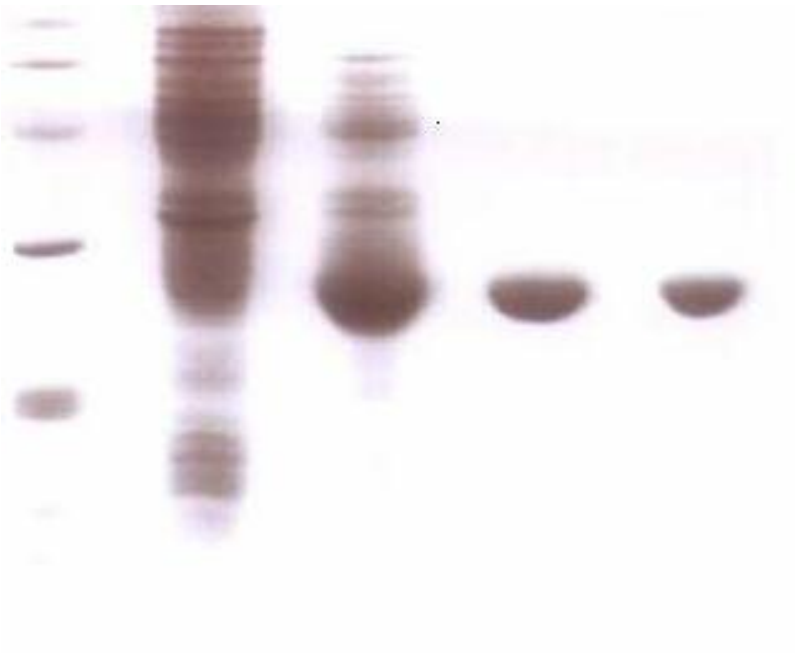


Figure 14: SDS-PAGE showing the induced, purified and dialyzed 23kDa protein. Lane 1: SDS-PAGE Broad range marker, Lane 2: uninduced DE3 cells containing the pET construct, Lane 3: induced DE3 cells containing the pET construct, Lane 4 and 5: Dialyzed protein.

fragment. This fragment was directly cloned into the TOPO vector. Sequencing results showed that the DNA fragment contained the complete ORF for the acpD protein. There were ten base pair and two amino acid differences were observed in the azoM when compared to the sequence from *E. faecium* ATCC 6569. The amino acid changes were seen at the 88 and 173 residue. These changes do not lie in the coenzyme binding motif sites and therefore no effect on the activity of the enzyme.

The pET vector is a powerful system used for the overexpression of recombinant proteins in *E. coli*. The target genes are cloned under the control of the T7 transcription and translation signals and expression is induced based on the hosts T7 polymerase. The pET15b vector was used for subcloning of the *acpD* gene. The pET vector was cut with the restriction enzymes *NdeI* and *BamHI* and the insert containing the corresponding sites were ligated into the vector. The plasmid containing the target gene was then transformed into DE3 cells where, upon induction with IPTG and under the influence of the host T7 polymerase the protein was overexpressed. Figure 14 (lane 3) shows that the protein AzoM was overexpressed successfully.

Purification of AzoM

The crude supernatant had a concentration of 40.2mg/ml, exhibited azoreductase activity and yielded a yellow coloration which persisted even after dialysis and concentration of the protein (Data not shown). When run on an SDS-PAGE gel, the protein showed an approximate size of 23kDa (Fig. 14). After dialysis, the concentration of the protein was 12.5mg/ml.

The crude supernatant and the dialyzed protein exhibited a yellow coloration. An absorption spectra of the purified protein, showed peaks at 377nm and 457nm

