5. DISCUSSION

5.1. Total heterotrophic bacterial population

Azo dyes were selected from the list of reactive dyes and used for microbial decolorization. Reactive dyes were the only textile colorants designed to bond covalently with cellulosic fibers are extensively used in the textile industry ever since of their wide variety of color shades with high wet fastness profiles, ease of application of brilliant colors and minimal energy consumption (Aspland, 1997). Reactive dyes were developed to resist fading on exposure to sweat, soap, water, light or oxidizing agents making them stable and resistant to degradation (Seshadri et al., 1994).

Total heterotrophic population from reactive dye contaminated textile wastes showed that $62 \times 10^6$ CFU/g in soil which was higher. The result of the present study clearly demonstrates that soil samples from the dye contaminated site have a higher bacterial population compared to sludge and textile effluent sample. Hence, the isolates from dye contaminated soil were naturally having resistance to withstand in the toxic chemicals contaminated site (Mariappan et al., 2003) found the bacterial population of dye contaminated soil was high and it ranges from $13.2 \times 10^7$ to $32 \times 10^7$ CFU/g.

The significant amount of bacterial load in the soil due to the continuous enrichment of wastewater and the potential strains multiplies by decomposing organic matter of the soil even at higher dye concentration (Jadhav et al., 2008). Further consequence, the dye removal strains gains facility to utilize the synthetic dyes and toxic compounds as a substrate for their growth (Khehra et al., 2005). Chen et al. (2003) found the strains from sludge sample collected near to lake-mud and effluent treatment plants have ability to utilize the dye as their carbon source for growth and degradation. Similarly, Elisangela et al. (2009) reported that dye decolorizing microorganisms from sludge of textile effluent treatment site have maximum dye removal capacity rather than the strains of various sources. Hence, microbial diversity of the dye effluent contaminated soil was observed to be highly restricted. Because
Chen et al. (2003) early stated that microorganism isolated from site near to textile industry which have ability to survive in the presence of toxic dyes.

5.2. Bacterial genera in textile effluent and contaminated soil

Fungal diversity in the collected sludge, soil and effluent was less when compared with bacterial diversity, since the fungus needs acidic pH condition for their growth and multiplication. On the other hand the dye contaminated soil, effluent and sludge was alkaline in nature due to usage of wide variety of chemicals, surfactants, salts and dyes in the dyeing process that impacts the bacterial diversity of textile wastes. Majority of the bacterial genera in dye contaminated sources belongs to Bacillus sp. (47.91%) and Enterobactericeae group (17.70%) followed by Lactobacillus sp. (4.16%), Pseudomonas sp. (6.25%), Alcaligenes sp. (10.41%), Aeromonas sp. (3.12%), Staphylococcus sp. (4.16%) and Micrococcus sp. (6.25%) found to be resist with azo dyes which were in accordance with (Sudhakar et al., 2002). Predominant bacteria present in the dye contaminated soil and sludge was Bacillus sp. eventually decolorizes and degrading azo dye was reported by (Dawarker et al., 2010).

5.3. Dye degrading strains

The twenty bacterial isolates were selected for further studies on the basis of its ability to form a zone of inhibition around the colonies in the medium containing 250 mg/l of dye concentration. The previous studies stated higher and lower dye concentrations greatly influenced the removal of color using microorganisms (Sushama et al., 2009). In the case of actinobacteria, dye concentration at 100 mg/l was found to be toxic for Streptomyces krainskii (SUK-5) reported by Mane et al. (2008). Zhou and Zimmerman (1993) already suggested that the ability of microorganisms to decolorize textile dyes depends upon the chemical structure of the dye and their adaptability with xenobiotic compounds through biological activity.

Secondary screening was carried out in the liquid culture with three reactive dyes namely RNL, blue RGB and red RGB based on the report by Arunprasad et al. (2010). In the present study showed that decolorization of three dyes with twenty strains arises under an anoxic condition. The distinct rate of this strain on dye decolorization percentages demonstrates that ability of dye degrading character was varied in the strains. Accordingly,
Learoyd et al. (1992) found that the different reductive capacity of bacteria in single/pairs with different sensitivity of dye to reductase enzyme. Similarly, various enzymes involved in microbial dye decolorization were laccase, manganese peroxidase (MnP), manganese independent peroxidase (MnP), lignin peroxidase (LiP) and tyrosinase. Many researchers have been studied and confirmed that the azoreductase enzyme plays a vital role in the breaking of azo bonds leading to decolorization (Khan, 2011).

5.4. Phylogenetic analysis

In the phylogenetic tree construction neighborhood joining method were widely employed to interpret the genetic relationship of microbial strains. Venkata Mohan et al. (2012) documented that phylogentic analysis of the dye decolorization strains belongs to four classes of family were β- proteobacteria (50%) followed by Bacilli (16%), α-proteobacteria (16%) and actinobacteria (16%). The gram positive facultative anaerobic bacteria Bacilli sp. releases the azoreductase enzymes were responsible for cleavage of azo bonds (Maier et al., 2004).

The laccase enzyme synthesizing Bacillus licheniformis (LS04) were also identified by 16S rRNA sequencing to decolorize the reactive black 5, reactive blue 19 and indigo dye (Lu et al., 2012). The dye decolorizing Lactobacillus sp. was identified by 16S rDNA sequencing and the 99% genetic relatedness identity exist in between the Lactobacillus (Lab2) with other Lactobacillus casei and Lactobacillus paracasei (Elbanna et al., 2012). Dye degrading Exiguobacterium (ESL52 and TSL7) was identified by nucleotide sequencing of 16S rRNA gene. Previously Exiguobacterium sp. was studied only for their resistance in extreme sites with high pH, temperature and higher salt concentrations (Ponder et al., 2008).

