5. DISCUSSION

5.1 SCREENING AND IDENTIFICATION OF DYE DECOLORIZING BACTERIA

C.I. Acid Blue 193 and C.I. Acid Violet 90 were selected as model sulphonated azo dyes for the optimization of decolorization process. Only few studies have been successful in isolating microorganisms capable of utilizing dye as their sole carbon source (Sarnaik and Kanekar, 1999). The obligate necessity of co-substrates for growth of dye-decolorizing bacteria is necessary, therefore isolation was attempted by employing peptone and yeast extract as co-substrates (Vijaya et al., 2003; Maier et al., 2004). Isolation and decolorization of the dye by microbes occurs if growth medium are supplemented with carbon and energy sources (Coughlin et al., 1997). In the decolorization medium, peptone (nitrogen source) or yeast extract (growth factor) is necessary to activate the coenzyme-producing metabolic pathways to induce the azoreductase activity for dye decolorization (Chang et al., 2001). Isolation of dye-decolorizing bacteria employing glucose and yeast extract has also been reported (Mathew and Madamwar, 2004; Moosvi et al., 2005; Junnarkar et al., 2006).

5.2 PHYLOGENETIC ANALYSIS OF 16S rRNA

The 16S rRNA sequence analysis is now employed as a framework for the modern classification of bacteria (Ki et al., 2009). There are previous reports that some strains of Bacillus sp. are able to degrade a number of toxic compounds. Reports are available on the decolorization by Bacillus sp. ADR isolated from soil for C.I. Reactive Orange 16 (Telke et al., 2009) and the bacterial strain Bacillus odysseyi SUK3 for Reactive Blue 59 (Patil et al., 2008). Mabrouk and Yusef (2008) studied decolorization of Fast Red by Bacillus
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subtilis HM and the degradation product was p-aminoazobenzene (Zissi et al., 1997). Other strains of Bacillus sp. which were able to decolorize or degrade dyes are Bacillus sp. AK1 (de Souza et al., 2001), Bacillus thermoleovorans US105 (Ben Messaoud et al., 2002), Bacillus fusiformis KMK5 (Kolekar et al., 2008), Bacillus sp. (Khalid et al., 2008), Bacillus cereus, Bacillus fusiformis and Bacillus sphaericus (Desai et al., 2008)

5.3 DECOLORIZATION EXPERIMENTS

5.3.1 Effect of incubation time on decolorization of AB 193 and AV 90 by Bacillus cereus KTSMD-03 at static and shaking conditions

Under static conditions, the decolorization of AB 193 and AV 90 by Bacillus cereus KTSMD-03 was more efficient than at shaking condition. Bacteria reduce azo dye under anaerobic condition through an electron transport process which depends on multi-components of electron transport chain system (Hong et al., 2007). Under anaerobic conditions, azo dyes act as electron acceptors and high decolorization rates were achieved (Song et al., 2003; Singh et al., 2007; Asad et al., 2007 and Handayani et al., 2007). The reason for decreased decolorization at shaking condition could be due to the competition in oxidation of reduced electron carriers with either oxygen or azo groups as the electron acceptor (Mabrouk and Yusef, 2008; Dawkar et al., 2010). Under shaking conditions, the aerobic respiration of the strain might dominate the utilization of NADH and inhibit azoreductase for obtaining electrons from NADH to decolorize azo dyes (Stolz, 2001; Chang et al., 2004). Microbial growth and bacterial decolorization are independent for decolorization of dyes as azoreductase is repressed under aerobic conditions (Chang et al., 2004). Decolorization of azo dyes cannot take place in extreme anaerobic conditions that is under oxygen-free nitrogen sparging. This clearly implies that bacteria
needs small amount of oxygen to maintain basic cellular activity for
decolorization (Chen, 2002). Similar results were observed in case of the
following bacterial strains: *Shewanella putrefaciens* AS96, *Comamonas* sp.
UVS, *Bacillus subtilis*, *Vibrio harveyi* TEMS1 and *Pseudomonas* sp. SUK1. All
these strains have shown promising results for dye degradation under static
conditions (Khalid *et al.*, 2008; Jadhav *et al.*, 2008; Gurulakshmi *et al.*, 2008;