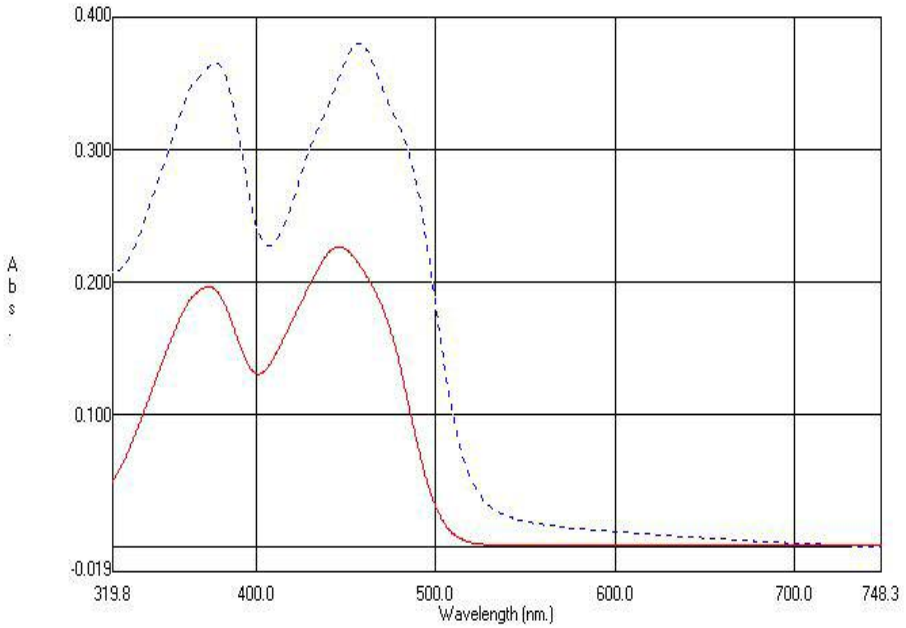


Figure 15: Spectrophotometric scan of the pure protein showing the presence of a flavin associated with the protein. Dotted line represents the protein and the solid line represents FMN. Peaks for the protein are observed at 377nm and 457nm.

which is similar to the peaks for free flavins (372nm and 445nm) with a slight shift (Fig. 15).

Enzyme Assay

To test azoreductase activity, two experimental approaches were used. The first experiment tested the decrease in dye concentration, using different concentrations of enzyme (100µg, 250µg and 500µg). Using 0.1 mM NADH, 17µM of Methyl red and increasing concentrations of the enzyme, an increase in thereduction of Methyl red was observed (Table 5). For example at 100µg of enzyme 4.8 µM of Methyl red was reduced (lowest). When compared to 500µg of enzyme, a reduction of 14.5µM (highest) of methyl red occurred. To determine if the enzyme was responsible for the decrease in dye concentration, a few drops of HCl (2µl, 36%) was added, followed by boiling for 30 minutes. When the reaction was carried out in the presence of denatured enzyme, there was no reduction of Methyl red demonstrating azoreductase activity of the enzyme (Table 5). In addition different dye and cofactor (NADH) concentrations were tested with the same enzyme concentrations and a similar conclusion resulted (Table 5). Interestingly, the different dye and NADH concentrations caused some change in the reduction of the concentration of Methyl red, suggesting some enzyme conditions were not optimal (Table 5). Based on these results, the optimal enzyme condition was 17 µM dye and 0.1mM NADH.

The second experimental approach focused on the enzyme activity (specific activity), using units (U) which is defined as one unit of enzyme= the amount of enzyme required to degrade 1 µM azo dye per minute. Using data from Table 5, the specific

activities (SA) for all conditions were calculated in order to analyze enzyme rate (See Table 6). The SA is represented as U/mg of enzyme. The SA results for 100 μg enzyme, 17 μM dye and 0.1 mM NADH provided the best condition for maximum enzyme rate, which was approximately 27 units per mg enzyme (Table 6). Again, when the protein was denatured, no SA was detectable.

To further demonstrate azoreductase activity, the data was analyzed using a time course measurement over a 2 minute period. The wavelength used to measure the absorption of the dye was 430nm. During the 2 minute time course, readings were acquired every second. In figure 16, when the reduction of the dye was carried out in the presence of different concentrations of the enzyme, the degree of dye reduction was the greatest with the 500 μg enzyme. As the enzyme concentration was decreased the degree of dye reduction lessoned. Because the rate of the enzyme reduction was high, the 500 μg enzyme dye concentration at time 0 (12.253 μM) was far from the concentration of the control at time 0 (19 μM). But, as the enzyme concentration was reduced, the difference between the 100 μg and control at time 0 was less. Figure 17 shows the enzyme reaction profile for 100 μg enzyme compared to the control. The time course data was able to demonstrate enzyme activity.

