1. INTRODUCTION

Rapid growth in industrialization, urbanization and man’s urge for color, the dyestuff usage is being increased day by day. Even though the use of dyes makes the world more beautiful, it represents a serious pollution problem worldwide. Dyes have been widely used in textile, dyeing, cosmetics, paper, leather, color photography, pharmaceutical, food and other industries because of their ease and cost effectiveness in synthesis, firmness and variety in color (Mohan et al., 2002; Sathiya Moorthi et al., 2007). Dyes are compounds that absorb light with wavelengths in the visible range, i.e., 400 to 700 nm (Van der Zee, 2003). They are composed of a group of atoms called chromophores which are responsible for the dye color and an electron withdrawing or donating substituent which are referred as auxochromes that cause or intensify the color of the chromophores (Christie, 2001). The most important chromophores are azo (-N=N-), carbonyl (-C=O), methane (-CH═), nitro (-NO₂) and quinoid groups. The auxochromes may belong to the classes of reactive, acid, direct, basic, mordant, disperse, pigment, vat, anionic, sulphur, solvent and disperse dyes (Welham, 2000).

Azo dyes comprise the largest chemical class of synthetic dyes which are more versatile. They play a significant role in almost every type of application and are widely used as colorants (Wong and Yuen, 1996). More than 60-70% of 10,000 dyes predominately used in the textile industry are azo dyes (Carliell et al., 1995). In case of acid dyes, azo group is the most important chromophore. It has been estimated that almost one million metric tones of dyes are produced annually in the world, of these azo dyes represent about 70% by weight (Zollinger, 1987, Dos Santos et al., 2003). These dyes are characterized by the
presence of reactive groups that form covalent bonds with OH-, NH- or SH-groups in fibres (cotton, wool, silk, nylon) (Christie, 2001). Various attractive forces are involved in binding of dyes to fibres, and often more than one type of chemical bonding can operate with the same dye-fibre combination. The relative strength between the dye and fibre is due to van der waals, hydrogen, ionic or covalent bonds (Ingamells, 1993; Guaratini and Zanoni, 2000).

Anthraquinone dyes constitute the second most important class of textile dyes. They have a wide range of colors in almost the whole visible spectrum, especially violet, blue and green colors are commonly used (Christie, 2001; Fontenot et al., 2003).

More than commercially 100,000 dyes are available with over $7 \times 10^5$ of tons of dyestuff produced annually (Robinson et al., 2001; Wang et al., 2002). In addition to that, solitary dyeing operation can utilize numerous dyes from diverse chemical classes resulting in a complex wastewater (Correia et al., 1994). In 1978, it was predicted that 2%, (or 9,000 tones) of the 4,50,000 tones of dye manufactured worldwide is discharged as effluent from manufacturing operations (Brown, 1987).

Most of the dyes are visible in water and the concentrations are as low as 1 mg l$^{-1}$. The release of colored effluents into the environment is undesirable; not only because of their color, since the dyes from wastewater and their breakdown products are toxic and/or mutagenic to life (Weisburger, 2002). Textile-processing wastewaters contain dye concentration in the range of 10-200 mg l$^{-1}$ and are usually regarded as highly colored. Though dyes are designed to be chemically and photolytically stable, they are highly persistent in natural
environments. The ecotoxic nature of the dye affects the ecosystem through the food chain. Moreover, dyes also serve as one of the major sources of heavy metals (Wagner, 1993) in water and soil (Zehra et al., 2009) and their persistent nature causes misbalance in the ecosystem. The toxic nature of textile dyes causes environmental pollution in developing nations, since the effluents from textiles are often untreated and discharged into river and open fields (Bakshi et al., 1999).