Few reports are available for the degradation of environmental pollutants, pesticide and heavy metal pollution by Exiguobacterium sp. (Edlund and Jansson, 2008). Brevibacillus sp. from sewage sludge degrades textile dyes into many aromatic amines were reported earlier (Ng et al., 2010). 16S rDNA sequencing technique was used to identify dye decolorizing Bacillus fusiformis isolated from textile dye contaminated soil (Kolekar et al., 2008).
5.5. Removal of dyes using individual bacterial strains under batch mode conditions

The growth of microorganisms mainly depends upon the nutrients supplemented in the media. Nutrients are necessary for their microbial growth and cell metabolic activities. Based on the nutrient availability and starving condition, the bacteria were adapted to alternative metabolic pathway to utilize the available substrates as a food to increase the dye removal mechanisms. Generally, the percentage of decolorization was best in co-substrate amended media. This result was correlated with starch and yeast extract in the medium as an amendment (Moosvi et al., 2005).

The absence of the supplementary carbon and nitrogen source in the bacterial culture was unable to decolorize the dye represents the essentiality of co-substrate for growth and decolorization (Nigam et al., 1996). Microorganism requires organic carbon sources for their decolorization mechanisms, without any extra carbon source for the degradation of dye was very difficult and they cannot utilize the dye as a growth substrate. Only few researches attained in isolating the cultures capable of utilizing dyes as a sole carbon source (Saranaik and Kanekar, 1999).

Microorganisms are proficient in utilizing a variety of complex materials including dye as their sole carbon source or either nitrogen. However, the aerobic decolorization of azo dyes can also be carried out in the presence of external carbon source and presumably does not use any azo dyes as the sole carbon source or energy source (Padmavathy et al., 2003). In the present study, 6 strains have the capability to utilize the dye as a sole carbon source along with nitrogen source under anoxic condition.

This report was contrasted to the previous reports that under aerobic condition, the azo dyes were non degradable as a result of most of the bacteria which use dyes as a sole source of carbon to be difficult (Zimmerman et al., 1982). The previous reports point out that the ability of microbes to utilize the dye as an additional source to induce the metabolic activity (Jadhav et al., 2010). Further, Hu (1998) reported that, the decolorization by *Pseudomonas luteola* was directly proportion to the yeast extract concentration 0.3 g/l in the
medium. Amendment of yeast extract leads to regeneration of NADH to act as electron donor for an enhanced decolorization (Ausubel et al., 1997).

5.5.1. Decolorization of dye in the medium amended with co-substrates

5.5.1.1. Carbon sources on bacterial dye decolorization

To establish the effect of carbon source by adapting the experimental design of one factor at a time was a valuable method to analyze the role of single factor at a time by keeping other factors are constant. Effect of carbon sources on the decolorization of remazol golden yellow decolorization with bacterial strains shows that starch and lactose enhanced the dye removal. The bacterial isolate (M1) decolorize the dye in the presence of lactose was only 56.41%, whereas fructose, starch and lactose showed 91 to 93% of dye decolorization by Pseudomonas sp. It was clear evidence reported by Jyoti et al. (2010). Bacterial isolates TSL9, ES37 and M1 use lactose as a carbon source for the efficient decolorization. Most of the reports stated that lactose can be a most needed carbon source for decolorization of synthetic dyes (Junarker et al., 2006).

Sushama et al. (2009) used an efficient strain Bacillus laterosporus for decolorization of navy blue-3G in the synthetic medium containing glucose and starch as a carbon source. It was closely related to our bacterial isolate ESL52 on dye removal. In the recent reports, decolorization assisted with starch was slow compared to glucose causes low activity of the glycoside hydrolase enzyme on decolorization although the mixture of starch and glucose attains good yield of color removal (Boonyakamol et al., 2009). The accessibility of glucose in the medium as a carbon source causes reduction of bacterial activity by decrease in the pH leads to ends in the formation of organic acids during fermentation.

In another report says that the presence of glucose enhances the decolorization (Kapdan and Kargi, 2002). Khehra et al. (2005) shows that glucose act as a co-substrate to increase the decolorization of RV5R with an increase in the concentration of dye up to 2.5 g/l. This was mainly due to the production of NADH and FADH from glucose metabolism enhanced the decolorization efficiency. Previously, Tony et al. (2009) reported that decolorization of remazol golden yellow dye in starch contained basal medium with different Bacillus sp.
Padhmavathy et al. (2003) reported that starch as a carbon source enhances nearly about 77% of remazol golden yellow removal with the aid of Pseudomonas sp. at 250 mg/l dye concentration. Georgiou et al. (2005) reported that application of potato-starch wastes to enhance the decolorization of textile effluents in large scale treatment. It was found that 0.1% of the starch as carbon source showed faster removal of RFB dye (Chen et al., 2003).

5.5.1.2. Nitrogen sources on bacterial dye decolorization

Effect of nitrogen source suggests that the addition of organic nitrogen source yeast extract, beef extract favors better decolorization and increases the color removal. Other nitrogen sources namely peptone, urea, ammonium sulfate and ammonium nitrate showed a poor decolorization rate in this study. Yeast extract was considered as an essential organic nitrogen source in the media to regenerate the NADH in the decolorizing broth; this act as an electron donor for reduction of dyes by bacteria (Hu, 1994). Reports have also been shown that decolorization of Ranocid Fast Blue was best in the media containing yeast extract (Mathew and Madamwar, 2004).

Our study signifies that the inquisitiveness of peptone and other inorganic nitrogen source on color removal was observed during dye decolorization was interrelated with the study reported by Sushama et al. (2009). However, the literature survey supports that addition of yeast extract act as a best co inducer for bacterial dye removal (Chen et al., 2003; Moosvi et al., 2005). The color removal rate of a wide range of dyes increased with addition of yeast extract as nitrogen source was reported by many literatures.

Azo dyes are deficient in carbon source; therefore color removal was feasible only with the co-metabolite incorporation. In contrast, the addition of carbon source reduces the dye removal rate instead of increasing decolorization was observed possibly by assimilation of added carbon source with over usage of dye as the carbon source (Saratale et al., 2009). The presence of carbon and nitrogen hold either inhibitory or stimulatory effect on decolorization of direct orange 39, results in the variation of the time required for decolorization process (Jyoti et al., 2010).
In the effect of carbon and nitrogen source on decolorization of RNL results did not show significant relationship with previous literature because decolorization take place up to 12 days was slow when compared to prior reports. Bacterial strains gradually decrease the decolorization of dye from the 7th day of incubation period. In the aspect of added carbon source decreases the level of bacterial decolorization seems do not utilize the extra carbon rather than complex dye molecule as source of carbon (Saratale et al., 2009).