Effect of pH and Temperature on the activity of the enzyme

Changes in the pH resulted in identifying the optimal pH for the activity of the enzyme (Fig. 18). The optimal pH was between 7.0 and 8.0, with an average specific activity of 17.4 U/mg. Low specific activity was recorded for pH 4.0 (7.8 U/mg) an pH 9.0 (0.9 U/mg). The optimum temperature at which the enzyme was active was found to

be 38^0C (Fig. 18). The activity of the enzyme increased from 14.75 U/mg at 12^0C to approximately 333 U/mg at 38^0C after which there was a drop in the activity to 310 U/mg at 53^0C. Table 7 summarizes the pH and temperature data.

Reduction of Methyl Red under optimal conditions

Based on the pH and temperature experiments, a time point measurement of the complete reduction of methyl red was taken. Using 13 μg of enzyme, 0.1mM NADH and 17 μM of methyl red incubated at 38^0C, it took approximately 10 minutes to reduce most of the dye (Fig. 19). The specific activity (U/mg) calculated using the first two minutes was 279.85 U/mg. However the units calculated for 10 minutes was 108.2 U/mg. The varied specific activity and incomplete reduction of the dye suggests other factors besides the methyl red concentration is limiting or inhibitory.

TABLE 5

RESIDUAL DYE REDUCTION

Enzyme conc. (μg)	Methyl red (17μM)		Methyl red (31 μM)	
	NADH (0.1mM)	NADH (0.5mM)	NADH (0.1mM)	NADH (0.5mM)
100 μg	12.283±0.056	15.531±0.008	30.940±0.007	29.316±0.022
250 μg	5.966±0.053	14.532±0.020	24.909±0.043	28.018±0.011
500 μg	2.583±0.040	2.969±0.047	13.767±0.079	5.739±0.039

Table 5: represents the mean residual dye reduction of different concentrations of the azo dye Methyl red (17 μM and 31 μM) with three concentrations of the enzyme and different concentrations of NADH (0.1mM and 0.5mM). The experiments were carried out in triplicates. The values represent the final concentration (μM) of Methyl red after 2 minutes.

TABLE 6

ENZYME SPECIFIC ACTIVITY (U/mg)

Enzyme conc. (μg)	Methyl red (17μM)		Methyl red (31 μM)	
	NADH (0.1mM)	NADH (0.5mM)	NADH (0.1mM)	NADH (0.5mM)
Control	-0.050	ND	ND	0.103
100 μg	27.005	8.94	1.634	9.575
250 μg	23.516	7.14	10.551	6.541
500 μg	20.67	10.965	13.687	21.29

Table 6: This table represents the specific activity of the enzyme and shows that the highest concentration of dye and NADH can reduce the activity of the enzyme (experiments were done in triplicates).