5.3.2 Effect of pH on decolorization of AB 193 and AV 90 by *Bacillus
cereus* KTSMD-03 at static condition

Decolorization of AB 193 and AV 90 was optimum at pH 7. The dye
decolourization varies with pH. At the optimum pH, the surface of biomass gets
negatively charged, which enhances the binding of positively charged dye.
Binding occurs through electrostatic force of attraction and it results in a
considerable increase in color removal (Daneshvar *et al.*, 2007). Below the
optimum pH, H⁺ ions compete effectively with dye cations, causing a decrease
in color removal efficiency. At alkaline pH, the azo bonds will be deprotonated
to negatively charged compounds and it results in obstruction of azo dye
decolorization. In acidic pH, the azo bond will be protonated (-N=N- → [-NH-
N=]+ which leads to decreased dye decolorization due to change in chemical
structure (Hsueh and Chen, 2007). Similarly azo dye decolorization was
exhibited at pH 7 in case of *E. coli* and *P. luteola* (Chang and Lin, 2001). Dye
decolorization of Scarlet R, Direct Fast Scarlet 4BS and RB-5 was maximum at
pH 7 (He *et al.*, 2004; Dafale *et al.*, 2008). Most of the azo dye reducing species
of *Pseudomonas luteola*, *Bacillus* and *Enterobacter* sp. EC3 (Chang *et al.*, 2001;
Kalme *et al.*, 2007; Wang *et al.*, 2009) were able to reduce the dye at neutral pH.
Due to the difference in genetic determinants for dye decolorization and
bacterial physiology, the optimal pH varies with species and dyes (Chang and Lin, 2001).

5.3.3 Effect of temperature on decolorization of AB 193 and AV 90 by Bacillus cereus KTSMD-03 at static condition

Enhanced dye decolorization of AB 193 and AV 90 was observed at 35°C but it drastically decreased with increase in temperature (40°C). Reduced color removal beyond 35°C may be due to the loss of cell viability or thermal deactivation of decolorizing enzymes (Panswad and Luangdilok, 2000; Cetin and Donmez, 2006). Decreased decolorization was exhibited at 50°C under static condition since the bacterium poorly grows at this temperature. It implies that the bacterium is mesophilic and the possible reason is that the enzyme responsible for decolorization has its activity between 30-40°C. Results obtained are also correlated with earlier studies by Khalid et al. (2008) where the decolorization of Methyl Red and RBR X-3B by Vibrio sp. and Rhodopseudomonas palustris was maximum around 30-35°C (Adedayo et al., 2004; Liu et al., 2006). Reports also show that Klebsiella pneumoniae RS -13 and Acetobacter liquefaciens S-1 had no decolorization of methyl red at 45°C (Wong and Yuen, 1998).

Previous reports indicate that rapid decolorization of Remazol Black B, Direct Red 81, Acid Orange 10, Disperse Blue 79, Navy Blue HER and Acid Blue 113 were observed at 37°C (Meehan et al., 2000; Junnarkar et al., 2006; Kolekar et al., 2008; Gurulakshmi et al., 2008;). Low temperature and high pH appear to reduce microbial activity. This implies that the temperature in the microenvironment has a significant role on decolorization activity of the microbes.
5.3.4 Effect of carbon sources on decolorization of AB 193 and AV 90 by *Bacillus cereus* KTSMD-03