Time consuming for our bacterial color removal was due to lack of interaction with other individual parameter affects the strain growth and its decolorization rate. Similar results were observed for decolorization of reactive green 19A by M. glutamicus (Saratale et al., 2009). Reduction in remazol golden yellow dye removal directly proportional to depletion of nutrients in aqueous medium to affects the metabolism of microorganisms. Moreover accumulation of degraded products together seems to be a new color in the medium. Other cause includes the chronic toxicity of a dye to bacterial cells viability and inadequate biomass concentration of dye (Jadhav et al., 2008). Further the chemical structure of the dyes also affects the rate of decolorization by inhibition of enzymes responsible for dye removal.

An increase in the concentration of dye also makes the medium in acidic nature. Dyes with low molecular weight and simple structure were degraded easily in mass quantities, excluding in the case of dyes with complex ring structure make it hard to break leads to the time consuming process (Elisangela et al., 2009). Numbers of cultures were isolated from soil samples utilizing synthetic dye RV5R in the presence of glucose and yeast extract as co-substrate however, a few strains failed to decolorize by successive transfer on second and third time (Nigam et al., 1996).

Our study revealed that the addition of 1% starch and yeast extract in the medium improved the color removal. This result was inconsistent with earlier reports of Jang et al. (2007). They reported that 1% glucose increase the decolorization process nevertheless nitrogen source does not have an effect on the color removal of decolorizing medium. The optical density and percentage of the decolorization value of all the substrates implies the need, intake and applicability of each co-substrate for dye decolorization.
The availability of microbial decolorization of remazol golden yellow with high efficacy was very less. The present study clearly indicates that individual co-substrates have different impact on the utilization of dye. It also has inherits in the growth pattern of bacterial isolates in the respective fermentation medium. Effect of nutrients on bacterial isolates was an appropriate technique supportive to generate the furthermore favored culture conditions to attain the maximum decolorization.

5.5.2. Decolorization of dye under optimized condition using individual bacterial strains

In addition to bio-treatment of textile wastes, several environmental factors such as pH, temperature, amount of oxygen and co-metabolic nutritional sources were influenced in biodegradation dyes (Kuhad et al., 2004). The use of optimized culture along with carbon and nitrogen source to increase the decolorization rate of remazol golden yellow seems to be a dynamic approach. The optimization study has to be found favorable point of each individual factor for a satisfied operational condition of the process (Ravikumar et al., 2006). Generally statistical tools includes design of experiments with response surface methodology (RSM) were employed to achieve the best optimized condition of a process. Few reports recommend, the culture medium optimization with different substrate affects the growth of bacteria and dye decolorization (Kim et al., 2008).

It is essential to understand the relationship amongst variables have to be investigated to find out the most suitable model. If the values don't fit into the given measure considered as the model is a failure due to the experimental data of the model are not used in the regression analysis. The significance of P-value mainly indicates the mutual interaction of the each individual factor (Karvela et al., 2009). \( R^2 \) value is closer to 1, the model exhibit a good level of correlation between the experimental and predicted values (Pan et al., 2008). The variation of the response data and residual error were determined in ANOVA.

The random noise formed in the model were confirmed and explained in the residual plot which lies close to the straight line of the points (Ayed et al., 2010). The plot is good compact to interpret the influence of factors and to find in which order the data present in a sequence of line. It is also useful in experiments to identify the runs were not randomized.
(Harbi et al., 2010). The 2-D, 3-D response surface and contour plot useful to examine the interaction exists between the variables in central composite design. The percentage of dye decolorization at various conditions was represented in the number of lines of inside contour plot (Hasan et al., 2009).

5.5.2.1. *Micrococcus endophyticus* (ES37)

The occurrence of 0.71% (w/v) yeast extract enhances the decolorization of remazol golden yellow dye with bacterial isolate *Micrococcus endophyticus* (ES37). This was supported with the study carried out by Nigam et al. (1996). They noticed that yeast extract 0.5% improves the bacterial reduction of azo dyes effectively. In contrast, it has been reported the yeast extract concentration less than 100 mg/l couldn’t enhance nor affect the decolorization. Glucose is not required together with yeast extract since decolorization process was non-specific with respect to the electron donors (Vander Zee and Villaverde, 2005). Many authors failed to analyze the role of yeast extract as a source of redox mediators. An earlier report also suggested that the reduction of azo bond were mainly carried out in riboflavin of the yeast extract reduced by electron carrier e.g. NADH present on the cell surface and re-oxidized by azo dye, excluding yeast extract contains amino acids and vitamins enhances the bacterial growth (John et al., 2008).

*Micrococcus* sp. reported to decolorize various classes of dyes were 2-nitrotoluene, nitrobenzene, melamine formaldehyde and azo structures (Saratale et al., 2009). The optimum pH of color removal was neutral to slightly alkaline and the strong acid or alkaline pH values attributes rapid color removal reduction. Altering the pH within a range of 7-9.5 has a very little effect on dye reduction process (Elisangela et al., 2009). Already it has been accounted that bacterial strains reduces the azo dye into aromatic amines and increases the pH of the medium about 0.7-1.0 (Hu, 1994). *Micrococcus glutamicus* decolorizes the azo dye up to 63% within 72 hours (Saratale et al., 2010).

5.5.2.2. *Exiguobacterium aurantiacum* (ESL52)

The pH of the medium mainly influences in the removal of dye by creating the electrostatic interaction with charged group of dye and bacteria. Earlier reports also stated
that additional carbon source is required for the growth of bacterial culture and decolorization of dye (Mohana *et al*., 2008). Yeast extract requires as a nitrogen source to generation of NADH acts as an electron donor in microorganisms to reduce the azo bond (Khehra *et al*., 2005). Ola *et al*. (2010) reported the maximum decolorization of dyes cibacron red P4B and cibacron black PSG achieved in the optimized media condition with finest carbon and nitrogen source. Similar to this study, the optimization of reactive red 195 removals by *Bacillus cereus* utilized maltose and peptone as a substrate to enhance the decolorization up to 97% (Modi *et al*., 2010).