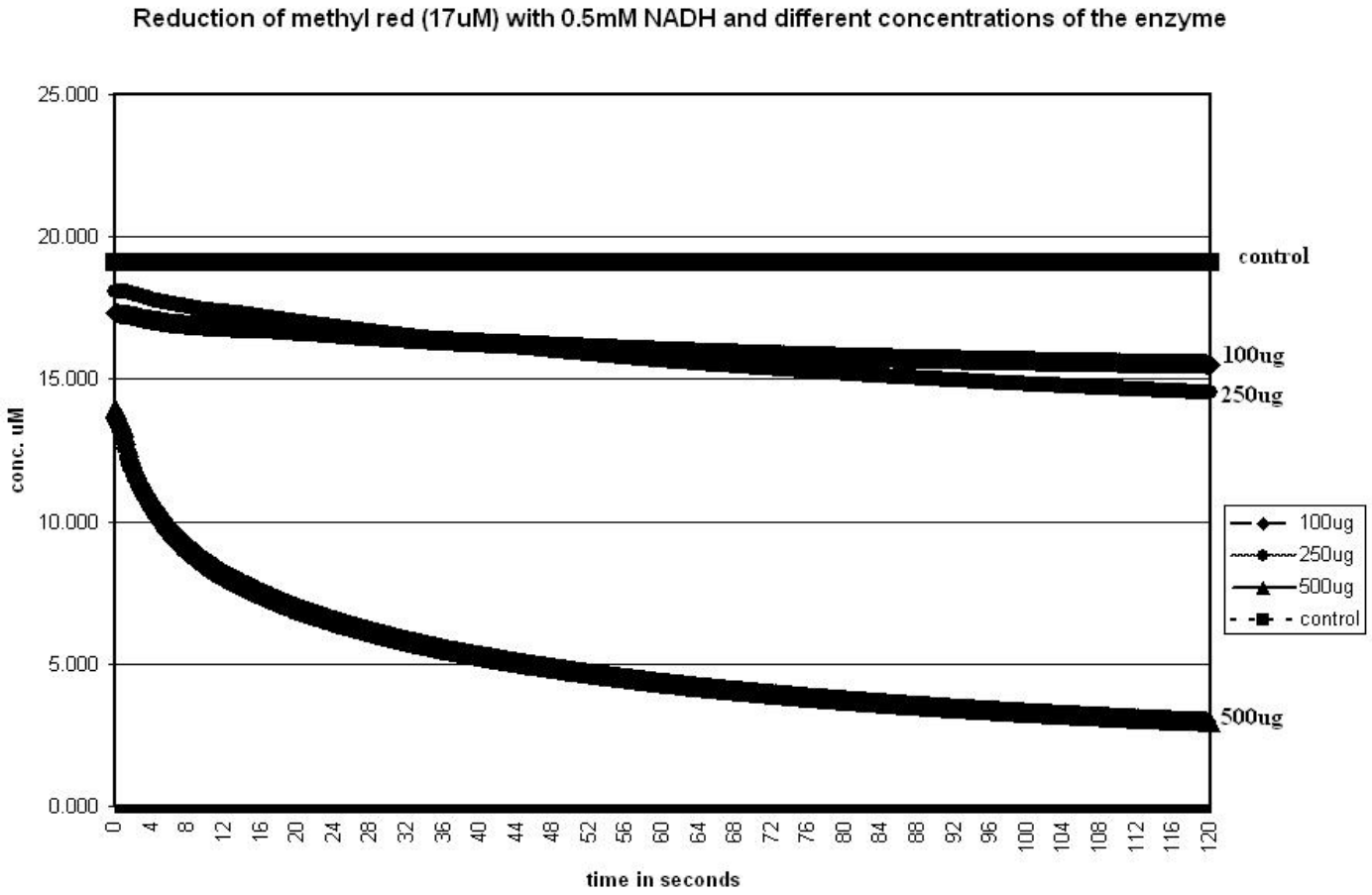


Figure 16: Reduction of Methyl red in the presence of different concentrations of the enzyme. Experiments were done in triplicates and the graph represents the mean and standard deviation values.