Dyes are usually deficient in carbon content and biodegradation without any carbon source is found to be very difficult (Padmavathy *et al.*, 2003). Decolorizations of azo dyes are dependent on carbohydrate metabolism. Enhanced decolorization of AB 193 and AV 90 was achieved at 2% and 1% of glucose. This phenomenon might be due to the role of glucose as a co-substrate and also as sources of electron donors, which are needed for cleavage of azo bond (Mendez-Paz *et al.*, 2005; Khan and Husain, 2007; Dafale *et al.*, 2008). The metabolism of glucose results in production of reduced metabolites (NADH and FADH) which leads to maximum color removal. With increase in glucose concentration, the dye decolorization also increases. Low glucose concentration cause insufficient growth of microbes thereby leading to decrease in decolorization efficiency. Earlier reports on decolorization studies indicate the necessity of glucose to enhance the decolorization process (Dos Santos *et al.*, 2005; Moosvi *et al.*, 2007; Hong *et al.*, 2007; Jadhav *et al.*, 2007; Ozdemir *et al.*, 2008). Bacteria utilize most of the energy generated from glucose for the reductive cleavage of azo dyes.

Sucrose is also used as co-substrate for decolorization of dye. Sucrose at 0.5% concentration increases the color removal for both AB 193 and AV 90. With further increase in sucrose concentration decreased color removal was observed. Mohana *et al.* (2008) reported that addition of sucrose as co-substrate enhanced polyazodye Direct Black 22 decolorization. Kapdan *et al.* (2000) demonstrated better decolorization (34%) in the presence of sucrose than glucose at the end of 63 h of incubation period. Jin *et al.* (2007) studied complete
decolorization of effluent when 1% sucrose was supplemented. Ozdemir et al. (2008) reported the decolorization of AB 210 to about 91.7% when sucrose was used as co-substrate. The effect of carbohydrate concentration on dye removal indicates that the sugar source might have synergistic as well as antagonistic effect on dye removal by microbes (Aksu and Donmez, 2000).

Utilization of starch as a co-substrate by microbes could be encouraging from commercial point of view (Moosvi et al., 2005). Starch is cheaper than glucose, hence it can be used as a good alternative to glucose for degradation of azo dyes (Brown and Laboureur, 1983). Starch at 1% concentration did not appreciate better decolorization of AB 193 whereas AV 90 was decolorized to 93.96%. Likewise, Steffan et al. (2005) noticed ethyl orange degradation with 1% starch as an additional energy source to activate co-metabolism by cleaving starch molecule and utilizing the formed products as a source of carbon to enhance color removal. Previous reports showed improved decolorization of dyes when starch was used as co-substrate (Sumathi and Manju, 2000; Oranusi and Ogugbue, 2005a; Junnarkar et al., 2006; Ozdemir et al., 2008). Azo dyes are readily cleaved by reduction of azo bond under anoxic conditions which requires electron donors like glucose, starch and volatile fatty acids (Mohanty et al., 2006).

5.3.5 Effect of nitrogen sources on decolorization of AB 193 and AV 90 by Bacillus cereus KTSMD-03

Peptone is considered to be one of the best co-substrate for bacterial growth and decolorization (Liu et al., 2006). Peptone concentrations of 1% and 2% showed maximum decolorization of AB193 (61.85%) and AV 90 (64.82). The presence of peptone regenerates NADH and this acts as an electron donor.
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for the azo dye reduction. In addition, peptone significantly enhances the strain’s activity of azo dye decomposition. Chen et al. (1999) reported that color removal rate was increased with rise in peptone concentration (40 g l⁻¹), with further increase in concentration, decreased color reduction was seen and substitution of inorganic nitrogen source (NH₄Cl) for peptone showed poor bacterial growth and reduced color removal efficiency. Similar studies on decolorization of dye with peptone have also been reported (Ramya et al., 2008; Saratale et al., 2009b). Bhatti et al. (2008) reported Cibacron Red decolourization in the presence of various nitrogen sources including peptone.