Statistical design revealed that the higher amount of decolorization achieved in the lower concentration of starch 0.49% (w/v) was agreed with the study carried out by Rama and Abedin (2008). In their study, the decolorization of crystal violet was 96% by *Fusarium solani* with low concentration of starch. The addition of low concentrated starch in the media enhances the decolorization of crystal violet 96% and increase the growth rate of *Fusarium solani* (Mohana *et al*., 2008). The standard point of pH for remazol golden yellow dye decolorization found to be pH 7.10 comparable to the study of Chang and Lin (2000). They observed that the optimal pH 7-8 for an effective dye reduction and in the pH 6.5 reduces the decolorization activity.

**5.5.2.3. Exiguobacterium aurantiacum (TSL7)**

Most of the bacterial strains require optimal pH for their dye decolorizations. Kamle *et al*. (2007) attained maximum decolorization in an alkaline pH. In the addition of starch with medium rapidly removes the dye frequently, as the textile waste water naturally contains a higher load of starch obtained from sizing process (Dos Santos *et al*., 2007). The halotolerant *Exiguobacterium* sp. decolorize the anthraquinone dyes by enzymatic activity and biocatalysis (Tan *et al*., 2009).

**5.5.2.4. Bacillus firmus (TSL9)**

The usage of high quantity of chemicals attributes the alkaline nature in the textile effluents. Hence, the pH plays a vital role in transport of complex dye molecule to cross the bacterial cell membrane in decolorization process (Kodam *et al*., 2005). The effects of pH
and temperature on microbial dye decolorization were extensively explored (Pearce et al., 2003). Kaushik and Malik, (2009) analyzed the effect of pH on various classes of dyes in microbial mediated decolorization. *Bacillus subtilis* generally exhibited maximum decolorization of acid blue 113 at pH values near to 7-8 (Gurulakshmi et al., 2008).

According to Praveen Kumar and Bhat Sumangala (2012) *Bacillus cereus* and *B. megaterium* efficiently removed 93.64% of Red 3BN dye in the amendment of sucrose, peptone as carbon and nitrogen source. Modi et al. (2010) found that maltose and peptone was the most excellent carbon and nitrogen source coordinated with *Bacillus cereus* to decolorize 97% of reactive red 195. *Bacillus firmus* with respect to dye decolorization was less investigated. Kalyani et al. (2007) found the decolorization of various dyes with *Bacillus firmus* decolorizes the dye up to 75 - 125 mg/l effectively.

Padhmavathy et al. (2003) reported *Bacillus firmus* act as a potential candidate in the textile industry effluent treatment. *Bacillus firmus* completely decolorizes the dye in the existence of starch within 21 hours (Ogugbue et al., 2012). The inoculums size provides the sufficient biomass to the decolorization of dye in an intermediate forms. *Streptomyces globosus* decolorizes reactive red dye with 1 to 4% inoculums size (Nermeen et al., 2011). Mabrouk and Yusef (2008) confirmed that 80% decolorization of textile dye direct black 22 achieved in the higher concentration of inoculums size and glucose.

An increase in the carbon, inoculum size boost the rate of decolorization, furthermore addition inhibits the synergistic reactions of existing factors (Tan et al., 2009). Dafnopatidou and Lazaridis (2008) stated *Bacillus firmus* halo tolerant strain decolorizes the dye at high salt concentration ranging from 0 – 60 mg/l. Tripathi and Srivastava (2012) found *Bacillus megaterium* decolorize the dye orange G up to 94.4% by adapting an ideal statistical tool as central composite design. *Bacillus thuringiensis* (4G1) were able to yield 98.23% of decolorization of methylene blue MB under an optimized condition (Nermeen and El-Sersy, 2007).
5.5.2.5. *Brevibacillus laterosporus* (TS5)

The decolorization of dye mainly depends upon pH of solution and optimal pH 6-10 favor for colour removal (Chen *et al.*, 2003). The main requirement of dye decolorizing bacteria is pH tolerance feature, in the fabric industry azo dye bind with the cotton fiber in an alkaline pH (Aksu and Tezer 2000; Kumar, 2009). Azo dye navy blue 3G decolorization by *Brevibacillus laterosporus* (MTCC2298) showed 80% dye removal within 48 hour under static condition at broad range of pH 7-11 (Jirasripongpun *et al.*, 2007). Gomare *et al*. (2009) detected *Brevibacillus laterosporus* (MTCC2298) decolorize the dye golden yellow HER at the concentration of 50 mg/l under static condition within 48 hours. Distinguish to the *Brevibacillus laterosporus* decolorize the dye reactive red 198 using pyruvate as a carbon source and yeast extract as an electron acceptor needed for the degradation and decolorization of dyes (Elisangela *et al.*, 2012).

5.5.2.6. *Lysinibacillus fusiformis* (M1)

Waghmode *et al*. (2012) found that lactose and dextrose act as additional carbon source inhibit the decolorization of Rubin GFL and textile effluent. Commonly utilized nitrogen source yeast extract excellently supports to the microorganisms for their various dye removal (Khehra *et al.*, 2005). Chen *et al*. (2003) reported *Lysinibacillus* sp. produce a higher level of dye removal with an increase in the yeast extract concentration. Elisangela *et al*. (2009) found *Staphylococcus arlettae* decolorize the dyes CI reactive red 198, black 5, yellow 107 and blue 71 effectively in addition of 1% yeast extract.