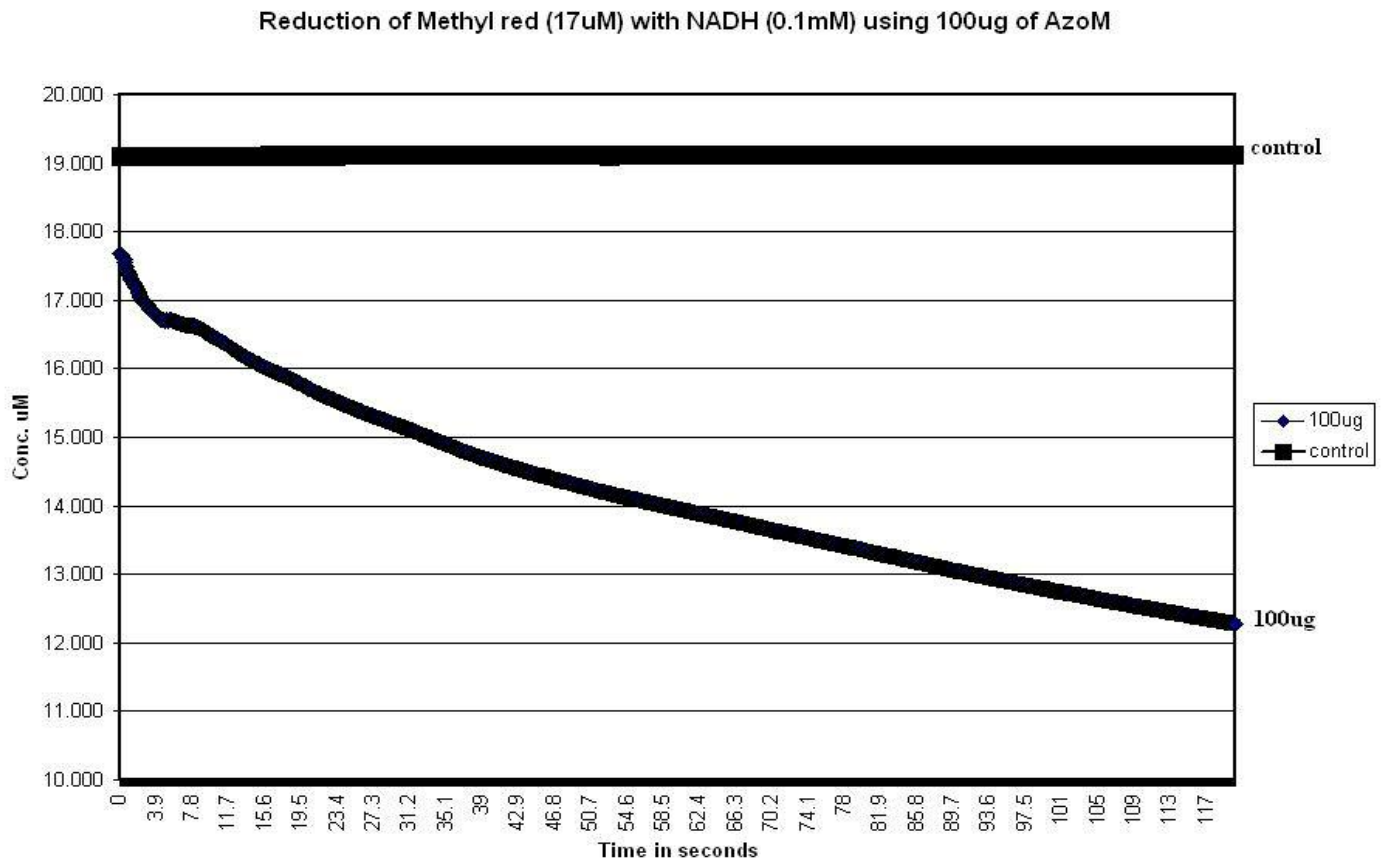
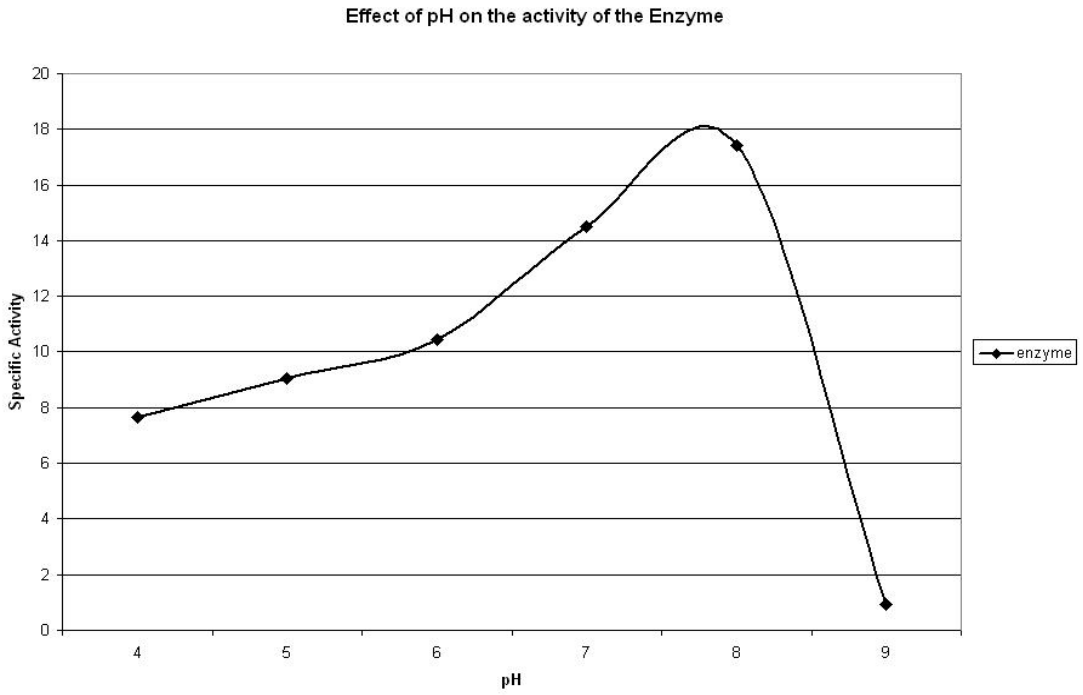