Yeast extract in the medium activates the necessary coenzyme for the metabolic pathway of azoreductase and serve as key components for decolorization (Chang et al., 2001). Supplementing yeast extract (0.5% and 1%) in the medium exhibited moderate decolorization of AB 193 and AV 90. Yeast extract provides a complex organic substrate that can be used by bacteria as an electron donor for decolorization (Xu et al., 2006; Mohana et al., 2008; Saratale et al., 2009b). The growth of bacterium is related to the concentration of the yeast extract and the major role of yeast extract in the medium may be as an N-balancing compound (Hu, 1998). Yeast extract is generally selected for improvement of decolorization because it is cheaper than tryptone or peptone (Chang et al., 2000; Chen et al., 2003). Sponza and Isik (2002) reported that yeast extract serves as a nitrogen source for the growth of azo dye degrading bacteria and also for decolorization. Increased dye decolorization was reported when yeast extract (0.5%) was present in the medium (Nigam et al., 1996). Bhatt et al. (2005) demonstrated decreased decolorization of dye at high concentration of yeast extract. Previous reports showed that decolorization of azo dyes was high with yeast extract as co-substrate under micro-aerophilic
condition (Chen et al., 2003; Sandhya et al., 2005; Joshi et al., 2008). The bacterial culture grew well with yeast extract and produces sufficient biomass which then reduces the dyes to the corresponding amines resulting in decolorization (Nigam et al., 1996).

Most pure cultures of bacteria like *Pseudomonas luteola* (Hu, 1998; Chang et al., 2001), *Klebsiella pneumoniae* (Wong and Yuen, 1996) *Aeromonas hydrophila* (Chen et al., 2003) and different mixed cultures like *Paenibacillus* sp. and *Micrococcus* sp. (Moosvi et al., 2007), *Bacillus* sp. and *Clostridium* sp. (Knapp and Newby, 1995) have exhibited effective dye decolorization in presence of yeast extract. In contrast, Bhatti et al. (2008) observed decreased dye decolorization in the presence of different nitrogen sources including peptone and yeast extract and highest decolorization was observed with urea.

5.3.6 Effect of different combination of co-substrates on decolorization of AB 193 and AV 90 by *Bacillus cereus* KTSMD-03

Decolorization of dyes was enhanced when carbon and nitrogen sources are available in the growth medium (Sheth and Dave, 2009). The decolorization rate of azo dyes is increased by using various combinations of co-substrates which generate redox mediators that catalyze the reaction rate by shuttling electrons from the biological oxidation of primary electron donors or from bulk donors to the azo dyes as electron acceptors (Rau et al., 2002; Khan and Husain, 2007). Glucose, sucrose and peptone in the medium completely decolorized AB 193 whereas AV 90 was decolorized to higher extent with 1% starch when compared with other combinations. The combinations of glucose and sucrose showed better decolorization of dye and decreased decolorization was observed with sucrose and starch (Mohana et al, 2008). Kapdan et al. (2000) demonstrated
complete decolorization of dye with lactose and sucrose in the medium. Similarly, when medium was supplemented with carbon sources namely glucose and pyruvate; starch and casein; ethanol and formate it produces more electrons to reduce the dye and hence it is encouraging from commercial point of view (Junnarkar et al., 2006; Franciscon et al., 2009; Costa et al., 2010). *Bacillus subtilis* decolorized Acid Blue 113 to a maximum level when starch and peptone were supplemented in the medium (Gurulakshmi et al., 2008). Joshi et al. (2008) observed enhanced decolorization in combination of glucose (0.6 g l\(^{-1}\)) and yeast extract (0.06 g l\(^{-1}\)). Combination of yeast extract and glucose for color removal was also used by other researchers (Khehra et al., 2005; Moosvi et al., 2005; Mohana et al., 2008). Carbon sources with yeast extract viz., lactose and yeast extract; glycerol and yeast extract were found to be the most effective carbon-nitrogen source for dye decolorization (Kapdan et al., 2000; Chang et al., 2000). In contrast, glucose and yeast extract strongly inhibited decolorization of the dyes (Khalid et al., 2008). Increased dye decolorization was noticed when peptone (20 g l\(^{-1}\)) or tryptone and yeast extract (2 g l\(^{-1}\)) were supplemented as nitrogen sources were reported (Chang et al., 2000; Ozdemir et al., 2008; Sheth and Dave (2009). Saratale et al., (2009a) demonstrated lowered decolorization of Navy Blue HER with urea and NH\(_4\)Cl as a nitrogen source. The optimization of medium components is important to predict nutrient supplementation for effective dye removal (Mohana et al., 2008).
5.3.7 Effect of initial dye concentration on decolorization of AB 193 and AV 90 by *Bacillus cereus* KTSMD-03