Absence of yeast extract in the medium reduces the level of decolorization to 50% for reactive yellow 107 and reactive red 198. *Pseudomonas aeruginosa* decolorize the dye in both acidic and alkaline pH, thus far significant decolorization of 98.88% was achieved in pH 7 (Joe *et al.*, 2011). Even though individual optimized microbial culture decolorizes the dye remazol golden yellow in a maximum level has many drawbacks in applying industrial scale in the view of post solid-liquid separation. In addition, number of complex chemicals made it critical to treat and environmental concern (Jacob and Azariah, 2000).
5.6. Decolorization of dye by bacterial consortium CD-3

Aslim et al. (2002) used antagonistic activity to formulate the microbial consortium holds strains for broad spectrum enzyme synthesizing on removal of complex chromophore dye. Microbial consortium is an alternative preference than existing physical and chemical methods for dye decolorization and mineralization (Saratale et al., 2010). Decolorization of dyes with pure culture was impractical, as the isolated culture would be dye specific and their application in large scale wastewater treatment plants with a variety of contaminants was not feasible (Murugesan and Kalaichelvan, 2003).

Efficient biodegradation of dyes in synthetic medium can be accomplished when catabolic activity of individual strain was complement with each other in a mixed culture community. In most studies, the microbial consortia have been found more effective than pure cultures. Individual strains Bacillus odyssey (SUK3), Morganella morganii (SUK5) and Proteus sp. (SUK7) completely removed reactive blue 59 at 50 mg/l within 60 hours, yet mixed culture of these strains carried consortium PMB11 attains the complete decolorization of dye within period of 3 hours (Patil et al., 2008).

The effect of formulated consortium showed a good result in dye removal compared with pure cultures infers the potentiality of consortium CD-3 for textile effluent decolorization. Transformation of dye into intermediates by single strains, which can act as redox mediators for an efficient transmit of reducing equivalents from other strains in consortium HM-4 stated with (Khehra et al., 2005). Microbial consortium consisted with a white rot fungus and Pseudomonas sp. showed a maximum dye removal rate under static culture, which might be attributed to the synergetic reaction of individual microorganisms (He et al., 2004).

Whereas the bacterial strains triggered the suitable genes results in degradation of toxic compounds (Chen et al., 2003). Microbial communities in the consortium combined with each other for their metabolic activities to degrade the dye completely and mineralize it (Khadijah, 2009). Similar to this study, Nikhil et al. (2005) screened seven bacterial consortia for the decolorization studies. Bacterial consortium SpNb1 containing Brevibacillus laterosporus and Galactomyces geotrichum effectively decolorized up to 96.75% rubine GFL
(50 mg/l) dye within 30 hours while individual strains does not decolorize the dye significantly (Waghmode et al., 2012).

Moosvi et al. (2005) made similar observation with bacterial consortium JW-2 comprises *Paenibacillus polymyxa*, *Micrococcus luteus* and *Micrococcus* sp. showed complete decolorization of textile dyes with low amount of yeast extract, starch. It was reported the intermediates of dye caused in one strain to act as a precursor for another strain tends to complete mineralization of intermediates in consortium decolorization (Kadam et al., 2011).

5.6.1. Decolorization of various dyes by microbial consortium

The dyeing industries employed with various groups of dyes with diverse structure directs to multiple dye possessed effluent generation (Saratale et al., 2009). *Bacillus firmus* isolated from the textile industry effluent have ability to decolorize the diverse classes of dyes safranin, methylene blue, neutral red, nigrosine, crystal violet and basic fuchsin within 48 hours of incubation (Ogugbue and Sawidis, 2011). Some of the dye contains sulphate group in their chemical structure inhibits the enzymatic activity of dye leads to increase in the electro negativity of resist force between negative ions of dye to bacterial surface (Suzuki et al., 2001). Dye contaminated soil bacteria *A. hydrophila* reduce the various dyes solophenyl red (72%), basic fuchsin (42%), safranin (32%) and nigrosine (18%) under static condition (Ogugbue et al., 2012).

In the study bacterial consortium CD-3 have ability to decolorize the ten dyes in different rate. The percentages of decolorization with different dyes were varied; depend upon the molecular structure and functional group includes chromium, cobalt and copper made them complex to degrade it (Wang et al., 2009; Li and Guthrie, 2010). Some of the dyes impede the DNA synthesis of micro organisms; hence, the rate of decolorization related with different dyes was varied (Guo et al., 2007). Dissimilar dyes reactive black 5, reactive yellow 107, reactive red 198 and direct blue 71 were completely decolorized by bacterial isolate *Staphylococcus arlettae* within in 48 hours at the concentration of 100 mg/l (Elisangela et al., 2009).
Related to this incubation consortium holds *Sphingobacterium* sp. (ATM), *Bacillus odyssey* (SUK3) and *Pseudomonas desmolyticum* (NCIM2112) have ability to decolorize red HE8B, red M5B, remazol red, orange 3R, rubine, golden yellow HER and direct blue GLL at the concentration of 0.8 mg/l (Tamboli et al., 2010). Similar to this study with seven days incubation decolorize the mixture of dyes (35%) by using consortium at 100 mg/l concentration (Senan and Abraham, 2004).

5.6.2. Requirement of oxygen in the dye decolorization

All the 6 bacterial isolates exhibit maximum decolorization only in the static condition. Azo dye degradation was normally opposing the attack of bacteria under shaking only. Aerobic reduction of azo dye removal with low efficiencies was achieved since oxygen is a more effective electron acceptor than azo dyes (Liu et al., 2007). The main mechanism of dye degradation involves synthesis of azoreductase in bacterial strains were reported. In aerobic condition the azoreductase active sites were inhibited in the presence of oxygen related to competition with oxidation of reduced electron carrier (NADH). Hence, the oxygen molecule was transferred to the upper surface of broth carried cells.

In contrast, mostly cells in bottom of the flask decolorize the dye in anaerobic condition (Chang et al., 2004). Therefore, the bacterial strains favor static/anoxic conditions for an effective color removal. The bacterial consortium CD-3 contains the *Exigiobacterium auranticum* (TSL7), *Brevibacillus laterosporus* (TS5) and *Bacillus firmus* (TSL9) effectively decolorizes 69% of remazol golden yellow dye in static condition within 24 hours compared to shaking process. Similarly, *Enterococcus gallinarium* strain decolorizes the direct black 38 up to 63% under static condition within 24h was reported by Bafana et al. (2008).