Figure 17: Reduction of Methyl red in the presence of 100ug of the enzyme possessed the best enzyme activity. Experiments were done in triplicates and the graph represents the mean and standard deviation values.



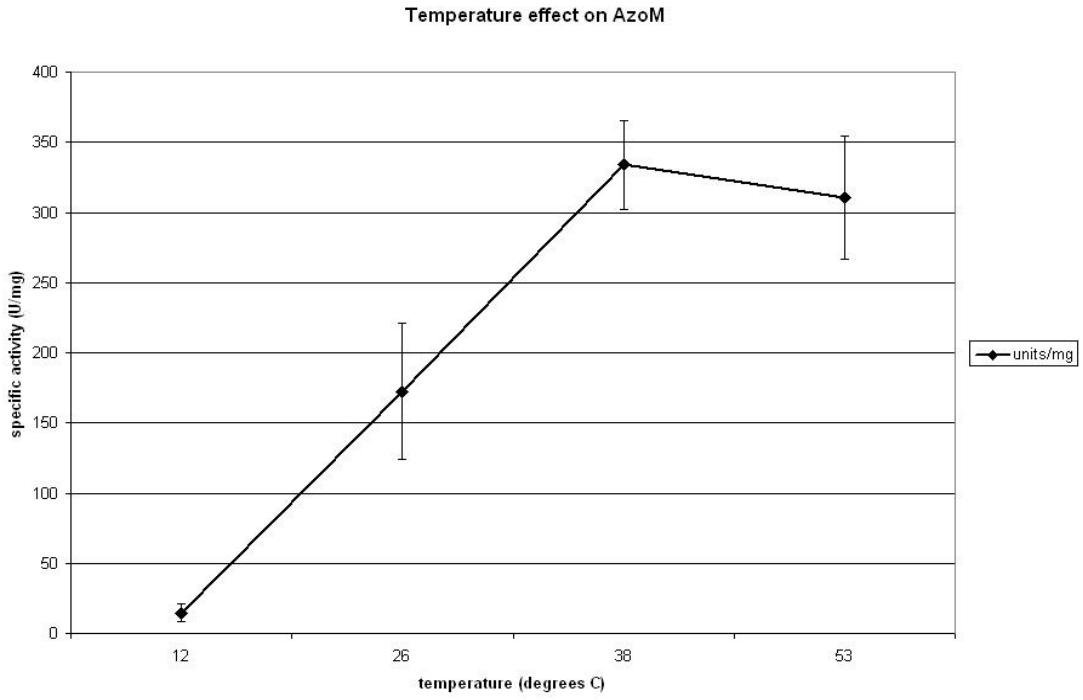


Figure 18: Effect of temperature and pH on the activity of the enzyme.