The dye at high concentration has inhibitory effect on azo bond reduction and causes reduced decolorization. This is due to the toxicity of dye to bacterial cells by inhibiting the metabolic activity or saturation of cells with dye products or electrons fail to reach the azo bond chromophores or inactivation of transport system or the blockage of active sites in azoreductase enzymes by the dye molecules (Sponza and Isik, 2002; Pearce *et al.*, 2006; Vijaykumar *et al.*, 2007). It was noticed that at low concentrations (50 mg l\(^{-1}\)), the rate of decolorization is efficient and reached upto 91% and 94% for AB 193 and AV 90, respectively. With further increase in dye concentration decreased color removal was noticed. The decolorization of dye was inversely related to the concentration of the dye which is due to the inhibitory effects of high dye stuff in the solution (Khehra *et al.*, 2005; Kalme *et al.*, 2007; Vijaykumar *et al.* 2007; Ramya *et al.*, 2008; Ozdemir *et al.*, 2008; Telke *et al.*, 2009). Reduced decolorization efficiency might be due to the toxicity of dyes (Kapdan *et al.*, 2000; Verma and Madamwar, 2002).

Generally azo dyes contain one or more sulphonic–acid groups on aromatic rings, which may act as detergents to inhibit the growth of microorganisms (Chen *et al.*, 2003). High concentration limits bacterial growth by adsorption to the cell walls of bacteria (Ali *et al.*, 2009). AV 90 was decolorized better than AB 193, since decolorization of individual dyes varies due to their structural differences (Nigam *et al.*, 1996). The characteristics of substituents and their relative positions of azo bond play a vital role for the performance of microbes in azo dye decolorization (Liu *et al.*, 2006; Sandhya *et al.*, 2008). Handayani *et al.* (2007) reported that the *E. faecalis* decolorized Acid
Red 27 to a higher extent than Reactive Red 2 which is attributed by the structure of dyes. Different and complicated structure of dye molecules is one of the factors influencing microbial decolorization (Van der Zee et al., 2001; Miao, 2005). The complex structure of azo dye makes it difficult to degrade, in contrast the simpler structure is easier to degrade. It was noticed that no clear relationship can be observed between the position of substituents in the aromatic rings of the dye and efficiency of dye decolorization by microorganisms (Hu, 2001; Chen et al., 2003). The effluent from textile industries has variations in dye concentration and thus the ability of the organism to degrade the dye at wide range of concentration is an important factor for effective biodegradation (Gopinath et al., 2009).

5.4 PURIFICATION OF AZOREDUCTASE FROM Bacillus cereus KTSMD-03

Azoreductase is an enzyme responsible for azo reduction by bacterial species. The azoreductase activities for both the dyes (AB 193 and AV 90) were different and this implies differences in specificity of the enzyme azoreductase to different dyes (Vijaykumar et al., 2007). Azoreductases isolated from several bacteria have been shown to be inducible flavoproteins which use both NADH and NADPH as electron donors (Moutaouakkil et al., 2003). Azoreductase is localized in the cytoplasmic fraction and it requires NADH as a co-factor for the decolorization activity. Studies on NADH dependent azoreductases in Pseudomonas luteola (Chang et al., 2001), Escherichia coli (Rau and Stolz, 2003), Pigmentiphaga kullae K24 (Blumel and Stolz, 2003), Enterobacter agglomerans (Moutaouakkil et al., 2003) and Bacillus sp. strain SF have been reported (Maier et al., 2004).
Azoreductase from *Bacillus cereus* decolorized AB 193 and AV 90 using NADH as electron donor. It is not necessary to remove oxygen or preincubate with NADH as in the case of previous report (Maier *et al.*, 2004) and this confirms that the azoreductase from the strain KTSMD-03 is oxygen insensitive. Azoreductase activity studied in different dyes as substrate have been reported (Chang *et al.*, 2000; Blumel and Stolz, 2003; Ooi *et al.*, 2007; Pricelius *et al.*, 2007 and Punj and John, 2008).