Textile wastewaters are characterized by extreme fluctuations of chemical oxygen demand, biological oxygen demand, pH, color, salinity, temperature etc., (Gupta et al., 2005). The textile wastewater composition usually varies everyday, due to the usage of different colors and fixing processes employed in the textile processing units (Sharma et al., 2007). Dyes discharged into the environment from the manufactures and consumers (i.e. textile, leather and food industries) are generally in the form of dispersion or a true solution (Seshadri et al., 1994; Pearce et al., 2003). Discharge of colored effluents even if is less toxic, are often objected by the public on the postulation that color is an indicator of pollution (Bell et al., 2000). Majority of the dyes are recalcitrant and they can confer color onto the designated materials (Plumb et al., 2001). They resist fading upon exposure to sweat, soap, water, light or oxidizing agents (Banat et al., 1996; Robinson et al., 2001). As a result, color in the wastewater has been considered as pollutant that has to be treated before release into aquatic bodies (Anjaneyulu et al., 2005; Brown, 1987; Parshetti et al., 2006).

Recently, severe regulation coupled with improved enforcement regarding the release of colored wastewater has been recognized in several countries. Government legislation is becoming increasingly stringent,
particularly in developed and developing countries for treatment of dyeing industrial effluents (Robinson et al., 2001). Enforcement of this law will persist to ensure that textile and other dye utilizing industries treat their dye-containing effluent to the permissible standards. In India, color limits in industrial waters are being made stringent in the last few years (Raghavacharya, 1997).

Physical, chemical, biological or combinations of these methods are established for color removal from wastewater (Cooper, 1995; Tunay et al., 1996; Vandevivere et al., 1998; Shaw et al., 2002). Physicochemical methods employed are membrane filtration, coagulation/flocculation, precipitation, flotation, adsorption, ion exchange, ion pair extraction, ultrasonic mineralization, electrolysis and advanced oxidation. Chemical methods include chlorination, bleaching, ozonation, fenton oxidation and photocatalytic oxidation. Biological techniques usually comprise microbial biosorption and biodegradation under aerobic, anaerobic, anoxic or combined anaerobic/aerobic treatment processes. Individual method for removal of dye depends upon several factors such as dye type, composition of wastewater, dose and cost of required chemicals, operation cost, fate of chemicals in the environment and handling costs of generated waste products (Van der Zee et al., 2001). Each technique has its own limitations and hence the usage of one individual process is not sufficient for dye removal (Raghavacharya, 1997).

Application of physical/chemical methods generates significant amount of sludge and also easily causes secondary pollution due to excess chemical usage. It is also expensive and has limited applicability (Vandevivere et al., 1998). Hence it is necessary to develop a biological method (aerobic and anaerobic) for treatment of a wide range of dyes in wastewater. Aerobic systems
generally require oxygen to perform the degradation process, but anaerobic process takes place in the absence of air and under static conditions (Stolz, 2001). In aerobic condition, the enzymes mono and dioxygenase catalyze the incorporation of oxygen into aromatic ring of organic compounds prior to ring fission. In many monooxygenases, the reduction of NADH or NAD(P)H is carried out through flavin by direct coupling with O₂ (Madigan et al., 2003).

Microorganisms capable of decolorizing azo dyes include gram-positive, gram-negative bacteria (Sani and Banerjee, 1999; Moosvi et al., 2005) and fungi (Verma and Madamwar, 2005). Even though azo dyes are aromatic compounds, their substituents contain mainly nitro and sulfonic groups, and are quite recalcitrant to aerobic bacterial degradation (Claus et al., 2002). The non-specific action of anaerobic bacteria facilitates the removal of wide range of textile dyes. Mostly the decoloration of reactive azo dye under anaerobic conditions is a co-metabolic reaction (Stolz, 2001).

Anaerobic reduction of azo dyes by bacteria is considered to be best (Stolz, 2001). It posses the following advantages: a) reactions takes place at neutral pH and are considered non-specific when low molecular weight redox mediators are available b) under static conditions, the depletion of oxygen is easily accomplished which allows obligate and facultative anaerobic bacteria to reduce azo dye and c) effluents provide additional carbon sources that generally increase the reduction rates. The major disadvantage of anaerobic azo dye reduction is that the aromatic amines formed from reductive cleavage cannot be further mineralized (Rafii et al., 1990; Van der Zee, 2003). Increased accumulation of aromatic amine may lead to carcinogenicity (e.g. naphthylamine or benzene derivatives) (Chung and Cerniglia, 1992).
Dyes can be removed by fungi through biosorption (Fu and Viraraghavan, 2001), biodegradation (Conneely et al., 1999) and enzymatic mineralization (Lip, MnP, MiP and Lac) (Wong and Yu, 1999; Wesenberg et al., 2003). White-rot fungus consists of diverse ecophysiological group such as basidiomycetous fungi responsible for aerobic lignin depolymerization and mineralization which play a central role in the carbon cycle (McMullan et al., 2001). This property is predominantly due to their relative non-specific activity of their lignolytic enzymes, such as lignin peroxidase, manganese peroxidase and laccase (Forgacs et al., 2004; Campos et al., 2001; Maier et al., 2004).