TABLE 7

EFFECT OF TEMPERATURE AND pH ON ENZYME ACTIVITY (U/mg)

Temperature	Specific Acitivity	pH	Specific Activity
12°C	14.75	4	7.634
26°C	172.51	5	9.033
38°C	333.84	6	10.462
53°C	310.41	7	14.518
		8	17.417
		9	0.942

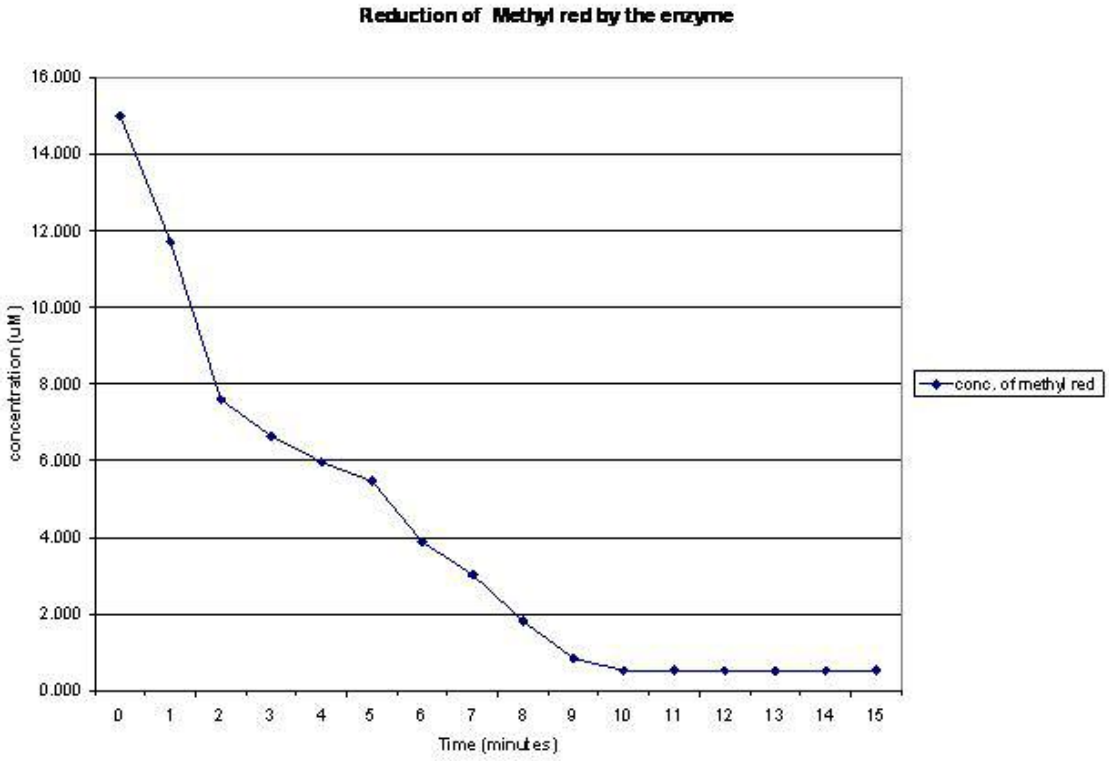


Figure 19: Time course reduction of Methyl red using optimal conditions for the enzyme. The optimal conditions were 0.1mM NADH, pH 8, 38^{o}C (Experiments were done in triplicates).

FINAL DISCUSSION

Azo dyes are used extensively in many industries. These azo dyes have been shown to be reductively cleaved by a wide range of microorganisms. Bacteria, both aerobic and anaerobic from different environments possess the ability to reduce azo dyes into genotoxic compounds. The human gastrointestinal tract is an important environment that harbors a complex mixture of bacteria. Evidence has shown that intestinal bacteria are linked to cancer formation based on azoreductase activity. Azo dyes are reductively cleaved to toxic aromatic amines, which are responsible for inducing certain cancers such as urinary bladder cancer.

A predominant bacterium in the gastrointestinal tract of humans is *Enterococci faecium* which is a Gram positive facultative anaerobe. Previous studies show that *E. faecium* possess the ability to reduce high molecular weight polymeric azo and nitro dyes such as Poly S, Poly T, Sunset yellow and Tartrazine (Brown 1981).