The azoreductase activity of various microorganisms in the presence of dye substrate differs and are given as follows, *Escherichia coli* - 0.014 (Nakanishi *et al.*, 2001), *X. azovorans* KF46F -0.027 (Blumel *et al.*, 2002), *P. kullae* K24 - 0.071 (Blumel and Stolz, 2003), *Staphylococcus aureus* - 0.14 (Chen *et al.*, 2005) and *Kerstersia* sp. - 0.069 (Vijaykumar *et al.*, 2007) U mg\(^{-1}\) of protein.

5.5 POLYACRYLAMIDE GEL ELECTROPHORESIS

Electrophoretic pattern of azoreductase from *Bacillus cereus* KTSMD-03 showed single band corresponding to a molecular weight of 28 kDa as determined from SDS gels and confirmed it to be of monomer. Similar results have been reported by Moutaouakil *et al.* (2003) where the molecular weight of the enzyme from *Enterobacter agglomerans* was found to be 28 kDa. Previous studies on microbial azoreductases also reported it to be monomers (Blumel *et al.*, 2002; Blumel and Stolz, 2003; Nachiyar and Rajkumar, 2005). Purified azoreductase enzyme on SDS-PAGE with a molecular weight of approximately 61.6 kDa, 21.5 kDa, 60 kDa, 58 kDa have also been reported (Maier *et al.*, 2004; Pricelius *et al.*, 2007; Bafana *et al.*, 2008a; Sandhya *et al.*, 2008). Azoreductase activity was noticed as clear zone on AB 193 and AV 90 dye on
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Stained gel in the presence of NADH. Zymogram pattern revealed a clear zone of azoreductase activity against the dye background (Nachiyar and Rajakumar, 2005; Bafana et al., 2008a). Mazumder et al. (1999) showed the location of the active enzyme which is indicated by a clear zone and reported that the azoreductase could use both NADH and NADPH as co-substrates but NADH was shown to be more efficient.

5.6. HPLC ANALYSIS OF AB 193 AND AV 90 METABOLITES

HPLC is an effective technique for the analysis of metabolites of degraded azo dyes due to its accuracy, high separation efficiency and simplicity procedure. HPLC analyses were performed to confirm the presence of degraded products. Some of the degraded products are separated and identified but there are few compounds which are unknown due to lack of standards for comparison. The azoreductase from the bacterial culture catalyzes the reductive cleavage of azo bond of AB 193 in the presence of NADH as electron donor and yields 1-amino-2-naphthol (Rt – 1.54 min) and sodium 4-amino-3-hydroxy-naphthalene-1-sulfonate (Rt – 5.69 min) as degraded products. Azoreductase cleaves AV 90 to sodium 4-amino-3-hydroxy-naphthalene-1-sulfonate (Rt – 5.49 min) and 4-amino-6-methyl-2-phenyl-2, 5 dihydro pyridazin-3-ol (Rt – 12.11 min) in the presence of NADH as electron donor. In our study, during degradation of AB 193, the presence of 1-amino-2-naphthol as one of the metabolite proved the involvement of a reductive process in the initial decolorization step. Similar to our report, 1-amino-2-naphthol has been identified as a degraded product on reduction of orange II (Zimmerman et al., 1982). Degradation of DR 28 to benzidine and 4-ABP by Bacillus velezensis was reported by Bafana et al. (2008a). The degradation of methyl red, resulted in appearance of two major peaks with retention times of 5.8 min and 7.1 min, respectively and was
identified as 2-aminobenzoic acid (ABA) and N, N’-dimethyl-p-phenylenediamine (DMPD) by HPLC analysis (Ooi et al., 2007; Chen et al., 2005).