Yeast is an economical and readily accessible source of biomass that is feasible for dye accumulation at lower pH values (Donmez, 2002). Yeasts can acclimatize and grow under diverse conditions of pH, temperature and nutrient availability as well as high pollutant concentrations. Yeast has been reported for rapid bioaccumulation of metal ions from solution, but little work has been carried out on the investigation of yeast to act as a bioaccumulator for textile dyeing effluents (Aksu 2003; Aksu and Donmez, 2000).

Few reports on the degradation of dyes by algae are available (Jiang and Bishop, 1994). Algae can play an important role in the removal of azo dyes and aromatic amines from the waste water. Degradation pathway involves reductive cleavage of the azo linkage followed by degradation (mineralization) of the formed aromatic amines (Banat et al., 1996).

Studies on biological degradation of dyes provoke to identify and isolate the enzymes responsible for the decolorization (Novotny et al., 2001). Azoreductase is a specialized azo dye reducing enzyme produced by microbes to
degrade dyes under aerobic conditions (Coughlin et al., 1999; Quezada et al., 2000). They are soluble cytoplasmic enzymes with low-substrate specificity (Robinson et al., 2001). Azoreductase isolated from various bacteria have been found to be an inducible (Nakanishi et al., 2001) flavoprotein (Ghosh et al., 1992) and utilize both NADH and NADPH as electron donors (Chen et al., 2005). This enzyme is a monomer with high substrate specificity to dye structure (Dykes et al., 1994; Ghosh et al., 1992) and is produced aerobically. Apart from these specific azoreductases, non-specific enzymes capable of catalyzing azo dye reduction have been isolated from aerobically grown cultures of *Shigella dysenteriae* (Ghosh et al., 1992), *Escherichia coli* (Ghosh et al., 1993) and *Bacillus* sp. (Suzuki et al., 2001).

The potential advantages of enzymatic treatment as compared to conventional treatments include: application on recalcitrant materials, operation at high and low contaminant concentrations over a wide pH range, temperature, salinity range, acclimatization to biomass and easy control process. The application process is simple and can be rapidly modified according to the character of the dye to be removed (Forgacs et al., 2004).

Biological treatment is the most popular and efficient method of industrial effluent treatment. However, the use of whole cells rather than isolated enzymes is advantageous, because purification processes are extremely higher and the cell offers protection from the harsh process environment to the enzymes. Moreover, the treatment of textile effluent has been studied extensively and much research has been focused on fungal aerobic systems, which are quite difficult commercially for upscale due to the sensitivity of the culturing conditions (Stolz, 2001; Wesenberg et al., 2003). On the other hand, bacteria have shown to be
ideal since it can withstand extreme environmental conditions (Kalyani et al., 2009) and hence it is necessary to investigate the potential bacteria to decolorize the dyes and to produce the novel enzyme which can bioremediate textile effluent for an overall effective treatment of dye wastewaters.

The objectives of the present study is

✓ To enrich the microorganisms present in tannery effluent that decolorize the dye C.I. Acid Blue 193 (AB 193) & Acid Violet 90 (AV 90)
✓ To isolate and investigate the potential bacterial species to decolorize azo dye
✓ To identify the efficient organism by 16S rRNA analysis
✓ To optimize culture conditions (pH, temperature, time and initial dye concentration) for efficient removal of dyes
✓ To optimize carbon and nitrogen sources concentration on decolorization of dyes
✓ To enhance decolorization of dyes in the presence of different combinations of carbon and nitrogen sources
✓ To isolate, purify and characterize azoreductase enzyme from isolated bacterial species
✓ To identify the degraded products and metabolites using HPLC