We established that *E. faecium* possessed the ability to reduce mono and diazo sulfonated dyes Direct Blue 15, Tartrazine and methyl red with no inhibitory effect on the growth of the bacteria. This indicates that the enzyme azoreductase is functionally expressed in *E. faecium*. When the supernatants were tested for activity, no activity was observed which showed that the enzyme is not extracellular but an intracellular protein.

Low amino acid sequence identities between azoreductases identified from different bacteria presented a challenge of how to identify new azoreductases from intestinal bacteria. We identified a new azoreductase gene from *E. faecium* based on amino acid sequence identity and the acpD domain commonality. The amino acid

sequence of the azoreductase (AzoA) that was isolated from *E. faecalis* was used to carry out a homology search which resulted in an acpD protein which showed 67% amino acid sequence identity with AzoA and 42% identity with the azoreductase (AzoR) from *E. coli*. Initially, the *acpD* gene of *E. coli* was said to code for the acyl carrier protein phosphodiesterase. The gene was isolated and characterized and encoded for the protein AzoR. The AzoA that was isolated from *E. faecalis* was also a putative *acpD* gene with azoreductase activity. The acpD commonality that exists was the basis for the identification, expression and purification of the new azoreductase (AzoM) from *E. faecium*. This approach can serve as a method for identifying other azoreductases from yet unidentified azoreductases from intestinal bacteria.

In conclusion, we have shown that the gram positive, facultative anaerobic intestinal bacteria *E. faecium* contains a gene which codes for an aerobic azoreductase. The enzyme (AzoM) was capable of reducing the water soluble mono azo dye methyl red in the presence of the cofactor NADH. There was no reduction observed in the presence of NADPH which shows that the enzyme is only specific for NADH only. Since a flavin moiety was bound to the enzyme there was no requirement for the exogenous addition of Flavins. The reduction of the dye by the enzyme was not linear. This may include several factors. These factors may include the NADH concentration and the build up of products which may affect enzyme activity. The optimal conditions for enzyme activity were found to be 38^{o}C and a pH of 8.

The strategy of using the acpD sequence identity as a means of identifying new azoreductases may be a potential and useful method of identifying additional azoreductase genes from intestinal bacteria. Identifying more azoreductase genes from

different intestinal bacteria will contribute toward our understanding of the complex interaction between bacteria and host.

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VITA

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Master of Science

Thesis: IDENTIFICATION AND ISOLATION OF AN AZOREDUCTASE FROM *Enterococcus faecium*

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Pages in Study: 78 Candidate for the Degree of Master of Science
Major Field: Microbiology and Molecular Genetics

Scope and Method of Study: Azo dyes are commonly used in the paper, textile, plastic, pharmaceutical, food, cosmetic, enamels, and drug industries. When these dyes enter the human body either by environmental contamination or from ingestion of food and drugs they are reduced to colorless end products by the intestinal microflora or the liver into aromatic amines which are potential carcinogens. The enzyme responsible for this reaction is azoreductase (Levine, 1991; Chung et al., 1992; Platzek et al., 1999). The human intestinal flora play an important role in the metabolism of xenobiotics and azo dyes with azo reduction being the most important reaction related to the toxicity and mutagenicity of these compounds. *Enterococcus faecium* which is part of the intestinal flora can reduce the azo dyes to colorless products. The *acpD* gene was identified, cloned, expressed and the recombinant protein was extracted and tested for azoreductase activity.

Findings and Conclusion: *Enterococcus faecium* showed to reduce the azo dyes Direct blue 15, Methyl red and Tartrazine, both visually and spectrophotometrically. When the *acpD* gene was isolated, cloned and expressed it yielded a protein of approximately 23kDa. Spectrophotometric analysis of the protein showed that Flavins are associated with the protein. Time course experiments revealed that the enzyme could reduce the monoazo dye Methyl red and the specific activity for the enzyme was highest with the use of 17μM dye and 0.1mM NADH. The optimum temperature and pH for the activity of the enzyme was found to be 38^{o}C and a pH of 8.

ADVISER'S APPROVAL: Dr. Gilbert H. John