The decolorization of remazol golden yellow dye under static condition was coordinate with the result of three *Halomonas* sp. decolorizes the dye effectively at a concentration of 5000 mg/l later than 4 days incubation (Asad et al., 2007). *Bacillus cohnii* (MTCC3616) was found to be decolorizes direct red-22 over 95% effective in static and 7% in shaking condition. There were direct connection exist between the dye concentration and time taken for decolorization. In case of shaking condition enhances the growth of bacterial
consortium though rate of decolorization was 5.74%. An aerobic condition causes decreased level of electron carrier available to the oxygen and azo dye (Dawkar et al., 2008).

Mono and di-oxygenase enzymes act on the organic compound aromatic ring fusion by donating oxygen in aerobic condition. Azo dyes and aromatic compounds were not responding in bacterial aerobic degradation related to electron withdrawing capacity of azo bonds and inhibit the oxygenase enzyme system (Madigan et al., 2003). The existence of oxygen inhibits the cleavage of azo bond, utilizes NADPH and blocks the electron transfer to NADH (Chang and Lin, 2001). The advantages of anaerobic reduction of dyes were lack of oxygen creates the static condition and facilitate the facultative anaerobes to degrade azo dye (Stolz, 2001). Pseudomonas sp. (OX1) found to be decolorizes the dye well in absence of oxygen was reported by (Lodato et al., 2007).

The present study result agreed with (El Ahwany, 2008) observed most effective decolorization of fast red acid dye by Oenococcus oeni (ML34) under static condition. Previous studies also suggests azo dye act as an electron acceptor supplied in the carrier of electron transport chain forms a low redox potential for better decolorization in an anaerobic condition (Stolz, 2001). Pseudomonas aeruginosa (NBAR12) decolorizes the dyes well in static condition rather than shaking indicates availability of oxygen sensitive reductase in the decolorization (Nikhil et al., 2005). Remazol black B dye decolorization studied with static and shaking condition, however 96% decolorization attained in static condition at the concentration of 50-300 mg/l (Joe et al., 2011).

5.6.3. Decolorization of dyes under optimized condition using microbial consortium

The environment condition must be favorable for the degradation of certain compounds include pH, electron acceptor, organic materials, inorganic materials, nitrogen and phosphorus are essential to carry out perspective of bioremediation. The optimized consortium increases the degradation capacity may be an effectual decolorization process was correlated with the assimilated consortium in effluent treatment (Younes et al., 2012).

The pH plays a major upshot on the efficiency of dye decolorization and the optimal pH for color removal was 6 - 10. An adaptation of microorganisms with varying pH enhances
the process of effluent treatment. The strong acid or alkaline pH values attributes reduction of color removal process. Variation of pH within a range of 7 - 9.5 has a very little effect on dye reduction process (Elisangela et al., 2012).

Dye concentration strongly influences in the rate of dye removal and also impacts the toxicity of dye molecule. Percentage of dye removal increased with increase in time irrespective of initial dye concentration. The dye concentration plays a vital role in decolorization as it stimulates the biological activity of microorganism. Toxicity of a dye inhibits the metabolic activities of bacterial cell or inadequate biomass for the uptake of higher concentration of dye leads to reductions in decolorization. Sulfonic acid groups of the dye molecule affect the active site of azoreductase and decrease the rate of decolorization (Kalyani et al., 2009).

Higher dye concentration act as an important factor to generate the resistance between the dye and microorganism (Ali et al., 2008). Bacillus subtilis decolorized acid blue 113 at different dye concentrations varying from 50 - 300 mg/l and the rate of decolorization increased with increase in dye concentration up to 200 mg/l (Gurulakshmi et al., 2008). Padamavathy et al. (2003) reported the Rhizobium radiobacter decolorize methyl violet with various concentrations from 10 - 100 mg/l and decrease in dye removal efficiency with an increase in dye concentration. Increase in the dye concentration enhances the resistance characters of strain to withstand the toxic dyes in higher saline condition.

The potential ability of bacterial consortia in azo dye decolorization than single culture was reported by Chen et al. (2003). Bacterial consortium from tanning and textile waste water were reported that 80% of bioaccumulation reactive black B dye at 59.3 mg/l concentration in the prevalence of Cr (VI) for 7 days (Kilic et al., 2007). Two aerobic bacterial consortia decolorize the maximum of synthetic textile dye under in optimized condition for 8 days (Kumar et al., 1997).

The textile dye direct black 22 with novel consortium was achieved 80% decolorization under in RSM mediated optimized condition (Mabrouk and Yusef, 2008). Correspondingly, the microbial consortium augmented with Sphingomonad paucimobilis,
Bacillus cereus (ATCC14579) and Bacillus cereus (ATCC11778) decolorizes methyl orange effectively in mixture of experimental design (Ayed et al., 2010). Similar to this study Anil Kumar et al. (2012) showed the maximum removal of acid blue 15 and methylene blue by the consortium consisted with Pseudomonas desmolyticum, Kocuria rosea and Micrococcus glutamicus. Mohana et al. (2008) used response surface methodology mediated optimization to decolorize the textile dye direct black 22 up to 80% using novel bacterial consortium.

The degradation rather than bio-adsorption mechanism involved with the consortium to decolorize the broth containing dye. The color less cell pellet from the decolorization has an evidenced of microbial degradation. Previously, Chen et al. (2003) stated the adsorption was identified by a colored cell pellet. Salt tolerance bacteria have ability to degrade azo dye waste water contain hypersaline nature Ng et al. (2010). The effluent released from textile industry contains high salinity due to usage of higher quantity of salts were ensured for the maximum fixation of dye to cellulose fiber in dying process (Carliell et al., 1995).

Earlier report states that the ability of bacteria obtained from different environment degrade wide range of reactive dyes at low (<5 g/l) salt concentration (Oturkar et al., 2011). The increase in concentration of salt affects the growth of microorganism, leads to plasmolysis and loss of cell metabolisms. Hence, the bacteria withstand in saline condition to decolorize dyes were a suitable candidate for an effective decolorization. Only few bacterial species capable to decolorize the dyes under high saline condition (Tan et al., 2009).