Jadhav et al. (2007) noticed prominent peaks at retention times of 1.75 min and 2.60 min with other three peaks (2.94, 3.96 and 3.95) which are different from the control peak of methyl red (2.1 min). In our study the metabolites formed after decolorization of dyes AB 193 and AV 90 showed peak with different retention time than the parent dye which confirmed biodegradation.

5.7. CHARACTERIZATION OF PURIFIED AZOREDUCTASE

The maximum activity of purified azoreductase was observed in pH range of 6 to 8. The enzyme had a typical bell shape over a broad pH range. Optimum azoreductase activity was observed at pH 7 for AB193 and AV 90. Above neutral pH, decreased activity was seen which is due to the inactivation of azoreductase. Azoreductases isolated from various Pseudomonas sp. had optimal activity at pH 7.0 (Nachiyar and Rajakumar, 2005; Pricelius et al., 2007). Maier et al. (2004) reported an optimum pH of 7.0 for azoreductase in the decolorization of Mordant Black 9, Mordant Brown 96 and Reactive Black 5.

Studies on the effect of temperature on enzyme activity (thermal activation) revealed 35°C as optimum for AB 193 and AV 90. Nachiyar and Rajakumar (2005) showed azoreductase activity at 35°C. Studies on azoreductases in Escherichia coli, Shigella dysenteriae Type 1 and Bacillus cereus showed maximum activity in the temperature range of 40-45°C (Pricelius et al., 2007). Moutaouakkil et al. (2003) observed thermal inactivation of
azoreductase above 40°C and the activity was completely lost at 60°C. In previous report, Ooi et al. (2007) showed that the enzymes were stable upto 55°C for azoreductase B and 50°C for azoreductase C after incubation for 1 h at pH 7.4. Enzyme activity is maintained at temperatures below 45°C and more reduced activity was observed at 70°C. These findings conclude that the physicochemical stable properties of azoreductase can withstand at higher temperature which can be further cloned for decolorization studies.

5.8 KINETICS OF PURIFIED AZOREDUCTASE

Azoreductase utilized NADH as an electron donor and the rate of reaction increased with increase in concentration of NADH which indicates that NADH plays an important role in dye degradation. The results suggest that the reaction mechanism is in a sequential pattern rather than a ping pong type. Similar report was observed where the enzyme reaction was sequential rather than a ping-pong manner (Moutaouakkil et al., 2003; Nachiyar and Rajakumar, 2005). In contrast to our report, few studies confirmed that the reduction catalyzed by azoreductase follows ping-pong mechanism (Nakanishi et al., 2001; Blumel and Stolz, 2003; Yan et al., 2004; Bin et al., 2004; Deller et al., 2006; Vijaykumar et al., 2007). In this study, the azoreductase from Bacillus cereus KTSMD-03 showed lower $K_m$ value towards AV 90 than AB 193 and this is due to the structural differences of the azo dyes. Hu (1998) reported that azoreductase from P. luteola had low $K_m$ values towards dyes Reactive Red 22, Reactive Violet 2 and Reactive yellow which might be due to structural features of azo dyes. The $K_m$ values of AB 193 (0.022 mM) and AV 90 (0.019 mM) are similar to the previous reports which correlate with $K_m$ of E. faecalis - 0.024 mM, with methyl red substrate (Chen et al., 2004), E. coli (AcpD) - 0.018 mM (Blumel and Stolz, 2003) and Enterobacter agglomerans - 0.029 mM (Moutaouakkil et al., 2003).
The $K_m$ value of NADH in the present study is similar to the $K_m$ of *E. agglomerans* (Moutaouakkil et al., 2003) and it was higher with $K_m$ values of *E. faecalis* (Chen et al., 2004). Vijaykumar et al. (2007) reported that $K_m$ and $V_{max}$ values of Amaranth, Fast Red E and Ponceau S vary in their azoreductase activity due to difference in dye structures.