In the present study percentage of dye decolorization differs with respect to salt concentration. The decolorization of dyes depends upon the bacterial growth, in the contradictory side exhibit direct correlation with salt concentration. An increase in salt concentration decreases the growth of bacteria, suppresses the enzyme activity of microorganisms and in turn subside the level of decolorization by inhibition of bacterial metabolism (Zilly et al., 2011). Marine derived lignin degrading fungus effectively decolorize the textile effluent with high salinity was documented by Raghukumar et al. (2008). Halophilic strains of Halomonas sp. has capability to degrade wide range of dyes with maximum 20% of sodium chloride concentration (Chen et al., 2003). The high
concentration of salt affects the cell membrane permeability and creates a competition between the dye and *B. firmus* active site (Diniz and Volesky, 2005).

Overall report of this optimization found that decolorization increases with increase in the concentration of sodium chloride up to moderate level and further amount of salt tolerance hinder the decolorization activity of the microorganisms. The increase in salt concentration decreases the level of microbial consortium decolorization efficiency was reported by Jadhav *et al.* (2010). Textile effluents have a high salt concentration as their major inorganic constituents at the end of dyeing and treatment process. Effect of salt tolerance on the bacteria for dye decolorization suggests, there was a steady increase in the rate of decolorization but excess salt in the medium inhibit the microorganism ability to decolorize the dye. This result was clearly supported by Mathew and Madamwar, (2004). They have tested up to 5.0% (w/v) salt tolerance on the bacterial consortium dye decolorization.

### 5.6.3.1. GC-MS analysis

Biodegradation results in the conversion of the high toxic dye molecule into non toxic intermediates (Younes *et al.*, 2011). Mostly dye has been converted in to aromatic amines at the end of anaerobic process. The sequential anaerobic / aerobic condition was essential for the complete degradation of aromatic amines in an aerobic reaction. The microbially treated dye solution was investigated for the presence of non-toxic metabolites by using gas chromatography mass spectrometry analysis. Earlier reports supports that degraded metabolites were mainly detected on this method (Jadhav *et al.*, 2008). Various enzymes are there in bacterial decolorization of azo dyes. Therefore for each microorganism enzymes involved in the process was different (Kalyani *et al.*, 2009).

Generally azo dyes were broken down in the symmetric or asymmetric axis with the active site of an enzyme (Kabra *et al.*, 2011). Remazol golden yellow was a mono azo reactive dye with aromatic ring and amide group. The first step of dye reduction was an asymmetric cleavage of oxidoreductase enzyme. The different enzyme mechanisms involved in the degradation of remazol golden yellow other than azoreductase. The results indicated it
was completely degraded into small units and major intermediate metabolite was 2-(p-methoxyphenylamino-2-phenyl) ethyl-2-oxazoline.

All the intermediates contain the functional groups as heterocyclic, halogens, imines and there were no toxic aromatic amines as an end product. It was supported by non toxic intermediates found in remazol orange decolorization by *Pseudomonas aeruginosa* BCH (Shekhar *et al*., 2012). Similar to this degraded metabolites, the dye Levafix Blue E-RA contains 2-[2-(3-hydroxy-2-peopenyl) phenyl]-1, 3-dioxane (Kalpana *et al*., 2012). The intermediate formed in degradation of disperse brown 3REL were (4-ethoxy-phenyl)-amine (Dawkar *et al*., 2008). This analytical study confirmed that the degradation of complex remazol golden yellow into smaller intermediates were non toxic one.

**5.6.3.2. Thin layer chromatography (TLC)**

TLC analysis was used to find out the presence of dye RED RBN intermediates in the medium by bacterial consortium Chen *et al*. (2003). The thin layer chromatogram (TLC) of remazol golden yellow dye degraded metabolites exhibited numerous bands in silica gel coated TLC sheet and there were no bands found in spot of cell free medium contains RNL dye. The first step involved as breakage of azo bond into aromatic amines in bacterial degradation of azo dye (Stolz, 2001). The formation of aromatic amines in decolorization by *P. aeruginosa* (NBAR12) was confirmed from the chromatogram and no amines were found in the cell free medium.

The lower number of bands formed in the chromatogram was found with an increase in the incubation period reveals the transformation of aromatic amines in to other metabolites. Stolz (2001) suggested the enzymes were azoreductase, peroxidases and flavin reductase involved in the degradation of dyes. The dye decolorization was maximum in the culture incubated at static condition suggest the limited oxygen requirement. Azo dye orange G was degraded by *Bacillus megaterium* (ITBHU01) and its spot obtained from microbial treated supernatant confirms the breakdown of large complex dye molecule into unknown nontoxic intermediates (Tripathi and Srivastava, 2012).
5.7. Decolorization of textile effluent using bacterial consortium

5.7.1. Role of natural microflora in dye decolorization

The decolorization of raw textile effluent with its indigenous microflora enhances the bacterial consortium CD-3 favored dye degradation process. It indicates metabolic activity of bacterial decolorization and found that adsorption mechanism does not involve. The present findings specified raw effluents indigenous microflora were adapted to grow in an alkaline nature combined with bacterial consortium CD-3 mediated decolorization process. Mixed culture efficiently decolorized the dye with presence of resident micro organism and other nutrients in dye solution could have helped in the removal of dye. This report was supported by Jalandani -Buan et al. (2009) explained that resident microorganisms alone could not have ability to decolorize dyes by themselves. The reason behind an indigenous microorganism present in the polluted water does not produce responsible enzymes to reduce azo dyes. Another reason is the natural micro floras was not exposed to azo dyes prior to experiments and were not adapted in the presence of textile dyes.

In addition, it was cross referred with the potential of sterilized effluent with bacterial consortium CD-3 action on textile effluent decolorization. In the present study natural organic matters, resident microorganisms drastically influenced in raw effluent decolorizing activity compared with sterilized effluent decolorization. Bacteria need to be adapted with the pollutant before they can degrade it (Baker and Herson, 1984). Accordingly, indigenous microflora isolated from Taiwan dye bearing waste water treatment plant decolorizes the textile azo dyes (Zhang et al., 2010).

5.7.2. Microbial decolorization of raw textile effluent

The physical-chemical characteristics of raw textile industry effluent from various parts of the world contain considerable variation. The process involved in the textile industry are multi-components, hence, the effluent are difficult to be decolorize by conventional treatment processes. COD values in the effluent were ranges from 10800 mg/l, TSS 600 mg/l. The BOD level in the raw textile effluent was 740 mg/l suggest that organic load in raw
effluent was biodegradable and comparatively higher than earlier reports (Younes et al., 2012) and permissible limit of textile waste water (Khelifi et al., 2010).

In fact, the chemical load of effluent was based on the process involved in textile processing industries. Generally, effluent contains high quantity of chemical pollutants and increases in the color, odor, appearance, total suspended solids, BOD, COD, total hardness, acidity or alkalinity (Ahn et al., 1999). Further the textile effluent contains a higher load of ions and metals due to the dyes required ions to fix into the fabrics (Kaushik and Malik, 2009). The ability of bacterial consortium CD-3 in bioreactor to decolorizes the dyes and removal of chemical oxygen demand in effluent was achieved more than 90% within 10 days of retention period.

The COD removal from the effluent was contrary with colour removal (Casu et al., 2012). In the present study the level of COD increases in the subsequent treatment of treated effluent was supported by Gopinath et al. (2009). They observed that the increase in the COD level in effluent indicates the breakdown of complex dye structure into organic compounds in term of mineralization. The BOD, COD, alkalinity, hardness and TDS of microbially treated effluent was less compared with untreated effluent reported by Santhy and Selvapathy (2006). In case of total dissolved solids, total solids, total suspended solid, BOD and COD were higher in the treated effluents than untreated effluents due to accumulation of microbial by products (Andleeb et al., 2010).

In the same way, single mode treatment was ineffective compared with combined mode of action on effluents. Madhuri (2012) found that the combination of electrochemical and active carbon absorption of wastewater removes 66% of color compared to single treatment process (59%). Similarly microbial consortium effectively decolorizes textile effluent containing BB150 dye with the mechanism of enzymatic degradation or by adsorption (Khouni et al., 2012). The presence of dissolved oxygen in the medium inhibits the decolorization of azo dye under anaerobic condition. Many azo dyes require oxygen as a terminal electron receptor (Pearce et al., 2003).
5.8. Application of treated and untreated effluent on the plant growth

The existence of toxic aromatic dyes has to be removed from effluents and its affect the growth of plants in the natural environment. Puvaneshwari et al. (2006) reported the colorless products of degraded dyes inhibit the growth of crops, causes serious health risk and distress in the diversity of water bodies. The effects of microbially detoxified pollutant compounds in the effluents were studied with various plant and mammalian cell studies. Yogesh Kolekar et al. (2012) analyzed the genotoxic effect of reactive blue 59 dyes and its degraded metabolite on earthworm coelomocytes recognized the intermediates were not toxic. The biodegraded direct black 38 were studied with cytotoxicity techniques to be found as nontoxic (Kumar et al., 2006).

In the plant phytoxicity studies have been found with more versatile advantages than mammalian and microbial cell systems. Plant study deals with environmental quality of treated effluents led to the alternative disposal method in term of nutrient source to plants because of its enormous micro and macro nutritional status (Araujo and Monteiro, 2005). Various dyes brilliant green, congo red, methylene blue and fast green were effectively treated by biological methods coupled with phytotoxicity evaluation (Ilyas et al., 2013). The widely applied phytoxicity includes seed germination and plant growth bioassay (Kapanen and Itavaara, 2001).

Maize (Zea mays) and green gram (Phaseolus aureus) seed germination and growth indicates the nontoxic nature of bacterial consortium CD-3 treated effluent to plants. Previously, Dawkar et al. (2010) identified that degraded metabolites of dyes were harmless and enhance the growth of plants Sorghum bicolor, Phaseolus mungo and Triticum aestivum. Corresponding to degraded metabolite of levafix blue E-RA dye were tested on plant V. radiator and B. juncea growth (Kalpana et al., 2012). Staphylococcus cohnii degraded dye ARB metabolite does not hinder the growth of plants T. aestivum and P. vulgaris (Yan et al., 2012).

Bacillus firmus decolorized malachite green dye solution enhance the germination of T.aestivum compared to control recommends the microbially treated effluents were suitable for irrigation purpose (Parshetti et al., 2006). Bacterial consortium degraded metabolites of
reactive blue 59 and green HE4BD were enhances the growth of plants compared to original dye (Saratale et al., 2010). Reactive red 195 degraded into metabolites with Georgenia sp. effectively enhance the growth of plants Sorghum vulgare and Phaseolus mungo (Madhuri, 2012).

Microbial by decolorized dyes remazol black B, remazol orange 3R, direct red 5B, navy blue HE2R, green HE4B and brilliant blue R enhances the root length of various plants P. grandiflora, B. malcolmii, Brassica juncea and T. patula with an induction enzymes laccase, lignin peroxidase, manganese peroxidase and tyrosinase (Kabra et al., 2011). Ayed et al. (2009) reported that germination, shoot and root length of plants Triticum aestivum and Sorghum bicolor were decreased in the untreated effluent compared to degraded metabolites as toxic to plants. Zhuo et al. (2011) found the effluent decolorized with Trametes pubescens does not inhibit the growth of plants than untreated effluent.

5.9. Toxicity of treated and untreated effluent on the growth of Rhizobium

In order to know the effect of textile wastes on microorganisms is an extremely important to determine the fittest level of wastes for land application by biological testing (Fuentes et al., 2004). The release of untreated textile effluent cause’s harmful effects in soil eco system was a major threat to the public concern. Rhizobium population in the textile effluent contaminated soil influences nitrogen fixation level and nutrient status of the soil. Some papers noticed that textile wastes are significantly reduced Rhizobium population and hinder the nodulation of legumes (Abd-alla et al., 1999). Similarly in our study, the untreated effluent reduces the Rhizobium populations. Reactive group of dyes with aromatic ring structure act as a detergent to affect the growth of microorganisms by inhibiting the DNA synthesis (Chen et al., 2003). According to Younes et al. (2012) acclimatized consortium treated effluent does not show any toxic effect on B. megaterium and E. coli growth compared to untreated effluent.